Implications of nitrogen nutrition for grapes, fermentation and wine

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Abstract
This review discusses the impacts of nitrogen addition in the vineyard and winery, and establishes the effects that nitrogen has on grape berry and wine composition and the sensory attributes of wine. Nitrogen is the most abundant soil-derived macronutrient in a grapevine, and plays a major role in many of the biological functions and processes of both grapevine and fermentative microorganisms. Manipulation of grapevine nitrogen nutrition has the potential to influence quality components in the grape and, ultimately, the wine. In addition, fermentation kinetics and formation of flavour-active metabolites are also affected by the nitrogen status of the must, which can be further manipulated by addition of nitrogen in the winery. The only consistent effect of nitrogen application in the vineyard on grape berry quality components is an increase in the concentration of the major nitrogenous compounds, such as total nitrogen, total amino acids, arginine, proline and ammonium, and consequently yeast-assimilable nitrogen (YAN). Both the form and amount of YAN have significant implications for wine quality. Low must YAN leads to low yeast populations and poor fermentation vigour, increased risk of sluggish/stuck/slow fermentations, increased production of undesirable thiols (e.g. hydrogen sulfide) and higher alcohols, and low production of esters and long chain volatile fatty acids. High must YAN leads to increased biomass and higher maximum heat output due to greater fermentation vigour, and increased formation of ethyl acetate, acetic acid and volatile acidity. Increased concentrations of haze-causing proteins, urea and ethyl carbamate and biogenic amines are also associated with high YAN musts. The risk of microbial instability, potential taint from Botrytis-infected fruit and possibly atypical ageing character is also increased. Intermediate must YAN favours the best balance between desirable and undesirable chemical and sensory wine attributes. ‘Macro tuning’, of berry nitrogen status can be achieved in the vineyard, given genetic constraints, but the final ‘micro tuning’ can be more readily achieved in the winery by the use of nitrogen supplements, such as diammonium phosphate (DAP) and the choice of fermentation conditions. This point highlights the need to monitor nitrogen not only in the vineyard but also in the must immediately before fermentation, so that appropriate additions can be made when required. Overall, optimisation of vineyard and fermentation nitrogen can contribute to quality factors in wine and hence affect its value. However, a better understanding of the effect of nitrogen on grape secondary metabolites and different types of nitrogen sources on yeast flavour metabolism and wine sensory properties is still required.

Keywords: nitrogen, fertilisation, grape, must, wine, Vitis vinifera, yeast, Saccharomyces cerevisiae, fermentation, flavour
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References
1.0 Introduction

Manipulation of grapevine nutrition has the potential to influence grape berry composition, and ultimately the composition of wine, which in turn can affect wine quality and value (Figure 1). This review will focus on nitrogen, which is an important macronutrient that plays a major role in many of the biological functions and processes of the grapevine and fermentative microorganisms (yeast and malolactic bacteria). Grapevine nitrogen status can therefore be expected to influence the concentration and composition of quality components in the grape berry. Grape berry composition is the primary determinant of must composition prior to fermentation. Therefore, the nitrogen concentration and composition of the grape, and thus the must in particular, has significant implications for wine quality. Yeast growth, fermentation kinetics and flavour metabolism are all greatly affected by the nitrogen status of the must, which when found to be sub-optimal can be further manipulated by addition of nitrogen in the winery (Bisson 1991, Kunkee 1991, Henschke and Jiranek 1991, 1993). Furthermore, the roles of yeast in the development of wine aroma, flavour and mouth-feel are becoming more clearly defined, as is the impact of nitrogen on the flavour metabolism of yeasts (Henschke and Jiranek 1993, Rapp and Versini 1996, Albers et al. 1998, Hernández-Orte et al. 2005, Swiegers et al. 2005). However, while yeast unquestionably contribute to wine composition, there are many other components of the final wine that may be affected by addition of nitrogen in the vineyard and/or in the winery that are not directly related to yeast fermentation (Figure 1).

Numerous studies on nitrogen supplementation and its impact on various aspects of the grape to wine continuum have been reported (Figure 1). However, most studies have only dealt with some components of this continuum. We know of no studies or reviews that have attempted to bring all this information together so as to gain a better understanding of how nitrogen supplementation can affect final wine quality. The aim of this paper is, therefore, to review the impacts of nitrogen addition in the vineyard and/or in the winery on grape and wine composition as an entirety and to establish the effect nitrogen has on the sensory attributes of the final wine. Armed with this information, it should become possible to better manage nitrogen inputs in the vineyard and in the winery to optimise productivity and wine value.

2.0 Vineyard to must

2.1 Vineyard

2.1.1 Factors affecting grapevine nitrogen status

Most of the published studies to date have used inorganic nitrogen rather than organic nitrogen sources because of the greater control in applying known amounts to the grapevine. Assuming the absence of other limiting factors (i.e. water deficit), inorganic nitrogen application provides an easily available source of nitrogen for the vine, and consequently increases the vine nitrogen status, which is typically monitored by petiole nitrogen analysis in Australian vineyards.

Yield and growth responses and the resulting impact on grape composition depend on the nitrogen status of the vine prior to the application of nitrogen in the vineyard. Figure 2 illustrates that many factors can influence the nitrogen status of the vine. Such factors include cultivar and/or rootstock (Ough et al. 1968a, Christensen 1984, Huang and Ough 1989, Stines et al. 2000), site – climate and soil (Huang and Ough 1989), season (Neilson et al. 1987, Bell and Robson 1999), cultural practices such as soil management (Bell et al. 1979, Maigre and Aerny 2001, Spring 2001), trellis system (Kliewer et al. 1991,
Miele et al. 2000), canopy shading (Smart et al. 1988, Perez-Harvey and Witting 2001), canopy temperature (Ewart and Kliewer 1977), and nitrogen form, timing and rate of application (Kliewer 1971, Bell et al. 1979, Chang and Kliewer 1991, Goldspink and Gordon 1991, Peacock et al. 1991, Spayd et al. 1994, Bell and Robson 1999, Conradie 2001). In summary, the grapevine’s response to a particular treatment or set of imposed conditions and the subsequent effects on grape berry composition is the result of a series of interactions between genetic characteristics, environmental conditions and cultural practices.

The focus of this review, however, is to explore the impact of nitrogen supplementation in the vineyard and/or in the winery on final wine quality, commencing with the effect that nitrogen application in the vineyard has on the composition of the grape berry at harvest (Figure 1). Therefore, discussion of the vegetative and reproductive response of grapevines to nitrogen application in the vineyard will be limited due to the large scope of that topic. Hence, the following section will briefly summarise the major effects that nitrogen application has on vine growth and yield and other factors that explain in part the apparently conflicting reports relating to the influence of nitrogen on grape composition at harvest, as discussed in Section 2.2. The initial nitrogen status of the vine will determine how the grapevine responds to nitrogen application. This will trigger a series of processes that can directly or indirectly affect grape berry composition.

2.1.2 Vine nitrogen status: Deficient to marginal
Adding nitrogen under conditions of low nitrogen nutrition increases the vine nitrogen status, which stimulates nitrogen metabolism and consequently protein synthesis (Perez and Kliewer 1978, 1982, Marschner 1995, Zerihun and Treeby 2002). As a consequence, an increase in leaf area (Kliewer and Cook 1971, Bell and Robson 1999) combined with an increase in chlorophyll formation (Kliewer and Cook 1971) stimulates the production of photosynthates (Marschner 1995). The production of photosynthates may or may not be sufficient to supply all the sinks and/or metabolic pathways that require carbohydrates at this stage. However, an increase in the amount of storage organs, i.e. roots, canes and trunk (Alexander and Woodham 1970, Kliewer and Cook 1971, Obink et al. 1973, Conradie and Saayman 1989b, Delas et al. 1991, Kliewer et al. 1991, Bell and Robson 1999), upon the application of nitrogen increases the capability of the vine to store nitrogen and carbohydrates. Reserves can be remodelled to support sinks and/or metabolic pathways that require more nitrogen or photosynthates than the vine can currently provide. Increased root growth (Alexander and Woodham 1970, Kliewer and Cook 1971, Obink et al. 1973) promotes the uptake of not only nitrogen but water and other essential nutrients. All these effects relating to vine growth have a positive effect on fruit composition when the application of nitrogen augments the limited carbohydrate and nitrogen supplies.

Total yield and many components of yield also increase upon the application of nitrogen when vine nitrogen status is low (Bell et al. 1979, Nielsen et al. 1987, Wolf and Pool 1988, Nielsen et al. 1989, Kliewer et al. 1991, Spayd et al. 1994, Bell and Robson 1999, Conradie 2001). A review by Kliewer and Dokoozlian (2000) indicated that, on average, a leaf area of 8–14 cm² (single canopy) and 5–8 cm² (double canopy) per gram of fruit produced is required to adequately ripen a crop to specification. Bell and Robson (1999) observed that on a single canopy the leaf area to fruit weight ratio of Cabernet Sauvignon vines increased from approximately 7 to 12 upon the application of nitrogen, with a positive effect on fruit composition (Bell 1994). However, it should be noted that the same effect was not observed when an excessive rate of nitrogen was applied.

2.1.3 Vine nitrogen status: Adequate
When the initial nitrogen status of the vine is adequate, further addition of nitrogen does not increase the growth and yield past the maximum value obtained by adding a lower amount of nitrogen (Nielsen et al. 1987, Conradie and Saayman 1989a, Nielsen et al. 1989, Kliewer et al. 1991, Spayd et al. 1994, Bell and Robson 1999). Similarly, an optimal balance between the leaf area to fruit weight ratio will be reached to ensure that optimal fruit ripening will occur for that cultivar within the confines of the characteristics that govern any particular site. Once maximum growth, yield and grape composition are attained the vine is considered to be adequately supplied with nitrogen. However, the rate of nitrogen application in the vineyard that results in maximum yield or growth may not be the rate at which grape composition is optimised. In the current market the focus is on ripening grapes to meet winery specifications, thus it is important that the effects of nitrogen on growth, yield and grape composition be considered together to reach a suitable compromise.

2.1.4 Vine nitrogen status: High
When using nitrogen applications in the vineyard to correct a deficiency or to maintain adequate vine nitrogen status it is important to remember that indiscriminate use of nitrogen has detrimental impacts on grape composition and yield. When vine nitrogen status is high, grape composition is primarily influenced by the consequences of increasing vine growth (e.g. sink-source relationships, canopy microclimate).

High vine nitrogen status may disrupt vine balance, leading to a limited supply of carbohydrates if the vine becomes overcropped or excessively vegetative due to further applications of nitrogen. However, in a number of studies that measured the effect of increasing rates of nitrogen application on both vine growth and yield it appeared that growth was maintained at the expense of yield (Kliewer 1971, Kliewer and Cook 1971, Kliewer 1980, Wolf et al. 1983, Ahmedullah et al. 1987, Wolf and Pool 1988, Tan and Crabtree 1990). The reduction in yield following high rates of nitrogen application in the vineyard can be explained largely by changes in the canopy microclimate resulting in shading in the renewal zone (Kliewer 1980).

If the source size (i.e. leaf area for carbohydrate synthesis) does not increase correspondingly with sink size
Implications of nitrogen nutrition

(i.e. crop and carbohydrate storage tissues), this could increase the competition for carbohydrates between competing sinks, resulting in lower concentrations of constituents associated with grape quality in the berry (Kliewer and Ough 1970). Kliewer (1977a) concluded that high rates of nitrogen application resulted in reduced grape colour and total soluble solid concentration because photosynthates had been diverted away from carbohydrate metabolism to amino acid and protein synthesis and storage. Additionally, changes in the vine microclimate (as discussed below) may exacerbate a source limitation, as light and temperature, in particular, can affect the rate of photosynthesis and hence the amount of photosynthates formed (Kriedemann 1968).

Within the confines of any given trellis system, increasing vine vigour increases canopy density, resulting in an alteration of the canopy microclimate (Smart et al. 1985, Wolf and Pool 1988, Bell and Robson 1999). Increasing canopy density results in a decrease in the ratio of non-shaded to shaded leaves in the canopy (Bell 1994). This has the potential to limit the production of photosynthates because an increasing number of leaves are not conducting photosynthesis at maximum capacity due to mutual leaf shading (Mullins et al. 1992). The end result is a source limitation that promotes competition between sinks and/or metabolic pathways for photosynthates and reduces stored carbohydrate reserves in the permanent parts of the vine (Buttrose 1969, Kliewer et al. 1972, Kliewer 1977a, Braun et al. 1989, Marschner 1995).


A reduction in temperature can result in an increase in titratable acidity and a decrease in pH, which were both largely due to an increase in the concentration of malic acid (Kliewer 1968b, Kliewer and Lider 1968, 1970, Buttrose et al. 1971, Kliewer 1971, Kliewer and Schultz 1973, Hale and Buttrose 1974, Ruffner et al. 1976, Crippen and Morrison 1986). Low temperature can also result in a delay in maturity (Tukey 1957, Kliewer and Lider 1968, 1970, Buttrose et al. 1971, Kliewer 1977a, Crippen and Morrison 1986), but increase the concentration of anthocyanins (Kliewer 1970b, Buttrose et al. 1971, Kliewer and Torres 1972, Kliewer and Schultz 1973, Hale and Buttrose 1974, Kliewer 1977a). The grape composition in a commercial vineyard reflects the effects of shading on both temperature and light. Therefore, for some berry components the impact of a change in microclimate may depend on which microclimate factor(s) is(are) the most dominant.

2.1.5 Other biotic factors

Phytohormones include compounds such as cytokinins and gibberellic acid (Marschner 1995). The synthesis and action of phytohormones in plants are influenced by many environmental factors, such as mineral nutrient supply, particularly nitrogen, as phytohormones play a dominant role in determining the equilibrium between the biosynthesis and breakdown of proteins (Marschner 1995). Phytohormone balance is also influenced by light and temperature regimes (Srinivasan and Mullins 1981), but the relationship between nitrogen nutrition, phytohormone levels and grape composition is not well understood.

Finally, grape berry size, or more accurately the skin to pulp ratio, plays a significant role in determining the concentration of quality components in the grape berry. Most studies have found that nitrogen application increases berry weight (Baldwin 1966, Nijjar and Chand 1969, Kliewer 1971, 1977b, Delas et al. 1991, Kliewer et al. 1991, Spayd et al. 1994, Bell and Robson 1999) or has no effect (Williams 1943, 1946, Morris et al. 1983, Wolf and

In conclusion, nitrogen application can influence grape composition at harvest in many ways. However, within given genetic and site constraints, the processes that drive final grape composition are largely determined initially by the vine nitrogen status at the time of nitrogen application. Therefore, grape quality components at harvest will benefit when vineyard nitrogen is applied (a) to alleviate a nitrogen deficiency when the vine nitrogen status is low to marginal, and (b) as a maintenance application, i.e. in small amounts to balance losses from a site in which the vine nitrogen status is adequate. In contrast, supplying nitrogen to vines with a high nitrogen status should be avoided, since grape quality components could be further devalued.

2.2 Grapes

2.2.1 Effect of nitrogen application on grape berry quality components

Table 1 documents the effect of nitrogen application on grape berry components and the extent of this effect expressed as a percentage of control vines, which received no nitrogen. The final column records the references from which this information was sourced.

The impact of vineyard nitrogen application on grape berry and/or juice composition in Table 1 illustrates that, in many cases, nitrogen did not always have the same effect on any one juice quality component within the confines of one study. This was because grapevine composition is created as a result of a number of interacting factors as Figure 2 schematically illustrates. In many studies, nitrogen is not the only variable. For example, Ough et al. (1968a), Wolf and Pool (1988) and Delas (1993) studied the impact of nitrogen application on *Vitis vinifera* cultivars on a range of differing rootstocks.

The only consistent effect of vineyard nitrogen application on grape berry quality components is an increase in the nitrogenous compounds, such as total free amino acids, arginine, proline, ammonium, and total nitrogen concentration (Table 1). Grape berry nitrogen components increase, but at a declining rate, as the application rate of nitrogen in the vineyard increases (Bell et al. 1979, Bell 1994). The observed decline in the rate of accumulation of grape berry nitrogen components might indicate that the conversion of nitrate to other nitrogenous compounds is limited at high nitrogen application levels. For example, increasing additions of nitrogen increase canopy density (Bell and Robson 1999) and nitrate reductase activity in leaves has been observed to decline in light limiting situations (Perez and Kliewer 1982, Smart et al. 1988). However, it might also indicate a diversion of nitrogen away from the fruit to support increased vegetative growth.

The impact of nitrogen on the sugar concentration in grapes has been inconsistent and a conclusive trend has not emerged. The majority of studies indicate that nitrogen application has either no effect or results in an increase in titratable acidity (summarised in Table 1). The increase in titratable acidity might be attributable to high malic acid concentrations, which rose upon the application of nitrogen in the majority of studies. This increase may be due to augmentation of the carbohydrate and nitrogen supplies required for the formation of acids in the early part of the season and/or reduced losses of malic acid during ripening as a result of lower respiratory demand of berries in shaded canopies (Ruffner 1982). Studies on the impact of nitrogen on both pH and potassium showed conflicting trends, but the majority of studies indicate that these components were not affected. Studies that have evaluated the impact of nitrogen application on the concentration of other nutrients in the berry, such as phosphorus, calcium, magnesium, chloride and sodium, are few in number and were not conclusive. Lastly, there is a distinct lack of information relating to the impact of nitrogen application in the vineyard on the formation of grape secondary metabolites, such as the polyphenols (e.g. anthocyanins and tannins) and the aroma and flavour compounds that are responsible for grape and wine varietal character. Nitrogen addition had an impact on total phenols and anthocyanins, but no clear trend was evident (Table 1). As for the tannins and flavonols, the results are too few to draw any conclusions. There is an important need for further research to elucidate the relationship between these important secondary metabolites and nitrogen application in the vineyard.

In summary, apart from the impact of nitrogen application on the grape berry nitrogen components, the trends for the other grape quality components are not clear cut. The underlying reason for this may be that different genetic, environmental and even cultural practices found in each study result in a variation in the initial vine nitrogen status. The most dominant mechanisms that operate as a consequence of a change in vine nutrient status will determine the grape berry composition in any given study.

2.2.2 Nitrogen evolution during grape berry maturation

Applying nitrogen to vines in the vineyard increases the concentration of nitrogen compounds in grape juices (Table 1), but has no impact on the pattern of evolution of the major nitrogen compounds during ripening (Bell 1994, Hilbert et al. 2003).

Total nitrogen concentration and content of grapes and juice increases during ripening (Figure 3a) (Bell 1994) and similar trends have been observed in other studies (Lafon-Lafourcade and Guimberteau 1962, Ough 1968, Kliewer 1970a, Solari et al. 1988, Kluba et al. 1978, Löhnertz and Schaller 1992, Hilbert et al. 2003). However, total nitrogen levels can plateau after an initial increase, and in some cases may decline towards the end of ripening (Lafon-Lafourcade and Guimberteau 1962, Kliewer 1971, Löhnertz and Schaller 1992, Hilbert et al. 2003).
Table 1. Impact of nitrogen application in the vineyard on composition and concentration of grape berry and/or juice components at harvest.

<table>
<thead>
<tr>
<th>Concentration of grape berry quality components†</th>
<th>Percentage change compared to non-fertilised vines (100%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total soluble solids</td>
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<tr>
<td>(+) 100.9 – 108.2</td>
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<td>2, 8, 24, 26, 31, 32, 44, 45</td>
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<td>(–) 84.7 – 99.4</td>
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<tr>
<td>Glucose and/or fructose</td>
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<tr>
<td>(+) 107</td>
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<tr>
<td>(–) 97.5</td>
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<tr>
<td>Titratable acidity</td>
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<td>Malate and/or malic acid</td>
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<td>(–) 84.4 – 89.5</td>
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<td>Tartrate and/or tartaric acid</td>
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continued
## Table 1. continued

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<tr>
<td>Tannin</td>
<td></td>
<td>5, 40</td>
</tr>
<tr>
<td>Flavonols</td>
<td></td>
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</tr>
<tr>
<td>Thiol Precursors (4MMP, 4MMPOH, 3MH)</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

†Upon the application of nitrogen, concentration of the grape berry quality compound increased (+), decreased (−) or did not change (0) per berry.

### References

The total amino acid grape and juice concentration increases from veraison to harvest (Figure 3b, Kliewer 1968a, Kliewer 1970a, Kluba et al. 1978, Solari et al. 1988, Hernández-Orte et al. 1999, Hilbert et al. 2003). However, in some instances the total amino acid concentration of juices reached a peak prior to harvest, after which it stabilised and/or proceeded to slowly decline until harvest (Kliewer 1968a, Solari et al. 1988, Hernández-Orte et al. 1999). In contrast, the ammonium concentration of grape berries declines during ripening (Figure 3c), an effect also observed by others (Lafon-Lafourcade and Guimberteau 1962, Kliewer 1968a, 1971, Ough and Stashak 1974, Hernández-Orte et al. 1999, Stines et al. 2000). The rate of increase is most dramatic in cultivars such as Cabernet Sauvignon, which are high proline accumulators (Ough and Stashak 1974, Stines et al. 2000). However, as was observed for total amino acids, there were some instances in which the proline concentration of juices reached a peak prior to harvest, after which it stabilised and/or proceeded to slowly decline until harvest (Lafon-Lafourcade and Guimberteau 1962, Ough and Stashak 1974, Hernández-Orte et al. 1999).

Arginine concentration in Cabernet Sauvignon berries has been shown to increase from veraison, but quickly reached a maximum and then began a slow decline as ripening continued to progress (Figure 3d). A similar trend was also observed by others (Lafon-Lafourcade and Guimberteau 1962, Kliewer, 1968a, Hernández-Orte et al. 1999, Stines et al. 2000). In some instances however, arginine continued to increase until harvest (Kliewer, 1968a, 1971, Kluba et al. 1978, Hernández-Orte et al. 1999, Stines et al. 2000, Hilbert et al. 2003). Interestingly, Stines et al. (2000) observed that the arginine concentration in berries did increase right up until harvest for the high arginine-accumulating cultivars, Gewürztraminer and Muscat Gordo. However, in contrast, arginine concentration in berries from Cabernet Sauvignon and Chardonnay, which are high proline-accumulating cultivars, generally stabilised and/or declined during the ripening period, having peaked pre-veraison (Stines et al. 2000). The reduction in berry arginine concentration towards the end of ripening could indicate the remobilisation of nitrogen to the storage organs (i.e. roots) in preparation for the following season. Nitrogen is stored primarily in the form of arginine. Alternatively, arginine could be converted to proline, which accumulates predominantly in the latter stages of ripening.

In conclusion, it appears that, with the exception of ammonium, all major forms of berry nitrogen reach a point, whether it is prior to, during or at the end of ripening, when a maximum concentration is achieved. It is only the pattern of evolution later in ripening that seems to be variable and is undoubtedly influenced by a combi-
nation of genetics, cultural practices and environmental conditions. Lasty, it must be reiterated that nitrogen application in the vineyard should only be considered when all other factors, such as vine vigour, canopy density, leaf colour and petiole nitrogen analysis, indicate a need for nitrogen. If a need is not indicated, it is likely that the benefits of good soil fertility are already being realised, and hence nitrogen application could introduce serious negative effects, such as excessive vegetative development and increased potential for fungal infection.

2.2.3 Components of YAN

Many independent studies have shown that the application of nitrogen to grapevines increases berry nitrogen concentrations, and those same effects are reflected in the resulting musts and wines (Ough et al. 1968b, Bell et al. 1979, Ough and Bell 1980, Bertrand et al. 1991, Dukes et al. 1991, Maigre 2002). Various grape components, which contribute to wine flavour and aroma, are also affected by nitrogen application, both directly and indirectly, as indicated in Table 1 and Figure 2. Therefore, changes in must nitrogen composition and concentration can potentially impact positively and negatively on the composition of the resulting wine. Many of these effects are mediated through yeast metabolism during fermentation.

In order to understand how grape berry nitrogen composition and concentration affects yeast growth and metabolism, and the transformation of grape-derived flavour precursor compounds, it is necessary to know what nitrogen components of the berry are important. Although grape must is relatively abundant in the content of nutrients required by yeast, the nitrogen component has been found to be highly variable, not only in concentration, but also in the types of nitrogen compounds present (Henschke and Jiranek 1993). Only some of the main nitrogen compounds of grape are important to yeast.

Saccharomyces cerevisiae, the principal yeast used for fermentation, preferentially uses simple nitrogen sources such as ammonium ions and free alpha amino nitrogen compounds, present in the form of primary amino acids (Cooper 1982, Monteiro and Bisson 1991b, Henschke and Jiranek 1993, Jiranek et al. 1995a). However, the secondary amino acids, such as proline and hydroxyproline are not metabolised to any great extent under usual winemaking conditions (Duteurtre et al. 1971, Ingledew et al. 1987). Low, but not high, molecular weight peptides can also be used but grape proteins cannot be used as a source of nitrogen since Saccharomyces cerevisiae lacks significant extracellular proteolytic activity. Therefore, the usable nitrogen fraction is often referred to as yeast-assimilable nitrogen (YAN). The remaining component of total nitrogen, which includes proline and hydroxyproline, larger molecular weight peptides and protein, will be referred to as yeast-non-assimilable nitrogen (YNAN). Depending on the analytical method, nitrate can also contribute to YNAN, since Saccharomyces cerevisiae lacks the ability to assimilate nitrate.

The analytical measure of yeast-assimilable nitrogen has become vitally important for managing fermentation, since plant tissue analysis of nitrogen is not a reliable predictor of grape berry YAN at harvest. Grape berry YAN is the primary determinant of potential must YAN composition and concentration (Figure 4). However, losses of YAN due to harvesting and transport conditions must also be considered. On reaching the weighbridge factors such as processing choice (influencing YAN extraction), treatment of the existing yeast population and supplementation of YAN in the winery all play a role in determining the final must YAN composition and concentration. This Section (2.2.3) and Sections 2.2.4 and 2.4.1 will focus on the major factors that affect potential must YAN composition and concentration, with the exception of YAN supplements in the winery, which will be covered in a later section (Section 3.1.2) of this review.

2.2.3.1 Ammonium

Ammonium is an important component of must YAN and, being one of the most preferred nitrogen source by yeast, it is readily assimilated (Cooper 1982, Bisson 1991, Henschke and Jiranek 1993, Jiranek et al. 1995a). The concentration of ammonium varies widely in grapes and has been reported to range between 5–325 mg N/L (Ough 1969, Ough and Kriel 1985, Bely et al. 1991, Henschke and Jiranek 1993, Butzke 1998).

Ammonium accounted on average for 40% (Bell 1994), 28% (Spayd et al. 1994) and 9% (Conradie 2001) of the YAN in juices at harvest sourced from vines that had received nitrogen additions in the vineyard. Similarly, ammonium accounted for 2–53% of the YAN component in juices prepared from berries obtained from a range of grape cultivars grown at the same location (Huang and Ough 1989). In Cabernet Sauvignon juice, ammonia accounted for 53% of the total YAN in comparison to 32% of the total YAN in the juice of Gewürztraminer berries (Huang and Ough 1989). Cabernet Sauvignon is a high proline accumulator relative to arginine and Gewürztraminer is a high arginine accumulator relative to proline (Kliewer 1970a). Therefore, the assimilable amino acid nitrogen contribution to YAN is lower in a high proline accumulator like Cabernet Sauvignon, resulting in a greater contribution by ammonia to YAN compared to a cultivar that accumulates more arginine relative to proline, like Gewürztraminer.

![Figure 4](image-url)
Nitrogen application to vines increases the concentration of ammonium in the juice of berries compared with ammonium levels in juices from vines receiving no nitrogen (Table 1). However, the impact of vineyard nitrogen on ammonium as a percentage of YAN at harvest is variable. In two of three studies, nitrogen application had very little effect on the ammonium concentration as a percentage of YAN rose by 10% for vines supplied with 112 kg N/ha compared to juices from vines that received no nitrogen (Spayd et al. 1994). Ammonium as a percentage of YAN declined during ripening (Bell 1994), but this was not surprising, as the ammonium concentration in grapes declined between veraison and harvest as previously discussed. This trend was unaffected by the application of nitrogen.

However, while the berry is the major source of ammonium before the fruit enters the winery, the amount of ammonium in must is routinely manipulated in the winery by the addition of ammonium salts, especially diammonium phosphate (DAP), when a nitrogen deficiency is suspected or known (Agenbach 1977, Vos et al. 1980, Monk 1982, Bisson 1991, Kunkee 1991, Henschke and Jiranek 1991, 1993). DAP is added to ensure adequate development of the yeast population and fermentation to the required level of residual sugar or dryness in a timely fashion, whilst inhibiting the formation of undesirable characters, such as hydrogen sulfide (H₂S) (refer to Sections 3.1 and 3.3). DAP increases the total YAN concentration by increasing the concentration of ammonium ions only. A balanced must, however, contains both amino acids and ammonium, as the amino acids play an important role in the formation of a number of the fermentation bouquet products which are by products of the fermentation process, as discussed in Section 3.3.

### Table 2

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration range reported in the literature (mg/L)</th>
<th>References</th>
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</thead>
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<tr>
<td>Alanine</td>
<td>10 – 227</td>
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<td>Arginine</td>
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<tr>
<td>Asparagine</td>
<td>1 – 171</td>
<td>3, 5, 6, 9, 11</td>
</tr>
<tr>
<td>Aspartic acid</td>
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</tr>
<tr>
<td>Citrulline</td>
<td>0.1 – 83</td>
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<tr>
<td>Cysteine</td>
<td>1 – 8.2</td>
<td>6, 10</td>
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<tr>
<td>Glutamine</td>
<td>9 – 449</td>
<td>1, 2, 3, 5, 8, 10, 11</td>
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<tr>
<td>Glutamic acid</td>
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<td>Glycine</td>
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<td>Histidine</td>
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<tr>
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<td>Ornithine</td>
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<tr>
<td>Phenylalanine</td>
<td>2.8 – 138</td>
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<td>Tryptophan</td>
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<td>Tyrosine</td>
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<tr>
<td>Valine</td>
<td>7 – 116</td>
<td>1, 2, 3, 4, 5, 6, 8, 9, 10</td>
</tr>
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</table>

centration as a percentage of YAN. In contrast, Bell (1994) found that the assimilable amino acid concentration as a percentage of YAN generally increased upon the application of nitrogen at two out of three sites, but this effect was not significant. In a different study, however, the assimilable amino acid concentration as a percentage of YAN in juices from vines supplied with 112 kg N/ha declined by 10% compared to juices from vines that received no nitrogen (Spayd et al. 1994).

L-arginine and L-proline generally make up the greatest proportion of the total amino acid concentration present in the grape (Kliewer 1970a, Ough and Bell 1980, Bell 1994, Stines et al. 2000). Arginine is an important nitrogen source for yeast, but its accumulation by yeast is regulated by the presence of more preferred nitrogen sources, such as ammonium (Middelhoven 1964, Grenson and Hou 1972, Monteiro and Bisson 1992a, Henschke and Jiranek 1993, Jiranek et al. 1995a, Salmon et al. 1998). DAP addition, therefore, inhibits arginine utilisation until the ammonium has been metabolised. Arginine serves as a nitrogen source for yeast due to its catabolism by arginase to form L-ornithine and urea, which under appropriate conditions ultimately form glutamate and ammonium, respectively: the latter two compounds are precursors for general amino acid biosynthesis in yeast (Large 1986). The excretion of urea, which results from arginine metabolism, by some strains has implications for ethyl carbamate formation in wine (Henschke and Ough 1991, Monteiro and Bisson 1991b, Ough et al. 1991), as discussed in Section 3.5. Arginine can also be directly incorporated into protein and a large proportion of arginine is translocated to the yeast vacuole as a nitrogen reserve (Watson 1976). L-Proline, however, is only utilised to a limited extent by yeast in the normal anaerobic environment of alcoholic fermentation due to the need for oxygen equivalents by proline oxidase, which catalyses the first step in its catabolism (Duteurtre et al. 1971). Proline typically accumulates in high arginine fermentations due to a block in glutamate formation from ornithine, which, in turn, results from arginine catabolism (Cooper 1982). The nitrogen of the remaining amino acids can be assimilated from grape juice, however there is evidence for selection based on the rates of their removal, at least when nitrogen concentrations are high (reviewed by Henschke and Jiranek 1993, Section 3.1.1).

2.2.3.3 Proline to arginine ratio
The differential accumulation of proline and arginine by different grape cultivars provides a characteristic index based on the ratio of the two amino acids (proline and arginine). This index, which reflects the proportion of non-assimilable (proline) to assimilable nitrogen (arginine), provides a useful indication of the likely nutritional value of the grape must of a particular cultivar to yeast.

Nitrogen application to vines invariably increased both arginine and proline concentrations in grapes (Table 1). Grape cultivars can be classified into categories based on their ability to accumulate one of these forms to a greater degree than the other. Two cultivars might have the same total amino acid concentration, but the variety that accumulates a larger concentration of proline relative to arginine will have a lower YAN. Thus, for example, Chardonnay, Cabernet Sauvignon and Semillon have a high proline to arginine ratio, whereas Grenache, Chenin Blanc, Pinot Noir, Gewürztraminer and Muscat Gordo have a low ratio (Kliewer 1970a, Kliewer 1977b, Stines et al. 2000). This ratio is, however, influenced by the level of grape maturity for some cultivars (Kliewer 1970a). For example, Grenache at an early stage of maturity (19.7º Brix) was classified as a high arginine accumulator, but by 24º Brix it accumulated predominantly more proline than arginine (Kliewer 1970a). In contrast, harvest date had little effect on the proline to arginine ratio of a high proline accumulator like Cabernet Sauvignon (Bell 1994).

Nitrogen application in the vineyard has a distinct effect on the proline to arginine ratio in the grape berry and thus on the final juice YAN. In the majority of studies increasing rates of nitrogen application in the vineyard reduce the proline to arginine ratio in berries from vines that are nitrogen-deficient or adequately supplied with nitrogen (Kliewer 1971, Kliewer 1977a, Bell et al. 1979, Ough and Bell 1980, Delas 1993, Bell 1994, Spayd et al. 1994, Conradi 2001, Rodriguez-Lovelle and Gaudillère 2002). In two studies, however, high rates of nitrogen application appeared to give no added benefit over moderate applications on the proline to arginine ratio (Bell et al. 1979, Ough and Bell 1980). Decreases in the juice proline to arginine ratio, upon the application of nitrogen in the vineyard, were observed for high proline- as well as high arginine-accumulating cultivars (Figure 5). Thus, for high proline-accumulating cultivars the proline to arginine ratio was always observed to be greater than one for the control vines (Delas 1993, Bell 1994, Rodriguez-Lovelle and Gaudillère 2002). However, the application of nitrogen in the vineyard increased the berry juice arginine concentration such that in some cases the proline to arginine ratio was less than one, whereas in other cases it was greater than one (Figure 5). The proline to arginine ratio value of the control vines did not appear to have a major impact on the degree to which the ratio would fall upon the application of nitrogen.

In contrast to high proline accumulators, the juice arginine concentration from high arginine-accumulating cultivars was always greater than the proline concentration in the juices from control vines, and hence the proline to arginine ratio was less than one (Kliewer 1971, Ough and Bell 1980, Kliewer 1977a, Bell et al. 1979, Spayd et al. 1994, Conradi 2001). The application of nitrogen in these studies had a consistent result, that is, the proline to arginine ratio always remained lower than one (Figure 5).

In conclusion, it appears that the application of nitrogen to vines has a positive impact on grape YAN, and hence potential must YAN, by increasing the contribution that readily assimilable arginine makes towards the total amino acid concentration.

2.2.3.4 YAN:YNAN ratio
The yeast-assimilable nitrogen (YAN) to yeast-non-assimilable nitrogen (YNAN) ratio has similar significance to the
proline to arginine ratio with respect to yeast nutrition (bearing in mind the ratios are the inverse of the other). But in addition, the YAN:YNAN ratio considers the contribution of ammonium and the remaining primary amino acids as a contributor to YAN. Therefore, the YAN:YNAN ratio will usually provide a better index of the nutritional value of the berry or juice for yeast growth during fermentation.

The proportion of YAN and YNAN as a percentage of total nitrogen changes during ripening and is influenced by nitrogen application in the vineyard and subsequent vine nitrogen status (Bell 1994, Spayd et al. 1994, Conradie 2001). Only a few of the many nitrogen studies measure and/or report all of the individual nitrogen components required to calculate YAN and YNAN. Given that vineyard nitrogen application increases all forms of juice nitrogen (Table 1), it is not surprising that the YAN and YNAN concentrations also rise (Bell 1994, Spayd et al. 1994, Conradie 2001). At a nitrogen-deficient site, the application of nitrogen increased YAN as a percentage of Cabernet Sauvignon juice total nitrogen and either had no effect or reduced the percentage of YNAN (Bell 1994, Spayd et al. 1994). As a consequence, the YAN:YNAN ratio increased in response to the application of nitrogen to nitrogen deficient vines. Treeby et al. (1996) observed a similar trend in Shiraz juices. However, in this study YAN was calculated as the total amino acid concentration minus proline, and YNAN was expressed as the proline concentration.

Conradie (2001) examined the response of two Vitis vinifera cultivars (Bukettraube and Heroldrebe) to the application of 50 kg N/ha at three different times during the season and found that the impact of nitrogen on the YAN:YNAN ratio varied with cultivar. With the exception of only one application, the less vigorous cultivar, Heroldrebe, exhibited the expected trend upon the application of nitrogen: that is, an increase in YAN as a percentage of total juice nitrogen and a decrease in the percentage of YNAN resulting in an increase in the YAN:YNAN ratio. However, adding nitrogen to the more vigorous Bukettraube vines resulted in a decline in juice YAN as a percentage of total nitrogen and an increase in the YNAN percentage. As a consequence, nitrogen reduced the YAN:YNAN ratio. The change in vine nutrient status upon the application of nitrogen to nitrogen deficient vines does not account for the difference, as the percentage of total nitrogen in blades and petioles was similar for both cultivars. On closer inspection the application of nitrogen more than doubled the Bukettraube juice YNAN component, whereas YAN rose by only 14%. The result was a lower juice YAN:YNAN ratio from those vines that received nitrogen compared to vines that received no nitrogen supplements. When nitrogen was supplied to the Bukettraube vines, proline as a percentage of YNAN declined and therefore it appears that there was a substantial increase in other YNAN nitrogenous compounds, such as proteins. Pocock et al. (2000) observed that the rate at which pathogenesis-related (PR) proteins, which contribute to haze formation in wine, accumulate during ripening varies considerably with cultivar. The detrimental impact of PR proteins on potential wine quality is discussed in Section 3.8.

Lastly, at a site where vine nitrogen status was adequate, the application of nitrogen increased the YAN and YNAN concentration (Bell 1994). However, YAN and YNAN as a percentage of juice total nitrogen and the YAN:YNAN ratio were unaffected by the application of nitrogen. Despite the anomaly in Conradie’s (2001) study, it appears that when vine nitrogen status is low, nitrogen addition may benefit potential must YAN by increasing the YAN:YNAN ratio of grapes entering the winery.
2.2.4 Location of nitrogen within the berry

Because must is prepared from different components of the grape berry for making different types of wine, it is important to consider how the different forms of nitrogen are distributed within the berry. This has particular relevance for YAN when taking into account the general differences in red and white winemaking techniques. For white winemaking, the pulp is the principal source of must components except when skin contact is used, whereas for red wine production the skins and seeds are included with the pulp.

In a study by Ough (1968), approximately 36–65% of the total nitrogen concentration (weight/weight or weight/volume) was located in the combined skin and seed sample. Bell et al. (unpublished data) found that the seed and skins of Merlot berries contained on average 83% of the total grape berry nitrogen concentration. However, on a content basis (mg per berry part), 80% of the total grape nitrogen was located in the pulp and skin. Nitrogen application increased the nitrogen content and concentration in all berry parts, but it was the nitrogen levels in the skin and pulp that were most responsive to nitrogen application in the vineyard (Bell et al. unpublished data). As a large proportion of the nitrogen is located in the skins and seeds, prolonged juice-skin contact before must pressing, prefermentation maceration and fermentation on skins might ensure that a higher nitrogen concentration is available to yeast (Ribéreau-Gayon 2000a) than that indicated by the nitrogen measurement that is taken on the free run juice, prepared either in the winery or the laboratory. However, it is important to note that a high total nitrogen concentration in the berry does not always ensure a high YAN concentration in the juice. This concept is illustrated later in this section.

Eighty-nine to ninety-two per cent of the total amino acid concentration was found in the skin and pulp of Riesling and Cabernet Sauvignon berries (Miele et al. 2000, Stines et al. 2000). However, the percentage in each berry part differed between sites. A French study found that 40% of the total amino acids were in the pulp and 53% in the skin of Cabernet Sauvignon berries (Miele et al. 2000). In contrast, an Australian study using the same cultivar showed that 77% of the total amino acid concentration was in the pulp and only 15% in the skins (Stines et al. 2000). The seeds contributed little to the total amino acid concentration. However, the percentage of total amino acids in the grape seed was more consistent, averaging 7.8% and 8.5% in the French and Australian study respectively (Miele et al. 2000, Stines et al. 2000). Although the bulk of the amino acids are found in the pulp and skins, the assumption is made that cultural practices and site-specific characteristics can modify the percentage of amino acids in each berry part. Presumably, such cultural practices include nitrogen application in the vineyard.

As previously discussed, the skin contains significant amounts of amino acids. Therefore, when skins are included in the must they can make a significant contribution to the yeast-assimilable amino acid nitrogen (AA-N) concentration of the ferment. In a study by Stines et al. (2000) grape berries were separated into seeds, skins and pulp and the AA-N concentration in each part was measured (Figure 6). Stines et al. (2000) observed that the skins accounted for approximately 19–29% of the yeast-assimilable AA-N concentration because they contained 15–23% of the total amino acid concentration in combination with a higher arginine to proline ratio than the pulp. Therefore, it might be expected that the contribution to YAN by the skins would be greater for a high arginine-accumulating cultivar. However, it appears that this is not always the case. For example, Miele et al. (2000) observed that the skin AA-N concentration in a high proline accumulator like Cabernet Sauvignon can still have a major influence on YAN in certain situations. These researchers showed that Cabernet Sauvignon skins accounted for 53% of the total amino acid concentration and consequently accounted for 50% of the final yeast-assimilable AA-N concentration. Of course must preparation and winemaking techniques during alcoholic fermentation play an important role in determining just how much of the total YAN will be liberated into the juice fraction. For example, the yeast-assimilable AA-N concentration might increase in the must if the fermentation time on skins is extended. It is well known that the rate of fermentation can benefit from the addition of ammonium and most amino acids throughout fermentation, and especially during the late stages (Bely et al. 1990a, Manginot et al. 1997, Blateyron et al. 2003). The delayed release of nitrogen during fermentation on skins or pulp, especially of incompletely crushed berries might, therefore, partly account for the lower incidence of fermentation problems observed with white or red wine fermentations that have high solids content (Henschke 1997a). Increased maceration during fermentation may also facilitate the liberation of YAN, as indeed it does for phenolics. This factor should be taken into account when considering DAP additions in the winery.

The cultivar will also have a major influence on the final YAN concentration of a must compared with free run juice. Stines et al. (2000) showed that the total amino acid concentration (includes proline) in the pulp of Cabernet Sauvignon berries was 1.5 times greater than that in the pulp of Riesling berries, despite Cabernet Sauvignon and
Riesling having approximately equal yeast-assimilable AA-N concentrations. Ough (1968) reported that the juice fraction contains the major amount of proline (50–80%), which later studies confirmed (Miele et al. 2000, Stines et al. 2000). The proline to arginine ratio of the Cabernet Sauvignon berries was approximately four times greater than the proline to arginine ratio of the Riesling berries at the same level of maturity. Therefore, Cabernet Sauvignon might have a higher total amino acid concentration, but the proportion of this component in the non-assimilable form is greater than for Riesling. This serves to illustrate that a high total amino acid concentration does not always result in a high yeast-assimilable AA-N concentration, especially if the cultivar is a high proline accumulator. When considering the nature of the cultivar in terms of its predominance towards arginine or proline accumulation, the stage of maturity must be considered. Stines et al. (2000) showed that Riesling accumulated approximately equal amounts of arginine and proline at 20.4º Brix. Kliewer (1970a) observed the same trend for White Riesling at 21.2º Brix. However, at a later stage of maturity (24.9º Brix), White Riesling had accumulated 3.8 times more proline than arginine, thus ensuring that a higher proportion of the total amino acid concentration is in a non-assimilable form compared to the earlier stage of maturity (Kliewer 1970a).

In conclusion, it is generally observed that nitrogen application in the vineyard, at least in nitrogen-responsive sites, has a positive effect on the final yeast-assimilable AA-N concentration in each berry part. However, it is important to remember that processing techniques play an important role in determining how much of the berry YAN will be present in the must and available to yeast. The skins in particular can contribute significantly to total available YAN, and therefore skin contact time should be considered when making decisions concerning DAP use in relation to must preparation and fermentation technique, bearing in mind that skin contact has other important winemaking consequences other than affecting YAN concentration.

2.4 Must
2.4.1 Effect of post-harvest processes on grape nitrogen compounds
From the moment of harvest, some grape berry constituents can be subject to degradation by chemical, physical or biological processes, which can potentially impact on the composition and quality of the resultant wine. Hand harvesting is considered the least invasive method, since the grape berry can remain intact during harvest and transport to the winery. On the other hand, mechanical harvesting can damage the structure of the berry and consequently activate a variety of degradative processes, such as oxidation and microbial metabolism. These processes can continue until some preventative action is taken either in the vineyard, during transport or in the winery (Figure 4).

The activity of polyphenol oxidases, which modify grape phenolics causing browning, is an especially well known reaction that occurs following fruit damage. Recently, the impact of mechanical harvesting on the release of haze-forming proteins has also been reported (Pocock and Waters, 1998, Pocock et al. 1998). However, the degradative role of microorganisms associated with the grapes and the harvesting equipment is less well appreciated, but can under certain circumstances lead to serious losses of grape berry nutrients, which are necessary for the proper growth and metabolism of the fermentative yeasts and other microorganisms used in secondary fermentations. One study suggests that wild yeast growth can outcompete the inoculated strains (Petering et al. 1993), whereas another suggested that fermentation kinetics can be altered, with the increased risk of stuck fermentation (Bataillon et al. 1996). Depending on grape berry temperature at harvest, any associated microorganisms can become activated and after a lag phase may commence accumulation of nutrients, including nitrogen compounds. The time and temperature of contact between microflora and must, and the numbers and types of microorganisms, will affect the rate of removal of nutrients. Mechanical harvesting carried out when ambient temperatures are low, the use of must chilling and the application of inhibitory chemicals, such as sulfites, can reduce both oxidative and microbial activities.

Direct study of the depletion of YAN in musts does not appear to have been reported, but the depletion of a critical yeast vitamin, thiamin, has been demonstrated (Bataillon et al. 1996) and may provide a useful marker for this process. Kloeckera species, which often quantitatively dominate must microflora (10³–10⁶ cells/mL) can accumulate thiamin up to one-tenth of their dry cell weight. Amino acids are present at greater concentrations than vitamins, but nonetheless for low nitrogen musts handled under conditions that favour microbial activity significant depletions could occur. The implications of failing to use sulfur dioxide as an antimicrobial agent during simulated must processing conditions highlights the activity of wild yeasts, even at temperatures considered by many to essentially prevent yeast growth (Petering et al. 1993). Storage of a mechanically harvested must, which contained 5 × 10⁵ cells/mL of wild yeast, at 10ºC in the absence of added sulfur dioxide for a period exceeding 30 hours compromised the ability of the inoculated yeast to dominate the microflora. Treatment of the must with sulfur dioxide at 100 mg/L suppressed the wild yeast for a period exceeding 50 hours.

In conclusion, the ineffective use of chemical and physical inhibitors at any stage after mechanical harvesting and inoculation can lead to depletion of important yeast growth factors and also increase the risk of ineffective inoculation, both of which can compromise subsequent wine composition and sensory properties (Figure 4). Given the transport of considerable quantities of must, often over large distances, more research is needed to establish effective procedures for conserving must quality.

2.4.2 Measurement of YAN
At this point it is important to reiterate the importance of measuring grape or must YAN prior to fermentation. Petiole nitrogen analysis, which is typically made at flow-
ering, is a useful tool for monitoring the nitrogen status of the grapevine, as long as visual assessment and vine performance is also taken into account. However, it does not necessarily give a reliable measure of the nitrogen concentration of the berry at harvest, which is important for fermentation. In addition, nitrogen depletion due to grape harvesting and must preparation factors, as summarised in Figure 4, further erodes its value. The various methods proposed for measuring YAN are summarised in this section.

Timing of sampling and sample preparation are important considerations for reliable YAN determination. In the previous section it was shown that the main nitrogen components of YAN, arginine and ammonium ions, are in significant flux. Therefore, samples should be taken as close as possible to the harvest date, typically within one to two weeks under cool to moderate conditions, but less under hot dry conditions during which berry moisture and nutrient levels can change more quickly. For cultivars from which clarified juices without skin contact will be used, a freshly prepared, bright juice sample will be suitable. For ferments requiring skin contact, it is desirable to measure YAN in the skin as well as the pulp (Stines et al. 2000); seeds should be omitted since little assimilable nitrogen is believed to be released during fermentation.

Due to the chemical diversity of the grape berry nitrogen compounds that can be assimilated by yeast, including primary amino acids, ammonium, amines, amides, purines, pyrimidines, vitamins and peptides, measurement of YAN has presented a challenge. Since the primary amino acids and ammonium quantitatively represent the major proportion of nitrogen assimilated, most methods quantify these compounds. Quantification of free amino acids by HPLC methods combined with the ammonium ion determination provides the most accurate measure, which is suitable for research studies but is not suitable for routine use. Chemical methods, which rely on a reaction with free alpha amino nitrogen are convenient for winery use. Several reagents/methods have been proposed: ninhydrin (Lie 1973, Nicolini et al. 2004), 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Crowell et al. 1985, Zoecklein et al. 1999), o-phenthaldehyde/N-acetyl-L-cysteine (NOPA) reagent (Dukes and Butzke 1998, Gump et al. 2002) and formol titration (Shively and Henick-Kling 2001, Gump et al. 2002). The latter two are most commonly used for routine analysis. The noxious nature of the formol reagent restricts the latter method to experienced analysts working in well-equipped laboratories. Ammonium can be quantified by enzymatic (Boehringer Mannheim kit) or chemical methods, or by an ammonia or ammonium ion selective electrode (McWilliam and Ough, 1974, Hebbard et al. 1993, Zoecklein et al. 1999, Turbow et al. 2002).

Several biological assays for measuring YAN have been proposed. Monteiro and Bisson (1991a) devised a yeast bioassay in which biomass yield was related to the YAN content of the juice sample. Another method has been designed around an apparatus for automatically monitoring fermentation by weight loss due to CO2 evolution (Bely et al. 1990a,b). Juice YAN over a wide concentration range (53–411 mg/L) correlated with maximum CO2 production rate (r = 0.965) and total fermentation time (r = 0.931). Blateyron and Sablayrolles (2001) determined YAN as the difference between total nitrogen, measured by the Kjeldahl method (Sheiner 1976), before fermentation and at the 80% progress point of fermentation when residual nitrogen is at its lowest concentration. Although these bioassays are useful research tools, their complex nature precludes routine application.

Spectroscopy combined with chemometrics shows potential for rapid and convenient analysis, with on-line capability. Generally, minimal sample preparation is required. Evaluation of the near infrared region (NIR; 700–2500 nm) of the electromagnetic spectrum showed a lack of sensitivity in measuring the YAN components such as ammonia nitrogen and alpha-amino nitrogen. However, the mid-infrared region (MIR; 970–3000 nm) in transmission mode gave greater accuracy (R2 = 0.94), thus showing more promise for routine YAN analysis (Dambergs et al. 2005). Until a spectroscopy method becomes generally available, the NOPA plus ammonium or formol titration methods provide useful alternatives.

### 3.0 Must to wine

The process of transforming grape must into wine by yeast in the absence of air (oxygen) is known as fermentation. In essence, a high concentration of sugar (glucose and fructose) is converted by a series of metabolic steps (glycolysis-alcoholic fermentation) to carbon dioxide (CO2) and a high concentration of ethanol, with a variety of by-products formed in relatively low concentrations. Fermentation is conducted principally by Saccharomyces yeast, and mainly cerevisiae strains (Fleet and Heard 1993, Henschke 1997b, Lambrechts and Pretorius 2000). These yeast are either naturally present on the grape berry biofilm or on the harvest/winery processing equipment, or added as a starter culture. As the initial population of yeast (1–5 × 1010 cells per mL) inoculated into grape must is typically not sufficient to carry out fermentation, they must multiply before an adequate rate of fermentation can be achieved. Yeast growth is, however, dependent upon the availability of a suitable source of nutrients in addition to sugar, especially nitrogen (Henschke and Jiranek 1993, Albers et al. 1998, Magasanik and Kaiser 2002). The latter nutrient is also an important fermentation activator, since upon its depletion the rate of fermentation can substantially diminish and even cease (Lagunas 1982, Salmon 1989, Manginot et al. 1997). Grapes typically provide all the essential nutrients for yeast, and represent the principal source of endogenous nitrogen, unless nitrogen supplements are added by the winemaker. The decision whether to supplement juice or must should be made on the basis of a prefermentation YAN measurement, fermentation conditions (i.e. yeast strain, temperature, aeration) and wine style (i.e. clean or ‘funky’) (Henschke 1997a). A neural network approach, which can take into account a variety of factors, could be used in the future (Insa et al. 1995).

In addition to the production of alcohol, fermentation leads to a large increase in the abundance of flavour-
active compounds (aroma, flavour and mouth-feel) (Rapp and Versini 1991, Jackson 2000, Lambrechts and Pretorius 2000, Guth and Sies 2002, Cole and Noble 2003, Swiegers et al. 2005) (Figure 7). Some are derived directly from the must with little or no modification, whereas others arise from the metabolism of compounds present in grapes, especially sugars and nitrogen compounds. The volatile compounds, which form part of the aroma profile of wine, constitute the ‘fermentation bouquet’, whereas non-volatile compounds, such as polyols and carboxylic acids, affect the flavour. In addition, yeast interact with a variety of grape secondary metabolites, which have considerable sensory importance. The hydrolysis of glyco-conjugates and cysteine-conjugates can contribute to the varietal character of wine and modification of various grape phenolic compounds can influence wine colour and mouth-feel (Strauss et al. 1986, Francis et al. 1992, 1996a,b, Selton et al. 1993, Rapp and Versini 1996, Selton 1998, Cole and Noble 2003, Francis and Newton 2005, Swiegers et al. 2005, Herderich and Smith 2005).

The nitrogen status of vines can also affect the forms and concentration of must nitrogen compounds that can influence the following: the residual nitrogen content of wine, which is important for secondary fermentations (tirage and malolactic fermentation) and can influence wine microbial stability; the accumulation of urea, a precursor of the carcinogen ethyl carbamate; the production of biogenic amines; and possibly the atypical ageing flavour. Furthermore, the nitrogen status of vines can affect susceptibility to some fungal infections and can modify the protein content of berries, and consequently, wine physical stability.

This section will review the scientific literature on the influence of vineyard and must nitrogen status on the formation of sensorially important precursor compounds in the grape berry, fermentation processes and the production and liberation of compounds that affect wine sensory attributes and stability, as well as compounds (ethyl carbamate and biogenic amines) that are regulated by wine-law.

Figure 7. A diagrammatic representation of the biotransformation of grape must compounds to wine compounds by fermentation with yeast. Grape must can be considered to contain three functional groups of compounds, ‘precursor flavour-active compounds’, ‘non-precursor flavour-active compounds’ and ‘nutrients’, which, after fermentation, contribute to the ‘appearance’, ‘fermentation bouquet’, ‘varietal character’ and ‘mouth-feel’ of wine. Biotransformation of nutrients by yeast: in addition to the major pathway of sugar fermentation to ethanol and CO\(_2\), the metabolism of sugar, nitrogen (amino acids and ammonium) and other nutrients produces volatiles (esters, higher alcohols, aldehydes and ketones, volatile fatty acids and thiols (hydrogen sulfide and mercaptans)) which contribute to the ‘fermentation bouquet’ or aroma of wine. Yeast polyols, carboxylic acids and polymers contribute to wine flavour. Non-precursor flavour-active compounds: some grape-derived compounds (some carboxylic acids, phenolics and aroma volatiles such as methoxyypyrazines) are not modified during fermentation. Biotransformation of precursor flavour-active compounds: these compounds are either hydrolysed (glyco- and cysteine-conjugates), biotransformed (monoterpenes, phenolic acids) or react (anthocyanins) with yeast metabolites (carbonyls). The grape-derived compounds, many of which are non-volatile and flavourless before fermentation, can then contribute to the sensory properties of wine.
3.1 Yeast growth and fermentation activity

Several studies have demonstrated that nitrogen application in the vineyard leads to increased yeast cell number in fermenting must when compared to the untreated control (Bell et al. 1979, Spayd et al. 1995). Fermentation rate also increases with an associated decrease in total fermentation duration (Ough et al. 1968a, Bell et al. 1979, Ough and Lee 1981, Dukes et al. 1991, Goldspink and Frayne 1997, Treeby et al. 2000, Chantelot et al. 2001, Spring 2002). This is not surprising, since yeast growth and fermentation kinetics are associated when other factors are optimal (Henschke and Jiranek 1993, Blateyron and Sablayrolles 2001, Cramer et al. 2002).

It is now well established in laboratory studies that, in the absence of other growth and fermentation limiting factors, must YAN concentration largely determines yeast cell population or biomass yield, and fermentation rate and duration (reviewed by Henschke and Jiranek 1993). YAN composition (of equivalent nitrogen concentration) also affects yeast growth rate with complex mixtures favouring higher rates than single compounds. Thus, mixtures of amino acids give higher rates of growth than the most preferred single nitrogen sources (i.e. ammonium, glutamine and asparagine), which in turn give higher rates than most individual amino acids; mixtures of ammonium and amino acids give variable rates relative to ammonium or mixed amino acids, depending on growth conditions (Cooper 1982, Henschke and Jiranek 1993, Albers et al. 1996, Ribéreau-Gayon et al. 2000a, ter Schure et al. 2000, da Cruz et al. 2002, Torija et al. 2003, Beltran et al. 2004). The greater efficiency of amino acid mixtures, especially balanced mixtures, compared with single nitrogen sources is linked to the ability of yeast to directly incorporate amino acids into protein, thereby minimising the need to maintain an energetically expensive amino acid synthetic capability.

Biomass formation is exponentially related to initial must YAN, but the quantitative relationship depends on many factors, including yeast demand for nitrogen, type of nitrogen source, fermentation temperature, oxygen availability, and amount of grape solids present (Agenbach 1977, reviewed by Henschke and Jiranek 1993, Blateyron and Sablayrolles 2001, Cramer et al. 2002). Depending on the strain, yeast have been reported to yield a biomass of 3–5 g dry wt./L, which corresponds to approximately 100 × 10^6 cells/mL, during the anaerobic fermentation of highly clarified media or grape juices containing an adequate source of YAN for biomass development (Salmon 1989, Henschke and Jiranek 1993, Jiranek et al. 1995a). Fermentation rate, measured as either sugar catabolic rate or CO₂ evolution rate, is related to yeast biomass or cell population size, but becomes uncoupled from growth during the latter stages of fermentation after growth has ceased. Fermentation rate is, as for cell growth, exponentially related to initial YAN concentration, and a direct relationship exists between fermentation rate and the amount of nitrogen utilised by yeast (Agenbach 1977). Therefore, fermentation duration is a function of initial YAN, when all other factors are non-limiting. Maximum fermentation rate, measured as maximum CO₂ evolution rate, is related to initial YAN, and this relationship provides a useful diagnostic tool for detecting (continuously) ‘slow’ fermentation (Bely et al. 1990a, Blateyron and Sablayrolles 2001). A kinetic model for nitrogen-limited wine fermentations which can predict a transition from normal to stuck fermentations as the initial nitrogen is decreased has recently been published by D. Block and colleagues (Cramer et al. 2002). Fermentation temperature as well as must assimilable nitrogen content is taken into account by the model of J.-M. Sablayrolles and colleagues, which predicts fermentation kinetics and duration accurately for multiscale wine fermentations, although atypical musts leading to sluggish fermentations are not well modelled (Colombié et al. 2005). It should be noted, however, that ‘sluggish’ or ‘stuck’ fermentations, i.e. those that commence normally but become slow or stop before must sugar concentrations have been depleted, have many causes, of which initial must YAN concentration only correlates poorly (Blateyron and Sablayrolles 2001). In fact, using metabolic flux analysis Varela et al. (2004) identified biomass as a major factor in determining fermentation rate in nitrogen-deficient fermentations. Furthermore, they could show that adding biomass taken from sluggish cultures significantly reduced the time to complete a problematic fermentation.

3.1.1 Nitrogen requirements of yeast

The nitrogen requirements and metabolism of *Saccharomyces cerevisiae* yeast have been extensively reviewed (Cooper 1982, Bisson 1991, Henschke and Jiranek 1993, Albers et al. 1998, Boullon et al. 1998, ter Schure et al. 2000, Magasanik and Kaiser 2002, Marks et al. 2003, Beltran et al. 2004) and will only be briefly mentioned here.

It is well established that yeast preferentially utilise certain nitrogen sources in a mixture and that the pattern of nitrogen compounds such as ammonium and amino acids accumulated depends on the nitrogen composition and concentration of the fermentation substrate. In general, nitrogen sources that favour high growth rates are preferentially assimilated because their metabolism readily yields ammonia, glutamate or glutamine, key components of yeast central nitrogen metabolism; these compounds are generally termed ‘good’ or ‘preferred’ nitrogen sources. Yeast use a mechanism called nitrogen catabolite repression (NCR), which mediates the selection of good nitrogen sources by the expression of appropriate transport systems (permeases) and the degradation of non-appropriate permeases (ter Schure et al. 2000, Magasanik and Kaiser 2002). Ammonium, glutamine and asparagine, are preferentially accumulated over others, such as arginine, alanine, aspartate, glycine and glutamate, and finally the poorer nitrogen sources, such as urea and proline. The branched-chain and aromatic amino acids seem to be a special case in that although they do not support high growth rates, they are typically accumulated early in fermentation (Henschke and Jiranek 1993, ter Schure et al. 2000, Beltran et al. 2005). Thus, the permeases for good nitrogen sources are induced during the NCR phase at the beginning of fermentation, such as Can1p (basic amino
The nitrogen component, and in some cases the carbon skeleton of degraded amino acids, and ammonium are ultimately used to synthesise glutamate or glutamine. These amino acids, which constitute the ‘yeast dynamic nitrogen pool’, are required for the synthesis of amino acids, purines and pyrimidines and their derivatives according to metabolic need (Large 1986, Magasanik and Kaiser 2002). α-Ketoglutarate, which is synthesised from acetyl CoA and oxaloacetate via the tricarboxylic acid cycle, combines with ammonium to produce glutamate. This reaction is catalysed by NADP⁺-linked glutamate dehydrogenase, encoded by GDH1. Glutamine is formed from glutamate by combination with ammonium as catalysed by glutamine synthetase, encoded by GLN1. Glutamate synthase, encoded by GLT1, catalyses the reaction between glutamate and α-ketoglutarate to yield glutamate. Glutamine synthetase and glutamate synthase together provide net synthesis of glutamate from α-ketoglutarate and ammonium. A fourth reaction, catalysed by NAD⁺-linked glutamate dehydrogenase, and encoded by GDH2, is required for the synthesis of glutamine when cells are exhausted for ammonium and are growing on glutamate or amino acids that can be degraded to glutamate, such as proline or γ-aminobutyric acid. Glutamate, from the nitrogen pool, supplies approximately 85% of the cell’s requirement for nitrogen. The amide group of glutamine provides the remaining 15%, which contributes to the synthesis of purines, pyrimidines and several amino acids (Cooper 1982, Magasanik and Kaiser 2002).

Since the metabolism of many amino acids contributes to flavour-active metabolites as well as to the yeast’s nitrogen pool, which is required for coordinating amino acid, purine and pyrimidine synthesis needed for cell growth, the nitrogen composition and concentration of the must or juice will not only affect wine flavour, but also fermentation activity. Yeast flavour-active metabolism will be considered in Section 3.3, whereas the remainder of this section will consider the impact of must nitrogen on yeast growth and fermentation kinetics.

Surveys of the nitrogen concentration of grape conducted world-wide, including Australia, have revealed that wide variability exists in relation to yeast requirements (Gockowiak and Henschke 1992, Spyad and Andersen-Bagge 1996, Butzké 1998, Stines et al. 2000, reviewed by Sponholz 1991 and Henschke and Jiranek 1993) (Table 2). In order to understand the consequences for grape and wine production, various studies have attempted to estimate the minimum concentration of juice nitrogen needed to achieve a satisfactory completion of fermentation as judged by low residual sugar, that is, the minimum concentration of nitrogen in juice at which the risk of slow or stuck fermentation is low. Due to the interplay of many factors, especially yeast strain (Jiranek et al. 1991, Manginot et al. 1997,1998, Blateyron and Sablayrolles 2001), and the different experimental approaches used, estimates range from 70–267 mg/L YAN, with a value of approximately 140 mg N/L for clarified musts of moderate sugar concentration being considered a practical minimal limit (Table 3).
Montpellier of the kinetic parameters that can be applied to describe fermentation showed that measurement of the maximum rate of carbon dioxide production, which occurs early in fermentation, provides a useful indication of the concentration at which nitrogen becomes limiting for risk-free fermentation (Bely et al. 1990a). A production rate of 1.3 g CO$_2$/h corresponded to the transition point between sluggish and adequate fermentation rate, which corresponded to a nitrogen concentration of approximately 140 mg N/L. This transition point was defined as the concentration of nitrogen at which only relatively small increases in the rate of carbon dioxide evolution were produced in response to nitrogen supplementation. Bely et al. (1991) suggested a second method for determining the minimum nitrogen requirement for complete fermentation. This estimate was calculated from the reduction in fermentation duration after a standard addition of nitrogen to a must. Musts that contained more than 140 mg N/L, by comparison, showed only a relatively small reduction in fermentation duration after a standard addition of nitrogen.

The threshold concentration of approximately 140 mg N/L has been confirmed by other investigators (Table 3). Cantarelli (1957) showed that a sharp reduction in fermentation rate corresponded to a must nitrogen concentration of less than 200 mg N/L, determined by supplementation studies of a nitrogen-depleted must. Reed and Peppler (1973) calculated the minimum nitrogen requirement of yeast from the nitrogen content of yeast. Agenbach (1977) defined the minimum nitrogen requirement of fermentation as that which just resulted in detectable residual nitrogen at the completion of fermentation. Bezenger and Navarro (1988) studied fermentation kinetics in model media supplemented with ammonium, whereas Mendes-Ferreira et al. (2004) also considered residual sugar.

From these different studies it can be concluded that the concentration of nitrogen at which the risk of incomplete fermentation of sugar (target concentration is typically <2 g/L) in clarified media of moderate initial sugar concentration is confined to a relatively small range. In practice, the threshold concentration of nitrogen will depend on the strain of yeast used, propagation conditions as well as the fermentation conditions chosen, and so this figure serves only as a rough guide. For example, Jiranek et al. (1991) observed that one of four Saccharomyces cerevisiae wine yeast strains was able to complete fermentation of a chemically defined medium that had an initial YAN of 78 mg N/L, whereas Bisson and Butzke (2000) have suggested that the initial YAN of grape juice should be 200–350 mg N/L, depending on must sugar content. No estimates for minimum YAN have been published for red wine (high solids) fermentations. However, anecdotally, the minimum concentration of nitrogen needed for complete fermentation of unclarified must is believed to be lower than for clarified must, due to the presence of a higher amount of grape solids. Furthermore, lower rather than higher must YAN may be beneficial for extending the duration of red fermentations and hence increasing the extraction of phenols, especially anthocyanins (Treeby et al. 2000). Grape solids, particularly skins, provide additional YAN (YAN concentration of skins is generally not measured when determining the YAN concentration of red berries or must; see Sections 2.2.4 and 2.4.2) and are a source of lipids, which are known to shorten the fermentation duration of clarified juices (Houtman et al. 1980, Ingledew and Kunkee 1985, Larue and Lafon-Lafourcade 1989). Oxygenation of must or ferments, which is often encouraged with red fermentations, is also well known to reduce the risk of aberrant fermentations (Ingledew and Kunkee 1985, Larue and Lafon-Lafourcade 1989, Sablayrolles and Barre 1986, Sablayrolles et al. 1996, Blatyeron and Sablayrolles 2001).

Choice of yeast provides another tool for managing the fermentation of low nitrogen musts. Jiranek, Langridge and Henschke (1991) first demonstrated that the wide variability in nitrogen demand between genetically distinct wine yeast strains affected fermentation performance. Whereas the fermentation rates were high and relatively similar for the four Saccharomyces cerevisiae strains studied, when an adequate supply of ammonium nitrogen (390 mg N/L) was provided, the rates were much lower and the differences between strains were large in the presence of a growth limiting concentration of ammonium nitrogen (78 mg N/L). Similar observations have been made by others (Mauricio et al. 1995, Gardner et al. 2002). Depending on the strain, maximum consumption of YAN varies widely from 329 to 473 mg N/L during the anaerobic fermentation of membrane-filtered Chardonnay juices/synthetic media containing an excess of YAN (Henschke and Jiranek 1993, Jiranek et al. 1995a). The oenological significance of maximum nitrogen consumption is not yet entirely clear, but relates to time of YAN exhaustion during fermentation and the consequential

<table>
<thead>
<tr>
<th>Minimum YAN (mg N/L)</th>
<th>Method</th>
<th>Reference</th>
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<tbody>
<tr>
<td>70–140</td>
<td>Residual N after complete fermentation</td>
<td>Agenbach (1977)</td>
</tr>
<tr>
<td>120</td>
<td>Calculated from N-content of yeast</td>
<td>Reed and Peppler (1973)</td>
</tr>
<tr>
<td>132</td>
<td>Kinetic studies in model media supplemented with N</td>
<td>Bezenger and Navarro (1988)</td>
</tr>
<tr>
<td>140</td>
<td>CO$_2$ production rate in response to N</td>
<td>Bely et al. (1990a)</td>
</tr>
<tr>
<td>140</td>
<td>Reduction of fermentation time after standard addition of N</td>
<td>Bely et al. (1991)</td>
</tr>
<tr>
<td>200</td>
<td>Fermentation rate in an N-depleted must</td>
<td>Cantarelli (1957)</td>
</tr>
<tr>
<td>267</td>
<td>Residual sugar in N-supplemented model media</td>
<td>Mendes-Ferreira et al. (2004)</td>
</tr>
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physiological responses, such as declining viability and fermentation rate, and sulfide production. However, Manginot et al. (1998) studied the nitrogen demand of various yeast strains by measuring the consumption of nitrogen during the steady state (stationary) phase of growth; a stage at which nitrogen addition stimulates the sugar catabolic rate but not cell growth. As a convenient form of strain comparison, yeast nitrogen demand could be characterised by the amount of nitrogen needed to maintain a constant rate of CO₂ evolution using a laboratory chemostat fermentor. By this technique, Julien et al. (2000) determined that wine fermentation strains varied widely in nitrogen demand, requiring 1 to 2.5 mg N per g CO₂ evolved. The characterisation of yeast in this way appears to be especially useful for winemaking since it is during the latter, non-growth stage of fermentation that the highest concentration of alcohol is present concomitantly with the lowest availability of nitrogen. Such strain characterisation provides the ability to select nitrogen efficient yeast for fermenting low nitrogen musts.

3.1.2 Managing must nitrogen deficiency by nitrogen supplementation
A considerable body of research has shown that must nitrogen deficiency is an important risk factor for sub-optimal fermentation (especially for completion of slow fermentations) and for hydrogen sulfide production (Cantarelli et al. 1988, Bely et al. 1990a,b, Sablayrolles et al. 1996, Blateyron and Sablayrolles 2001). The sensory implications of nitrogen addition have been shown conclusively that, from a practical point of view, DAP supplementation at the one-third to mid-point stage of fermentation is generally the most effective at resolving aberrant fermentation kinetics for musts initially low in YAN. Yeast growth is largely completed by this stage and induction of excessive thermal output is minimised. Thus, nitrogen consumption is relatively low and the added nitrogen is more likely to act during the remainder of fermentation to prevent sugar transport arrest (Busturia and Lagunas 1986, Salmon 1989, Salmon et al. 1993). This research also showed that a small amount of oxygenation (5–10 mg/L) of the ferment, at the end point of the yeast growth phase, was also effective. The oxygen appears to act by allowing sterol biosynthesis and providing a redox sink (Salmon et al. 1998, Fornàiron-Bonnefond et al. 2002). When oxygenation was combined with DAP addition, a high proportion of musts, which otherwise did not complete fermentation, were completed more quickly and with lower residual sugar concentrations (Blateyron and Sablayrolles 2001). The sensory implications of nitrogen concentration during fermentation are discussed in Section 4.0.

3.2 Grape vine and yeast biotechnology for improving nitrogen-use efficiency
Over the past two decades, the questions of how much nitrogen and when to add it have been addressed. The early studies of Agenbach (1977), Vos et al. (1980), Monk (1982) and others established the efficacy of ammonium salts in improving fermentation kinetics. Studies aimed at determining the optimum timing of supplementation only came later. For example, the work of Bely et al. (1990a), in which a nitrogen deficient medium was supplemented with DAP at different stages of fermentation, showed that nitrogen has in fact two main effects on fermentation. DAP added at any stage during the yeast growth phase increases the size of the yeast population, but has little effect on population size when added at later stages. With respect to fermentation rate, however, DAP addition is equally effective throughout fermentation, though the response kinetics decrease at later stages; this has been confirmed by others (Beltran et al. 2005). Thus, DAP addition throughout fermentation results in a shortening of fermentation duration, with the effect being greatest when added during the early stages of fermentation.

A comprehensive series of studies conducted by Sablayrolles and colleagues (Bely et al. 1990a,b, Sablayrolles et al. 1996, Blateyron and Sablayrolles 2001) have shown conclusively that, from a practical point of view, DAP supplementation at the one-third to mid-point stage of fermentation is generally the most effective at resolving aberrant fermentation kinetics for musts initially low in YAN. Yeast growth is largely completed by this stage and induction of excessive thermal output is minimised. Thus, nitrogen consumption is relatively low and the added nitrogen is more likely to act during the remainder of fermentation to prevent sugar transport arrest (Busturia and Lagunas 1986, Salmon 1989, Salmon et al. 1993). This research also showed that a small amount of oxygenation (5–10 mg/L) of the ferment, at the end point of the yeast growth phase, was also effective. The oxygen appears to act by allowing sterol biosynthesis and providing a redox sink (Salmon et al. 1998, Fornàiron-Bonnefond et al. 2002). When oxygenation was combined with DAP addition, a high proportion of musts, which otherwise did not complete fermentation, were completed more quickly and with lower residual sugar concentrations (Blateyron and Sablayrolles 2001). The sensory implications of nitrogen concentration during fermentation are discussed in Section 4.0.
biotechnology of *Vitis vinifera* has greatly advanced in the last decade, but the mechanisms and regulation of nitrogen uptake, metabolism and effects on the production of amino acids and flavour molecules, and their precursors, are still in their infancy (Roubelakis-Angelakis 2001). Some of the genes which encode metabolic enzymes in various plants have been identified and several homologues have been identified and studied in the grapevine (Loulakakis and Roubelakis-Angelakis 2001, van Heeswijck et al. 2001). The main focus of these studies has been on nitrogen assimilation and regulation. Information on nitrogen uptake by roots and interchange of nitrate and ammonium ions, and the distribution between tissues, especially the grape berry, is limited and needs to be established. Molecular expression and regulation studies of the nitrogen assimilation genes, glutamine synthase (GS1 and GS2), glutamate synthase (NADH-GOGAT and Fd-GOGAT) and glutamate dehydrogenase (GDH) have commenced in various grapevine tissues, including the grape berry (Loulakakis and Roubelakis-Angelakis 2001). Expression and regulation studies of proline metabolism have also commenced, due to the importance of proline as a potential nitrogen source for yeast in nitrogen-deficient musts (van Heeswijck et al. 2001). In light of the observation that some grape varieties have a high proline to arginine ratio in grape berries, as discussed in Section 2.2.3, the possibility exists to alter this balance in favour of arginine, which is readily assimilated by yeast. Proline is synthesised from glutamine/glutamate via γ-glutamyl phosphate and pyrroline-5-carboxylate, but can also be synthesised from ornithine, from which arginine is also derived (Stines et al. 2000, van Heeswijck et al. 2001;
The relative importance of the two pathways for proline production is uncertain at this time. However, studies under conditions known to affect proline accumulation, such as water stress, should be revealing. The complexity of nitrogen metabolism across the different tissues of the grapevine will mean that a useful understanding is still a long way off, however the strong relationship between grape berry YAN and wine quality should provide a strong impetus for further research.

The characterisation of wine yeast or the clonal selection of natural isolates for reducing the risk of sub-optimal fermentation, as discussed in the preceding section, provides strains that can be immediately applied to wine production. One important limitation of these classical approaches is, however, that a large number of strains must be screened not only for the target property of interest but also for the many other winemaking properties, such as growth and fermentation rate, alcohol tolerance and sugar attenuation, off-flavour production, and so on (Henschke 1997b, Rainieri and Pretorius 2000). Genetic engineering, on the other hand, facilitates the ability to modify strains that are already well characterised. The complexity of the task cannot, however, be underestimated, given that a recent study has shown that some 350 genes of a commercial wine yeast are regulated by diammonium phosphate (Marks et al. 2003). Nevertheless, studies such as this, made under appropriate winemaking conditions, are a powerful tool for identifying target genes. At least for the near future, however, while there is consumer and industry resistance to the use of genetically modified organisms (GMOs), such strains will not be used for producing wine unless a clear benefit can be unambiguously demonstrated to the consumer as well as the producer and there is no harm to the consumer and environment. Even so, yeast biotechnology is a powerful tool for unravelling the complexities of yeast metabolism and facilitates the development of ‘new/novel’ biochemical solutions. Conventional techniques, such as hybridisation, adaptive evolution under appropriate selective environmental pressure, and so on, can then be used more effectively to develop strains with improved performance (Querol et al. 2003, Pretorius 2004).

Several studies that are exploring the ability to improve the nitrogen-use efficiency of strains by the yeast biotechnology approach have recently been reported (Smyl et al. 1996, Salmon and Barre 1998, Poole et al. 2002, Gardner et al. 2005). The principal focus of these investigations concerns the metabolism of proline, which represents a large untapped source of nitrogen in grape must. Although the concentration of proline varies widely in grape must, Ough and Amerine (1988) reported a mean value of 650 mg/L, which represents 80 mg N/L. The uptake and metabolism of proline, a poor nitrogen source for yeast, is repressed by the nitrogen catabolite repression (NCR) mechanism that operates when better nitrogen sources are also present in the grape must (Cooper and Sumrada 1983, Beltran et al. 2004; see Section 3.1.1). NCR is mediated by at least three genes, \textit{GLN3}, \textit{GAPI1} and \textit{URE2} (Magasanik 1992), the latter of which represses the transcription of the genes involved in the assimilation of poor nitrogen sources in the presence of better sources. Salmon and Barre (1998) have isolated and characterised a variant of an industrial yeast, which carries a mutation in the \textit{URE2} gene, and have shown that proline metabolism is no longer subject to regulation by better nitrogen sources. By comparison to the parent strain, the \textit{ure2} mutant strain produces greater biomass and has higher fermentation capacity on various grape juices. Proline assimilation can therefore proceed in the presence of a low amount of dissolved oxygen. To further enhance proline utilisation during fermentation Poole et al. (2002) have over-expressed the existing proline permease \textit{PUT4} gene under the control of a constitutive promoter so that proline can be accumulated during the early stages of growth even when better nitrogen sources are present. By combining the over-expression of the \textit{PUT4} gene with the \textit{ure2} mutant gene in a single yeast, such strains should be capable of assimilating proline in the presence of better nitrogen sources during the cell growth phase of fermentation (Figure 9). However, the first step of proline catabolism involves cleavage of the proline ring by proline oxidase. This step requires a functional electron transport system, located in the mitochondria, and oxygen. While musts often initially contain dissolved oxygen it is typically depleted soon after inoculation, leaving insufficient for the oxidative catabolism of proline. In an attempt to obviate the need for adding oxygen during fermentation, Smyl et al. (1996) have cloned the gene encoding pyrroline-5-carboxylate dehydrogenase into yeast (Figure 9). This enzyme, which is located in the cytosol, uses cellular NAD\(^+\), derived from sugar metabolism, as an oxidant.

An alternative approach to improving the nitrogen use efficiency of strains is being undertaken by Gardner et al. (2005), who have screened a genomic library of mutants produced by insertion of a transposon, which serves to both disrupt genes and to facilitate their identification (Ross-Macdonald et al. 1997). Of some 5,000 mutants characterised for sugar catabolism in nitrogen-limited media, two candidate strains were identified that catabolised significantly more glucose than the parent strain in a low nitrogen medium. The affected genes were identified as \textit{NGR1} and \textit{GID7}. Deletion of these genes from haploid derivatives of wine yeast enhanced glucose catabolic activity when grown in limiting nitrogen conditions.

Wine chemical and flavour consequences resulting from the various genetic modifications described above are still to be evaluated.

3.3 Aroma, flavour and mouth-feel compounds
The appearance, aroma, flavour and mouth-feel of wine, are considered to be derived from four major sources; (a) primary grape-derived compounds formed in the plant cell; (b) secondary grape-derived compounds modified by processing; (c) fermentation compounds formed by yeast during alcoholic fermentation; and (d) age-related properties formed during wine maturation (Rapp and Versini 1991).

Fermentation represents the major process in the
development of flavour-active compounds since, by comparison to wine, the aroma and flavour of grape juice/must is relatively low (Rapp 1988, Jackson 2000, Lambrechts and Pretorius 2000). The complexity of flavour development during fermentation is still relatively poorly understood, however many of the important features are summarised in Figures 7 and 10. Three main routes of flavour development during fermentation can be identified, namely, some grape-derived compounds remain essentially chemically intact, others are metabolised to form flavour-active metabolites, and others undergo hydrolytic or biotransformation reactions either intra- or extra-cellularly, which modify their flavour-active attributes.

During the yeast growth phase, the metabolism of sugars leads to the formation of a variety of volatile compounds, including higher alcohols, fatty acids, esters, carbonyls, S-compounds and several organic acids, many of which contribute to wine aroma and flavour (Rapp and Versini 1991, Guth and Sies 2002, Francis and Newton 2005, Howell et al. 2005, Smyth et al. 2005, Swiegers et al. 2005). Nitrogen compounds also contribute to the formation of some of these compounds, especially higher alcohols and esters, and in addition, regulate the formation of other volatiles, such as hydrogen sulfide, thiols/mercaptans and monoterpenes (Henschke and Jiranek 1993, Rapp and Versini 1996, Albers et al. 1998). The impact that must nitrogen composition and concentration has on the uptake of amino acids by yeast has been summarised in Section 3.1.1.

In addition to the production of primary metabolites, yeast interact with a variety of non-volatile, flavourless grape-derived precursor compounds that have considerable sensory importance. The glyco-conjugates and S-cysteine-conjugates, upon hydrolysis by yeast enzymes, can strongly contribute to wine varietal character (Strauss et al. 1986, Francis et al. 1992, 1996a,b, Selton et al. 1993, Francis and Newton 2005, Swiegers et al. 2005). Examples of grape-derived aroma compounds include hexyl derivatives that elicit fruity and floral aromas and α-terpineol (Strauss et al. 1986). They exist in the extra-cellularly, which modify their flavour-active attributes.

As already mentioned, the proportion of volatile compounds present in wine is high compared to grapes, juice or must, due to the process of fermentation and the metabolic activity of yeast and bacteria. However, grapes contain a large pool of potential volatile aroma compounds that are mainly constituted as odourless, non-volatile glycoconjugates. These compounds have the potential to make an important contribution to the varietal sensory properties of wine (Francis et al. 1992, 1996a,b). Several classes of compounds are typically present as glycoconjugates, and include monoterpenes, norisoprenoids, aliphatics, benzene-derivatives and phenols. These odourless glycoconjugates are either glucosides, disaccharides or trisaccharides. They all contain a glucosyl moiety, but for the disaccharide glycosides, the glucose moiety is further substituted with α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-xylopyranosyl or β-apiofuranosyl sugars. The disaccharide glycosides represent a major source of aroma compounds (Williams et al. 1982).

The glycosyl-glucose (G-G) assay, which exploits the fact that all glycoconjugates contain glucose, was developed to provide a relatively simple indicator of the level of potential flavour compounds present in grapes, and hence, wine. G-G correlates with wine flavour intensity and wine quality score (Francis et al. 1999, Gishen et al. 2002). With only one exception, Treeby et al. (2000) observed that a summer or postharvest application of nitrogen significantly reduced the G-G concentration in wines of Shiraz grafted onto three different rootstocks. However, this was only reflected in a reduced wine palate intensity score for one scion-rootstock combination. This highlights a problem with the G-G assay method in that it measures all glycosidic compounds, especially in red grapes, and thus it measures compounds that do not affect flavour, as well as those that do (Gishen et al. 2002). Spectroscopy methods are increasingly being developed and can be expected to provide rapid and convenient estimates of grape quality factors in the near future (Gishen et al. 2002).

### 3.3.1 Glycosides

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### 3.3.2 Monoterpenes

Monoterpenes, which elicit a floral aroma in wine, are regarded as key impact compounds in the floral grape varieties, such as Muscat of Alexandria, Riesling and Gewürztraminer. The most important compounds are the monoterpenes diols, linalool, geraniol, nerol, citronellol, and α-terpineol (Strauss et al. 1986). They exist in the berry principally as glycoconjugates with only a small proportion present in the free form and are liberated during fermentation. A recent report also suggests that yeasts can produce monoterpenes which might contribute to wine aroma (Carrau et al. 2005).
Several studies have shown that a link exists between total monoterpene (free and bound forms), concentration in the grape, and canopy microclimate factors such as temperature and light (Reynolds and Wardle 1997, Marais et al. 1999, Hunter et al. 2004). Nitrogen application in the vineyard increases vegetative growth and consequently canopy density in comparison to vines receiving no supplementary nitrogen (Wolf and Pool 1988, Spayd et al. 1993, Bell and Robson 1999). Canopy density significantly affects canopy microclimate, particularly solar radiation interception (Marais et al. 2001, Smart 1991). A higher berry monoterpene concentration coincided with increased light intensity inside the canopy (Marais et al. 1999). In the same study, canopy management had no effect on temperature within the canopy, but the total monoterpene concentration in grapes was generally higher in cooler seasons and/or regions compared to those ripened in warmer conditions. Therefore, it is conceivable that nitrogen induced changes in canopy density might affect the berry and subsequently the wine monoterpene concentration. Unfortunately, this is an area that has not been well studied, as only one report was found that demonstrated the effect of nitrogen application in the vineyard affected the level of some of the monoterpenes in aged wine, all of the monoterpene concentrations were below the sensory threshold (Webster et al. 1993). It is not known whether nitrogen application affected the concentrations of free and bound monoterpenes entering the winery, as monoterpenes were not measured in the berry.

Monoterpenes exist in juice and must principally as mono- and disaccharide terpenes and are released by acidic hydrolysis and various glycosidic enzymes of grape and exogenous origin, such as commercial enzyme preparations added during the winemaking process (Rapp and Versini 1991). Monoterpenes exist in juice and must principally as mono- and disaccharide terpenes and are released by acidic hydrolysis and various glycosidic enzymes of grape and exogenous origin, such as commercial enzyme preparations added during the winemaking process (Rapp and Versini 1991). Monoterpenes exist in juice and must principally as mono- and disaccharide terpenes and are released by acidic hydrolysis and various glycosidic enzymes of grape and exogenous origin, such as commercial enzyme preparations added during the winemaking process (Rapp and Versini 1991). Monoterpenes exist in juice and must principally as mono- and disaccharide terpenes and are released by acidic hydrolysis and various glycosidic enzymes of grape and exogenous origin, such as commercial enzyme preparations added during the winemaking process (Rapp and Versini 1991). Monoterpenes exist in juice and must principally as mono- and disaccharide terpenes and are released by acidic hydrolysis and various glycosidic enzymes of grape and exogenous origin, such as commercial enzyme preparations added during the winemaking process (Rapp and Versini 1991).
microorganisms. Furthermore, the transformation of free terpenes by different yeast species, especially non-
Saccharomyces species, has been reported (Fernández-
González et al. 2003). This might be especially important in ‘natural ferments’, in which such yeast species present on grape berry biofilms and grape processing equipment are permitted to play a stronger role during fermentation (Heard and Fleet 1986, Fleet 2003).

In addition to the release of volatile compounds by hydrolysis of non-volatile glycoconjugates, various yeast species associated with fermentation can produce monoterpenes from sugar metabolism, albeit at a low concentration (Carrau et al. 2005). Although mutant strains of Saccharomyces with a genetic defect in the sterol pathway (Chambon et al. 1990) have been reported, Carrau et al. (2005) revealed that some wine strains of Saccharomyces cerevisiae are capable of significant production under certain fermentation conditions. Strikingly, the YAN and oxygen content of the fermentation medium influences monoterpane formation. High YAN concentration of the medium (400 compared with 180 mg N/L), which stimulates fermentation rate but not biomass yield, also stimulates monoterpane, but not sesquiterpene (nerolidol and farnesol) formation. To explain these results, based on blast searches performed on the Saccharomyces genome database, the authors hypothesised that monoterpenes might not be derived from the sterol pathway, as sesquiterpenes appear to be, but by an alternative pathway. This latter pathway, which involves the conversion of leucine to mevalonic acid, is located in the mitochondrion, and this fact could explain the non-coordinated synthesis of the two terpene groups. Assimilable nitrogen, as well as oxygen, is known to regulate mevalonic acid and sterol formation, and hence the concentration of intermediates, such as geranyl pyrophosphate, which can act as a terpene precursor (Novotny et al. 1988, Vaudano et al. 2004).

In summary, some strains of yeast, especially Saccharomyces, might contribute to the floral aroma of wine by de novo synthesis of monoterpenes, and this effect could be augmented by higher juice nitrogen in combination with microaerobic fermentation.

3.3.3 Pyrazines

The pyrazines are cyclic nitrogen-containing compounds that contribute significantly to the varietal aroma of a number of cultivars such as Cabernet Sauvignon and Sauvignon Blanc (Hashizume and Samuta 1999, Roujou de Boubée et al. 2002). Methoxypyrazines are responsible for the vegetative, herbaceous, capsicum-like aromas that these cultivars commonly display (Lacey et al. 1991). They can contribute positively to certain varietal wine aroma profiles. However, when present at high levels, methoxypyrazines may be considered as a negative sensory attribute, as their aroma may not be suitably balanced by other aroma compounds in the wine. Three methoxypyrazines (3-isobutyl-2-methoxy-5-pyrazine, 3-sec-butyl-2-methoxypyrazine and 3-isopropyl-2-methoxy-
pyrazine) have been identified in grapes and wines (Sala et al. 2004). 3-isobutyl-2-methoxy-pyrazine (IBMP) how-
ever, is the dominant compound present (Lacey et al. 1991, Sala et al. 2004). Unlike the monoterpenes, the methoxypyrazines are not bound in any way, and thus the IBMP concentration in the wine is primarily dependent on the IBMP concentration in the grape at harvest.

Roujou de Boubée et al. (2002) found that 95.5% of the IBMP was located in the skins of Cabernet Sauvignon grapes, and as it was easily extractable most of the IBMP was present in the free run juice. Similarly, the IBMP concentration was at its highest on the first day of maceration (Sala et al. 2004). These authors saw no further increase in the IBMP concentration after racking. Therefore, the impact of nitrogen application in the vineyard or winery on fermentation time on skins is less likely to affect the final IBMP concentration in the must, given the quick extractability of the methoxypyrazines.

As yet we know of no studies that have examined the effect of nitrogen application in the vineyard on the concentration of pyrazines in grapes and wine, and how the concentration of pyrazines would affect the sensory attributes of the final wine. However, as methoxypyrazines are nitrogen-containing compounds it is conceivable that increasing the vine nitrogen status might increase the formation of these compounds in nitrogen-responsive sites. Nitrogen application in the vineyard might also have an effect on the methoxypyrazine concentration via its impact on canopy microclimate. At similar Brix levels, the methoxypyrazine concentration of grape juices from three cool Australian regions was higher than those concentrations found in juices grown in hot areas (Lacey et al. 1991). Furthermore, Allen and Lacey (1993) showed that grape IBMP concentrations increased as the leaf layer number of the canopy increased; that is the IBMP concentration was higher in less exposed fruit. Therefore, increased bunch shading caused by nitrogen application in hot regions may slow the rate of methoxypyrazine decline during ripening. However, as discussed earlier, when nitrogen is supplied to vines with a high vine nitrogen status, the potential limitation in carbohydrate supply and reduction in nitrate reductase activity may result in decreased formation of these compounds in high density canopies.

3.3.4 Carboxylic acids

The acidity and pH of grapes and wines results from the types and concentrations of organic acids present. L(+)-Tartaric and L(−)-malic acids are the principal organic acids, with citric and D(−)-lactic acids making smaller contributions (Table 1). Succinic and keto acids are only present in trace concentrations in grapes but are higher in wines as the result of fermentation. Volatile fatty acids will be considered in the following section. This section will review the influence of must nitrogen on organic acid metabolism by yeasts.

Tartaric acid is essentially stable and is neither degraded nor produced by fermentation microflora. On the other hand, many yeasts can degrade or produce malic acid, and malolactic bacteria are capable of completely degrading it to lactic acid and CO₂ (Rankine and Fornachon 1964, Shimazu and Watanabe 1981, Radler 1993). The
role of nitrogen in the catabolism of malic acid by yeasts has not been reported, however sub-optimal assimilable nitrogen concentrations (< 300 mg N/L) in fermentation media favour its production (Schwartz and Radler 1988). Malic acid production appears to be common amongst *Saccharomyces uvarum* strains, whereas it is restricted to only some strains of *Saccharomyces cerevisiae* (Radler 1993, Giudici et al. 1995). In a small survey of *Saccharomyces cerevisiae* wine yeast, Enoferm M2 increased the malic acid concentration of Cabernet Sauvignon wines by up to 1.5 g/L, whereas ICV D254 consumed 0.5 g/L; the remaining strains produced smaller changes (Holgate 1997). Further work is needed to establish the role of must nitrogen in determining the malic acid content of wine.

Succinic acid production, but not catabolism, is common amongst yeasts and is the main carboxylic acid produced during fermentation, where it typically accumulates to 2 g/L (Thoukis et al. 1965, Shimazu and Watanabe 1981, Coulter et al. 2004). Its formation appears to be independent of that for malic acid, with the most likely pathway being the reductive branch (via oxaloacetate and malate) of the tricarboxylic acid (TCA) cycle during anaerobic fermentation (Roustan and Sablayrolles 2002, Camarasa et al. 2003). Studies on the role of nitrogen in regulating succinic acid formation, undertaken in synthetic media, suggest that the addition of certain amino acids to the medium can significantly stimulate succinate formation. Glutamate, to the greatest extent, and asparagine, proline, glutamine and threonine, to a lesser extent, stimulated succinate formation (Heerde and Radler 1978, Albers et al. 1996, Camarasa et al. 2003). These authors concluded that succinate formation from amino acids resulted directly from the metabolism of, at least, glutamate and aspartate, and probably not by regulating succinate formation from sugar via the reductive branch of the TCA cycle, which is the principal route for succinate formation. Thus, aspartate was deaminated to oxaloacetic acid and reduced to succinic acid via malic and fumaric acids, whereas glutamate was deaminated to α-ketoglutarate and decarboxylated and activated to form succinic acid (Camarasa et al. 2003). By a different mechanism, Bach et al. (2004) suggested that abnormally high concentrations of succinic acid could arise from γ-amino butyric acid, whose concentration in must appears to be affected by post-harvest factors. Because succinic acid production during fermentation is variable and can impact on the titratable acidity of wine (Coulter et al. 2004) a better understanding of succinate metabolism in fermentation yeast is needed.

The keto acids, pyruvic and α-ketoglutaric acid, are important in winemaking due to their abilities to bind SO₂ and to react with phenols (Rankine and Pocock 1969, Fulcrand et al. 1998). They are produced during the early stages of fermentation via sugar metabolism. These keto acids can also be formed from the corresponding amino acids, alanine and glutamate, by the Ehrlich pathway. In addition to the importance of yeast strain, the type and concentration of nitrogen in the medium affects the production of α-ketoglutaric acid; a low concentration (125 mg N/L) stimulates production to several hundred mg/L, whereas high nitrogen (1000 mg N/L) reduces production to typically 50–100 mg/L (Rankine 1968b, Radler 1993).

Organic acid metabolism by fermentative yeast and bacteria can impact both directly and indirectly on wine flavour, however, the role of nitrogen in regulating acid metabolism requires better definition.

3.3.5 Yeast volatile compounds

Esters, higher alcohols, volatile fatty acids and carbonyls are important contributors to the fermentation bouquet of wine (Francis and Newton 2005). These compounds principally arise as primary metabolites of yeast sugar and amino acid metabolism (Rapp and Versini 1991, Henschke and Jiranek 1993, Swiegers et al. 2005) (Figure 10). Therefore, the production of these flavour-active compounds during fermentation can be expected to be influenced by the amino acid composition of the must. The following section will consider the impact of nitrogen application in the vineyard and winery on the composition of these volatile wine compounds, with the main focus being on the relationship between must nitrogen content and their formation.

There is sufficient evidence to support the concept of a relationship between must nitrogen concentrations and the composition of yeast volatile aroma compounds in wine. For example, it has been long observed that wines with a combination of high concentrations of esters and low concentrations of higher alcohols are typically given higher sensory ratings (Wagener and Wagener 1968, Zeeman et al. 1980). Wines with high concentrations of esters, particularly ethyl esters, which contribute fruity aromas, and low higher alcohol concentrations, are associated with higher must concentrations of amino acids (Ough and Bell 1980, Vos 1981, Ough and Lee 1981, Rapp and Versini 1996, Guitart et al. 1999, Torrea and Henschke 2004, Hernández-Orte et al. 2005). Additionally, Sinton et al. (1978) observed a strong correlation between wine taste intensity and must nitrogen concentration ($R^2 = 0.750$) and Vos (1981) observed a correlation between wine aroma score and must nitrogen concentration ($r = 0.825$).

Surprisingly, very few studies have focused directly on the impact of nitrogen on fermentation volatiles. Few researchers have determined the sensory implications of must nitrogen on fermentation, apart from the effect that nitrogen has on the undesirable volatile sulfur compound, H₂S.

Not surprisingly, genetically dissimilar strains of yeast can affect the composition of wine volatile compounds (Rankine 1977, Zeeman et al. 1980, Soles et al. 1982, Cabrera et al. 1988, Vila et al. 1998) due to variability in the regulation of these complex metabolic pathways (see for example Bisson 2000, Marks et al. 2003). Aside from the importance of nitrogen composition and concentration of must, many fermentation variables that affect yeast growth, such as temperature, pH, sugar concentration, must clarification, aeration and vitamins, can also modify the concentrations of volatile compounds (Jiranek et al. 1993, Rapp and Versini 1996). Due to the complexity of the effects that nitrogen has on the formation of aroma...
volatile compounds by yeast, the following discussion will consider each group of volatile compounds separately.

3.3.5.1 Ethanol and glycerol
Ethanol concentration affects the sensory perception of wine flavour-active compounds (Guth and Sies 2002), but is also an issue of growing importance to the consumer (Swiegers et al. 2005). Within the normal range found in wine, changes in glycerol concentration appear to be of little sensory significance (Swiegers et al. 2005). Nitrogen regulates the accumulation of sugar and its metabolism in yeast (Boulton et al. 1998), but little has been reported on the regulation of sugar-alcohol conversion efficiency by nitrogen. It has been established that the type of nitrogen source affects the formation of sugar-derived metabolites, especially glycerol, and to a lesser extent acetic acid, α-ketoglutaric acid and succinic acid, which will affect the flow of sugar carbon to ethanol production (Albers et al. 1996).

Glycerol formation, under anaerobic conditions, which is an essential step for the maintenance of oxidised NADH in the cell, will lead to a lowered production of ethanol, due to the diversion of sugar-carbon to these non-ethanol metabolites (Swiegers et al. 2005). When yeast cells are growing on amino acids little surplus NADH is generated from protein synthesis and growth, resulting in minimal loss of ethanol formation due to minimised glycerol formation. However, growth on ammonium, for example, requires the cell to synthesise all of the amino acids, which results in increased NADH formation. The additional production of glycerol, in order to reoxidise the additional NADH, reduced ethanol yield by 14% in a chemically-defined medium (Albers et al. 1996). At least one report suggests that amino acid composition of a synthetic grape must can affect ethanol concentration (Hernández-Orte et al. 2002), however studies in authentic grape musts are required to establish the significance of this effect.

3.3.5.2 Higher alcohols
The beneficial role of higher (fusel) alcohols on wine aroma is uncertain, but Rapp and Versini (1991) reported that concentrations below 300 mg/L add a desirable level of complexity to wine, whereas concentrations that exceed 400 mg/L can have a detrimental effect on wine quality. The total concentration of higher alcohols determined in a number of viticultural studies ranged from 76.5 mg/L to 310 mg/L (Ough et al. 1968b, Ough and Bell 1980, Webster et al. 1993, Giorgessi et al. 2001, Maigre 2002). It is likely, however, that the desirable concentration range of higher alcohols in wines will depend on their type and style. Some answers might be gained from studies such as that undertaken by Smyth et al. (2005), who correlated the composition of wine volatiles with formal descriptive sensory data. For Riesling wines, they found that 2-phenylethanol was important, whereas for Chardonnay 2-methylpropanol and 2-and-3-methylbutanol were important.

In the majority of studies nitrogen applied in the vineyard decreased the higher alcohol concentration in wine compared to wine prepared from vines that received no nitrogen (Table 4). However, in any given study in Table 4, wines made with fruit from nitrogen-treated vines had higher concentrations of some higher alcohols and lower concentrations of others compared to wines from the control treatment. In addition, there are many higher alcohols, and not all of these compounds are measured in every study, and it is possible that the full extent of the effect of nitrogen on the higher alcohol concentration is underestimated.

Higher alcohols can be formed anabolically from sugar as well as catabolically from amino acids via the Ehrlich pathway (Thoukis 1958, Ingraham and Guymon 1960, Guymon 1972, Chen 1978, Nykänen 1986, Marchetti and Guerzoni 1987, Herraiz et al. 1989, Rapp and Versini 1991, 1996; Figures 10 and 12). Must nitrogen concentration plays an important role, as the association between initial nitrogen concentration of the must and the concentration of higher alcohols in the wine has been demonstrated repeatedly (Table 4). However, the relationship between higher alcohol production and amino acid assimilation throughout the fermentation cycle is not clear. The amino acids and their corresponding higher alcohols, and related compounds, are summarised in Table 5.

It is generally observed that the excretion of higher alcohols tends to occur towards the middle to later stages of fermentation, whereas the bulk of amino acids are consumed early in fermentation, during the yeast growth phase. Furthermore, the total consumption of structurally related amino acids does not account for the final concentration of corresponding higher alcohols produced in wine, and generally represents the smaller proportion of the total concentration of higher alcohols produced (Guymon 1972, Nykänen 1986, Rapp and Versini 1991). Thus, the greater proportion of higher alcohols are synthesised from sugars. When amino acids are absent and ammonium is the sole source of nitrogen, higher alcohols are nevertheless produced, though at lower concentrations (Herraiz et al. 1989). Gene expression studies, such as those by Marks et al. (2003), may provide a molecular rationale for the complex relationship observed between the concentrations of branched-chain amino acids, total nitrogen and DAP and the formation of higher alcohols. For example, they observed upregulation of genes encoding for branched-chain amino acid permeases as well as genes encoding enzymes involved in branched-chain amino acid metabolism in response to DAP addition, whereas the formation of higher alcohols is generally observed to be lessened as the initial must nitrogen concentration is raised (Rapp and Versini 1996). Quantitative gene expression analysis, such as that provided by serial analysis of gene expression (SAGE) provides another powerful molecular tool for unravelling the importance of, in particular, poorly understood metabolic pathways, in which the important genes are not well known (Varela et al. 2005).

Must nitrogen concentration has a strong impact on the final concentrations of higher alcohols in wine. In general, when the nitrogen concentration of must is low, a direct relationship between initial nitrogen concentration and the total concentration of higher alcohols exists,
whereas at moderate must nitrogen an inverse relationship with higher alcohols prevails (Äyräpää 1971). At high initial must nitrogen, the concentrations of total higher alcohols are at their lowest. Using isotopically labelled amino acids, Oshita et al. (1995) showed that during low amino acid availability, surplus keto acids, which are largely synthesised from sugars, are decarboxylated and reduced to higher alcohols, due to the lack of alpha-amino nitrogen availability from transamination reactions. Furthermore, feed-back inhibition regulating keto acid biosynthesis is absent, due to the low concentrations of amino acids in the medium. On the other hand, in the presence of sufficient amino acids in the medium, amino acid transamination reactions (needed for the biosynthesis of amino acids in short supply) lead to the relatively larger formation of higher alcohols essentially from corresponding amino acids by the Ehrlich pathway, relative to those formed from sugars. Since the proportion of the branched chain amino acids (leucine, isoleucine, valine and threonine, from which the higher alcohols are formed) is relatively low compared to other amino acids when the total nitrogen concentration is high, only low amounts of higher alcohols are therefore produced. It has also been suggested that in complex media yeast use a branched-chain α-keto acid dehydrogenase, which does not yield higher alcohols, thus ensuring a low production of higher alcohols in high nitrogen conditions (Sinclair et al. 1993). Surprisingly, in the presence of DAP, the genes associated with branched-chain amino acid metabolism and higher alcohol formation were generally upregulated (Marks et al. 2003). The apparent discrepancy between gene regulation and higher alcohol production still needs to be investigated.

The formation of phenylethanol follows a similar trend to the other higher alcohols, that is, an inverse relationship exists with its corresponding amino acid, phenylalanine. n-Propyl alcohol is, however, a clear exception, whose production is directly related to initial nitrogen concentration and yeast growth (Boulton et al. 1998). The production of n-propyl alcohol seems not to be influenced by the concentration of structurally related amino acids, threonine or α-aminobutyric acid (Guymon 1972,
Table 5. Yeast metabolites of amino acids and related compounds.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Keto acid</th>
<th>Aldehyde</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>2-Ketopropionic acid</td>
<td>Acetaldehyde</td>
<td>Ethanol</td>
</tr>
<tr>
<td>2-Amino butyric acid or Threonine</td>
<td>2-Ketoisocaproic acid</td>
<td>Isovaleraldehyde</td>
<td>2-Methyl-1-butanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(active amyl alcohol)</td>
</tr>
<tr>
<td>Serine</td>
<td>3-Hydroxy-2-ketopropionic acid</td>
<td>Glyoxal</td>
<td>2-Phenylethyl alcohol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tyrosol</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2-Keto-3-methylvaleric acid</td>
<td>2-Methylbutyraldehyde</td>
<td>2-Methyl-1-butanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(isoamyl alcohol)</td>
</tr>
<tr>
<td>Leucine</td>
<td>2-Ketoisocaproic acid</td>
<td>Isovaleraldehyde</td>
<td>3-Methyl-1-butanol</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3-Phenyl-2-ketopropionic acid</td>
<td>–</td>
<td>2-Phenylethyl alcohol</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>–</td>
<td>–</td>
<td>Tryptophol</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3-(4-Hydroxyphenyl)-2-ketopropionic acid</td>
<td>–</td>
<td>Tyrosol</td>
</tr>
<tr>
<td>Valine</td>
<td>2-Ketoisovaleric acid</td>
<td>Isobutyraldehyde</td>
<td>2-Methyl-1-propanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(isobutanol)</td>
</tr>
</tbody>
</table>

Nykänen 1986, Rapp and Versini 1991). Also, methionol, which appears to be formed from methionine by the Ehrlich pathway, shows a direct relationship with initial methionine concentration (Hernández-Orte et al. 2005). Hexanol is formed from a must-derived precursor, probably hexanal. Hexanal could be formed from grape linolenic and linoleic acids during must processing, since yeast are not able to synthesise it. The pattern of hexanol formation therefore depends largely on the formation of its precursor during grape harvest and must processing, and is not greatly influenced by initial must nitrogen content. In summary, since the mechanism of formation of the various higher alcohols varies, the relationship between immediate amino acid precursor and total nitrogen concentration in the must and higher alcohol concentration also varies.

3.3.5.3 Esters

Esters, which generally impart pleasant fruity and floral aromas, make a major contribution to the vinous character of wine, and provide the ‘scaffolding’ onto which the grape-derived aromas are projected to give wine its varietal character. Wine esters, which confer the generic or fermentation bouquet, are largely derived from the metabolism of sugars and amino acids by yeast, while several esters are derived from grape glycosides (Lambrechts and Pretorius 2000, Swiegers et al. 2005). Several esters that confer varietal character have been identified recently. Ethyl anthranilate, ethyl cinnamate, ethyl 2,3-dihydroxy-cinnamate and methyl cinnamate, which are found in Pinot Noir, are derived from grape precursor compounds and only released by fermentation (Moio and Etiévant 1995). The effect of must nitrogen concentration on the formation of this group of esters is not known.

Generally, nitrogen application in the vineyard results in an increase in wine ester concentration (Table 4). The acetate esters characteristically have a fruity aroma, whereas the ethyl esters of small acyl chain fatty acids are described as ‘fruity’ or ‘floral’, and those derived from the longer acyl chain acids are more ‘soap-like’ (Table 4; Rapp and Versini 1996). Although the principal esters of wine and their characteristic odours are well known, the aroma profile of wine appears to be defined by esters as a group rather than by individual compounds, unless an ester is represented in a relatively high concentration (van der Merwe and van Wyk 1981, Francis and Newton 2005). Giorgessi et al. (2001) found that wines made with fruit from nitrogen-treated vines scored consistently higher in ‘fruity’ aroma in comparison to wines from untreated vines, and that the fruity aroma sensory score was strongly correlated with the concentration of fruit esters in the wines. However, in the second season of the study nitrogen application had no effect on fruit ester or higher alcohol concentrations, despite augmenting the total nitrogen concentration in the wines from vines receiving no nitrogen. It was observed that the must total and amino acid nitrogen concentrations were much lower in both the plus nitrogen and control treatments compared to the previous year (Giorgessi et al. 2001). The second season may have been drier than the first, thus limiting nitrogen uptake by the vines.

The principal esters of wine are synthesised enzymatically by yeast from alcohols and fatty acids, but esterases and wine acidity compete by causing their hydrolysis (Lambrechts and Pretorius 2000) (Figure 10). The fatty acids are first activated with coenzyme A (CoASH) before esterification with alcohols. Thus, acetyl-CoA is condensed with higher alcohols by the enzyme alcohol acetyltransferase to form acetate esters (Peddie 1990). The ethyl esters are formed by the enzymatically catalysed reaction between ethanol and activated medium and long chain fatty acids. Because of the high concentrations of acetyl-CoA and ethanol, compared to other activated fatty acids and alcohols produced during fermentation, ethyl acetate reaches the highest concentration of the esters in wine after fermentation.

The concentrations of esters produced by different strains of yeast vary, but for some strains these variations are not sufficient to produce aroma profiles that are statistically different (Avedovech et al. 1992, Jane et al. 1996,
Lema et al. 1996, Dumont and Dulau 1997, Henick-Kling et al. 1998, Vila et al. 1998). This is not surprising, because no aroma character in wine can generally be linked to a particular ester of yeast origin (van der Merwe and van Wyk 1981, Francis and Newton 2005). The grape-derived esters, however, can have more distinct odours and can therefore have a distinctive impact (Moio and Etiévant 1995, Swiegers and Pretorius 2005). Nevertheless, several yeast strains, such as AWRI 350, have been identified that produce high amounts of several esters, including isoamyl acetate, hexyl acetate, ethyl hexanoate and ethyl octanoate, which can noticeably impact on the aroma profile (Rankine 1977, Soles et al. 1982, Lambrechts and Pretorius 2000). The nitrogen demand of yeast might also affect ester production; a strain with high nitrogen demand when compared with one of lower demand produced higher total esters and lower total higher alcohols, though some volatiles showed strong variation from the general trend (Torrea et al. 2003).

Improving our understanding of the cellular regulation of ester biosynthesis will be important to gaining better control over ester formation. The alcohol acetyltransferase genes, ATF1, ATF2, LgATF1 and EHT1, have been identified and partially characterised (Fujii et al. 1994, Swiegers and Pretorius 2005). The alcohol acetyltransferase enzymes encoded by these genes vary in the pattern of esters produced and it is likely that variability in the expression of these and related genes defines the ester profile. Environmental factors, such as nitrogen, in addition to oxygen and lipids, affect the pattern of esters in wine and it is likely that these effects are partly mediated through the regulation of the Ehrlich, fatty acid and ester synthetic pathways, and substrate and cofactor availability (Yoshimoto et al. 2002, Swiegers and Pretorius 2005). With respect to must nitrogen concentration, except at very high concentration, direct relationships have been found for ethyl acetate, 3-methyl-1-butyl and 2-methyl-1-butyl acetate (isoamyl acetate). However, for 2-phenylethyl acetate, the effect was variable and an inverse relationship for 3-methylthiopropan-1-ol was found (Bell et al. 1979, Ough and Lee 1981, Cantagrel et al. 1982, Seeber et al. 1991, Yoshimoto et al. 2002, Torrea and Henschke 2004).

There are some indications that the ester and higher alcohol profile of wine might contribute to varietal specificity (Etiévant et al. 1991, Seeber et al. 1991, Ferreira et al. 1996, Garcia et al. 1998, Ferreira et al. 2000, Smyth et al. 2005). The work of Smyth et al. (2005) has shown that Chardonnay wines contain the ethyl esters ethyl-2-methyl propanoate, ethyl-2-methyl butanoate, ethyl-3-methyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and the acetate esters hexyl acetate, 2-methylbutyl acetate and 3-methylbutyl acetate. While Riesling wines contained similar esters, only ethyl hexanoate was unimportant. However, concentration differences for the esters existed between wine types. In addition, the higher alcohols 2-methylpropanol, 2-methylbutanol and 3-methylbutanol and the acids hexanoic acid, octanoic acid and decanoic acid were only important for Chardonnay wines.

In an attempt to explain the varietal aroma profile link, model studies have been performed by Hernández-Orte et al. (2002) to establish whether the amino acid composition of must might contribute to the characteristic varietal volatile composition of wine. Wines were prepared from model fermentation media made up with the characteristic amino acid composition of eleven grape varieties. Statistically significant differences in the concentrations of some of the volatiles, ethanol, ethyl acetate, acetic acid, higher alcohols, acetate esters, methionol, isobutyric acid, ethyl butyrate, hexanoic acid and octanoic acid, were found. The concentrations of some of the volatiles correlated well with the aromatic composition of the equivalent wines. Development of models by chemometric analysis showed that threonine and serine affected corresponding fatty acid esters and alcohols, phenylalanine affected β-phenylethyl alcohol and methionine strongly affected methionol concentration. This study suggests that the amino acid composition of must could be more important to the aroma profile of wine than previously believed, and could have implications for the clonal selection of grape cultivars.

3.3.5.4 Volatile fatty acids
Volatile fatty acids, of which acetic acid accounts for more than 90%, have an important impact on wine quality. Concentrations vary widely (<0.2 to >2.0 g/L) and depend on wine type. Dry red wines typically have higher concentrations than dry white wines, whilst sweet whites tend to have the highest concentrations (Eglinton and Henschke 1999). The flavour threshold varies according to wine type and style, from 0.4 to 1.1 g/L.

Acetic acid is formed as a metabolic intermediate in the synthesis of acetyl-CoA from pyruvic acid (Figure 10). It is formed directly from acetaldehyde by aldehyde dehydrogenases. Five aldehyde dehydrogenases exist in yeast, being located in the cytoplasm and mitochondria, and are regulated independently (Navarro-Avino et al. 1999). The aldehyde dehydrogenases encoded by the genes ALD6, ALD4 and ALD5, are most important under anaerobic wine fermentation conditions (Saint-Prix et al. 2004). In addition to providing the acetic acid precursor for acetyl-CoA, the oxidation of acetaldehyde provides a redox sink, with surplus acetic acid being excreted (Eglinton et al. 2002, Saint-Prix et al. 2004). Many factors affect acetic acid production, including yeast strain, sugar concentration, nicotinic acid, inoculation rate, fermentation rate, fermentation temperature, pH, aeration and nitrogen (Henschke and Jiranek 1993). Yeast strain has a major impact on acetic acid concentration in wine, but the initial nitrogen concentration is important, for which an inverse relationship exists, except at high nitrogen concentrations exceeding 450 mg N/L, when high concentrations of volatile acidity are formed (Agenbach 1977, Tromp 1984, Dukes et al. 1991, Torrea and Henschke 2004). In high sugar musts (>320 g/L) an inverse relationship was found up to a YAN concentration of 200 mg N/L, above which a direct relationship resulted (Bely et al. 2003). Must nitrogen concentration is, therefore, of considerable importance in controlling volatile acidity in wine.
The medium-chain fatty acids also occur in wine, but at low concentration (Bardi et al. 1999). Hexanoic, octanoic and decanoic acids are important to the aroma profile of Chardonnay but not Riesling wines (Smyth et al. 2005). However, the impact of must nitrogen on their formation is not well described.

3.3.5.5 Carbonyls
The short-chained aliphatic aldehydes, particularly acet-aldehyde, and diacetyl constitute the principal carbonyls produced by yeast during fermentation. Acetaldehyde imparts a green, bruised apple or nutty aroma and diacetyl is characterised by a buttery aroma (Liu and Pilone 2000). Hexanal, which is present in grape must at high concentration but low in wine, imparts a green character. The aliphatic carbonyls are formed as intermediates in the formation of alcohol/higher alcohols, anabolically from sugar and catabolically from amino acids by the Ehrlich pathway (Figures 10 and 12, Table 5). Diacetyl is formed by the decarboxylation of α-acetolactate, and hexanal is derived from C6 lipid metabolism in grapes. As is the case for the regulation of the aliphatic higher alcohols, the availability of the respective amino acid affects the formation and excretion of the related aliphatic aldehyde. Yeast strain, fermentation temperature, aeration and sulfur dioxide are also important factors affecting formation of carbonyl compounds (Nykänen 1986, Rapp and Versini 1991). As for acetic acid production, which is derived from acetaldehyde, an inverse relationship between acetaldehyde production and must nitrogen has been reported (Dukes et al. 1991).

Diacetyl synthesis is regulated by the availability of nitrogen, especially valine and threonine (Dufour 1989, O’Conner-Cox and Ingledew 1989). When nitrogen is low, valine synthesis is activated, leading to formation of the intermediate, α-acetolactate. This compound spontaneously decomposes by oxidative decarboxylation to diacetyl, which, depending on fermentation conditions, is reduced to 2,3-butanediol via acetoin. The latter two compounds have much lower aroma thresholds than diacetyl. α-Acetolactate formation, and hence that of diacetyl, is suppressed when nitrogen availability is sufficient, due to the repression of valine uptake by threonine.

3.3.6 Hydrogen sulfide
Hydrogen sulfide (H$_2$S) is a highly volatile thiol with a very low odour threshold and an objectionable ‘rotten-egg’ odour. There are many causes of H$_2$S occurrence in grape must and wine (Monk 1986), but only those associated with yeast and nitrogen will be considered here.

It is well established that H$_2$S is formed metabolically by yeast from either inorganic sulfur compounds, sulfate and sulfite, or organic sulfur compounds, cysteine and glutathione (Rankine 1963, Eschenbruch 1974, 1978, Schütz and Kunkee 1977, Monk 1986, Henschke and Jiranek 1993, Rauhut 1993, Jiranek et al. 1995b, Spiropoulis et al. 2000). H$_2$S is usually formed in response to a metabolic requirement, such as that induced by growth, for organic sulfur compounds, which include cysteine, methionine, S-adenosyl methionine and glutathione. Some strains of yeast, however, appear to form unregulated amounts of H$_2$S and presumably represent metabolic defects, at least in the wine environment (Jiranek et al. 1995b, Spiropoulis et al. 2000). Grape must is typically deficient in organic sulfur compounds (less than 10 mg/L cysteine and methionine) (Henschke and Jiranek 1991). The deficiency of organic sulfur compounds in the must signals yeast to synthesise organic sulfur compounds from inorganic sources, which are typically plentiful in grape must (Henschke and Jiranek 1991, Hallinan et al. 1999) (Figure 11). H$_2$S is, therefore, a metabolic intermediate in the reduction of sulfate or sulfite needed for the synthesis of organic sulfur compounds (Rauhut 1993). When these reactions proceed in the presence of a suitable nitrogen supply, H$_2$S is sequestered by O-acetyl homoserine, which is derived from nitrogen metabolism, to form the organic sulfur compounds (Ono et al. 1999). However, under some conditions, when insufficient or unsuitable nitrogen sources are available, free H$_2$S can accumulate in the cell and diffuse into the fermenting must (Henschke and Jiranek 1991).

H$_2$S that is formed during the early to middle stages of grape must fermentation is associated with yeast growth and typically responds to nutrient addition, especially DAP (Henschke and Jiranek 1991, Jiranek et al. 1995b). Many factors have been reported to affect H$_2$S production, including yeast strain, sulfur source, nitrogen composition and nitrogen concentration of the test medium (reviewed by Spiropoulis et al. 2000). Surveys by different methods show that strains of yeast vary widely in their ability to produce H$_2$S, thereby making the yeast strain the most important factor in determining H$_2$S production (Rankine 1968a, Eschenbruch et al. 1978, Thornton and Bunker 1989, Thomas et al. 1993, Giudici and Kunkee 1994, Jiranek et al. 1995c, Spiropoulis et al. 2000, Mendes-Ferreira et al. 2002). However, the characterisation of strains has proven technically problematic due to poor understanding of the regulation of sulfur metabolism in yeast. Recent studies by L. Bisson at UC Davis have suggested that no one method is suitable for determining the potential of a strain to produce H$_2$S under winemaking conditions (Spiropoulis et al. 2000). This is largely due to the fact that the regulation of sulfur and nitrogen metabolism in yeast is complex (Mountain et al. 1991, Hinnebusch 1992, Gasch et al. 2000, Marks et al. 2003). Laboratory studies have shown that for a nitrogen responsive strain, the ability of an amino acid to regulate H$_2$S is related to its efficiency as a nitrogen source, though there are some notable exceptions (Jiranek et al. 1995b). As ammonium represses the Met10 gene, which encodes the alpha-subunit of sulfite reductase (Hansen et al. 1994), it is not surprising that DAP is widely used in the practical control of H$_2$S production, however, it is not always effective. Many factors can be involved, apart from yeast strain. An important factor, which is often overlooked, relates to the concentration of methionine. Methionine, in combination with other nitrogen sources, regulates amino acid transport into the cell and sulfur metabolism, especially the sulfate reduction sequence, which generates H$_2$S. Thus, the effectiveness of nitrogen sources, such as DAP,
depends on the concentration of methionine, at least for some strains, such as UCD522. Therefore, H$_2$S production was little affected by DAP addition when the concentration of methionine was low, and likewise methionine addition had minimal effect at low ammonium concentrations (Spiropoulis et al. 2000).

Henschke and Jiranek (1991) also observed a second phase of H$_2$S production that was associated with the final stages of fermentation. This phase was essentially unresponsive to DAP, although in two experiments DAP appeared to exacerbate H$_2$S production when fermentations were aerated (Thomas et al. 1993, Henschke and de Kluis 1995). Limited evidence for responses to aeration and vitamin addition have been observed (Henschke 1996). Nutrient levels are typically very low during the final stages of fermentation, suggesting the involvement of vitamins or degradation of S-reserves (Eschenbruch et al. 1978, Henschke 1996, Hallinan et al. 1999, Wang et al. 2003). Recent evidence suggests that intracellular glutathione can be degraded to cysteine and ultimately H$_2$S under nitrogen deficit conditions (Elskens et al. 1991, Hallinan et al. 1999). H$_2$S is known to be produced directly from cysteine by cysteine desulphhydrase when nitrogen is limited (Tokuyama et al. 1973).

Although recent investigations are improving our understanding of sulphur metabolism in yeast, especially in relation to H$_2$S production, the use of strain selection remains the most useful tool for minimising H$_2$S production in winemaking. Whilst H$_2$S production during must fermentation has many causes, measurement of must YAN and the use of nitrogen supplements (e.g. DAP) continues to be the best approach in the practical control of

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**Figure 11.** Sulfur metabolism in wine yeast.

Amino acids, including methionine, are accumulated from the medium and contribute to the cell’s total N pool and protein synthesis. When methionine becomes depleted (early in fermentation), the Sulfate Reduction Sequence (SRS) pathway is activated to accumulate and reduce sulfate, or sulfite, to hydrogen sulfide (H$_2$S). H$_2$S is incorporated into two amino acid precursors, O-acetyl homoserine (OHS) and O-acetyl serine (OAS), to form methionine and cysteine, respectively. If amino acids and ammonium become depleted in the medium, synthesis of OAH and OAS ceases but SRS pathway continues, releasing surplus H$_2$S and mercaptans from the cell. Addition of nitrogen (i.e. DAP) restores the nitrogen pool, allowing OAH/OAS synthesis, whereby methionine synthesis is restored and prevents H$_2$S release from the cell.

**Figure 12.** The Ehrlich pathway for the formation of higher alcohols from amino acids and sugar.

A deficiency of amino acids during growth activates their synthesis from α-keto acids, derived from sugars via glycolysis. If insufficient nitrogen is available for transamination reactions, surplus α-keto acids are excreted as higher alcohols. During amino acid sufficiency, transamination of amino acids can produce a surplus of α-keto acids, some of which are decarboxylated and reduced to alcohols.
In addition to hydrogen sulfide, a variety of other thiols exist in wine, and although they are usually present in only very low concentrations (< ng/L range), they generally confer highly objectionable aromas. However, several confer pleasant fruity aromas (Rauhut 1993, Darriet et al. 1995, Ribéreau-Gayon et al. 2000, Swiegers and Pretorius 2005). Many of these S-compounds are formed during fermentation and respond to the nitrogen status of the must in a similar manner to \( \text{H}_2\text{S} \). For the purpose of this discussion, the wine associated thiols will be divided into three groups: highly volatile thiols (bp <90ºC), which include hydrogen sulfide, methanethiol (MeSH), ethanethiol (EtSH) and dimethyl sulfide (DMS), low volatility thiols (bp >90ºC), including methionol and 2-mercaptoethanol, and the grape-derived thiols, 4-mercapto-4-methylpentan-2-one (4MMP), 4-mercapto-4-methylpentan-2-ol (4MMPOH), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA).

The highly volatile thiols, MeSH and EtSH, give wines rotten egg/cabbage and onion/rubber aromas, respectively, whereas DMS at low odour values is considered more desirable, including aromas reminiscent of asparagus, cooked corn and molasses (Rauhut 1993). Fermentation studies suggest that MeSH is derived from methionine whereas EtSH is believed to be formed by reaction between \( \text{H}_2\text{S} \) and acetaldehyde (Rauhut 1993). In addition, thioacetic acid esters formed during fermentation slowly hydrolyse to MeSH and EtSH (Rauhut et al. 1996). Rauhut et al. (1996) observed the formation of these thiols and their esters in conjunction with \( \text{H}_2\text{S} \) and noted that their production was suppressed by DAP in nitrogen-responsive strains. The origin of DMS is not clear but it might be formed by yeast from cysteine, cystine or glutathione or from dimethyl sulfoxide by yeast reductase (Rauhut 1993, Ribéreau-Gayon et al. 2000a). The concentration of DMS in wine is higher when grapes are sourced from cooler regions, such as Coonawarra and New Zealand (de Mora et al. 1986), suggesting that the grape-derived precursor compound is influenced by viticultural conditions. In the Coonawarra region for example, DMS evolution in wine has been controlled by canopy and crop level management. Supplementation of must fermenters with either cysteine, methionine or their combination confirmed their importance as DMS precursor compounds (Moreira et al. 2002), but whether this mechanism exists in grapes is unknown.

The most important low volatility thiol, 3-(methylthio)-1-propanol (methionol), which gives a raw potato or cauliflower aroma, is present in wines at up to 5 mg/L. It is derived from methionine by the Ehrlich pathway, which involves deamination to the keto acid, decarboxylation to the aldehyde and enzymatic reduction to the alcohol. Methionol, its corresponding acid and ester all increased upon methionine supplementation of fermenters, suggesting that methionine concentration might play a role as well as must total nitrogen concentration (Rauhut 1993, Moreira et al. 2002). 2-Mercaptoethanol, which confers a poultry-like aroma, is hypothesised to be formed from cysteine. However, in supplementation experiments using musts with different nitrogen concentrations, elevated 2-mercaptoethanol was observed in only one experiment (Moreira et al. 2002).

The volatile thiols 4MMP, 4MMPOH, 3MH and 3MHA have been identified as major contributors to the varietal aroma of Sauvignon Blanc wines, and are associated with aroma characteristics such as box tree, blackcurrant, grapefruit, passionfruit and citrus zest (Darriet et al. 1995, Tominao et al. 1995, 1998b, Dubourdieu 2000). The compound 4MMP is also present in wines made from Scheurebe, Gewürztraminer, Riesling, Colombard, Petit Manseng, Semillon, Cabernet Sauvignon and Merlot. These volatile thiol aroma compounds, however, are only found in trace amounts in grapes and must. Tominao et al. (1998a) identified the aroma precursors in Sauvignon Blanc musts as non-volatile, non-glycosylated, odourless S-cysteine conjugates.

Berry assimilable nitrogen increased upon the application of nitrogen to a soil that had a low nitrogen status (Choné 2003). Correspondingly, the concentration of the 4MMP, 4MMPOH and 3MH precursors in berries from vines receiving nitrogen were 1.7, 2.7 and 4.4 times higher, respectively, compared to the levels in berries from vines that received no nitrogen. When water was non-limiting, vines grown on soils with moderate levels of nitrogen had a higher vine nitrogen status and higher concentrations of 4MMP, 4MMPOH and 3MH precursors in the berry compared to those from vines growing in a soil with a low nitrogen status (Peyrot des Gachons et al. 2005).

Degradation of the S-cysteine thiol precursors by yeast during alcoholic fermentation releases the corresponding volatile thiols (Tominao et al. 1998a, Murat et al. 2001, Howell et al. 2004). The mechanism has been suggested to involve a cysteine \( \beta \)-lyase, which, in the case of S-(4-methylpentan-2-one), releases 4MMP, pyruvic acid and ammonium (Tominao et al. 1995). Yeast strains vary in ability to affect the concentration of 3MH, 4MMP and 4MMPOH in wine (Murat et al. 2001, Howell et al. 2004).

Commercial strains, *Saccharomyces cerevisiae* V1.3c and EG8, release more volatile thiols than strains VL1 and 522d. Strains of *Saccharomyces bayanus* and their hybrids made with *Saccharomyces cerevisiae* release even greater concentrations of volatile thiols. The role of must nitrogen status on thiol release is not known, but given that ammonium is released, it might prove important.

### 3.3.8 Phenolics

Phenolic compounds are an important constituent of white and especially red grapes and, consequently, wines. They play an important role in the sensory properties of wines, including colour, flavour and mouth-feel, and act as antioxidants, and thus have been associated with health benefits (Kinsella et al. 1993). There is, however, very
little information concerning the impact of nitrogen application in the vineyard on phenolic compounds, such as anthocyanins and tannins, which are important to the colour and astringency of red wines. Seven viticultural trials have considered colour and anthocyanin content of grape berries in relation to nitrogen application to vines, with variable results, whereas only one study has reported vineyard nitrogen effects on wine colour. No studies have investigated nitrogen supplementation of red fermentations on wine colour and phenolic composition.

Nitrogen application in the vineyard increased the assimilable amino nitrogen concentration of Shiraz juice for all rootstocks (5C Teleki, Schwarzmann, Ramsey), with the exception of 5C Teleki supplied with nitrogen postharvest (Treeby et al. 2000). When nitrogen was applied postharvest the colour density of young wines from vines receiving nitrogen was generally lower in comparison to those wines from untreated vines, even in the 5C Teleki treatment in which assimilable amino nitrogen was not affected. There was, however, no effect on the total anthocyanin concentration of these wines. This is not surprising, as the contribution of monomeric anthocyanins to wine colour quickly declines during fermentation and in young wine. The anthocyanins react with fermentation metabolites and with other phenolic compounds to form more stable pigments, such as polymeric pigments, pyranoanthocyanins and vitisins (Somers 1966, Fulcrand et al. 1998, Romero and Bakker 1999, Hayasaka and Asenstorfer 2002, Håkansson et al. 2003, Eglinton et al. 2004). These compounds play a major role in the long term colour density and stability of red wine. The total phenol concentration in Shiraz juices from the nitrogen-treated Schwarzmann and Ramsey vines rose in response to nitrogen application and certainly gave no indication that wine colour density might be reduced (Treeby et al. 2000). However, the increase in total phenol concentration might have been due to an increase in the tannin concentration in the juice rather than the anthocyanins, but this is only supposition as neither anthocyanins nor tannins were measured in the juice. It is more likely that the reduction in wine colour density was due to the decrease in fermentation time associated with a vineyard nitrogen application, which shortened the amount of time spent on skins (Treeby et al. 2000). Wine from Shiraz grafted onto a Schwarzmann rootstock had the highest juice free amino acid concentration of all Shiraz/rootstock treatment wines and was the only treatment where postharvest application of nitrogen had a significant negative impact on the sensory characteristics of the resulting wine.

There was very little effect of summer-applied nitrogen on the phenolic components of the wines from the same three rootstocks described above (Treeby et al. 2000). Wine from Shiraz grafted onto Ramsey vines had the highest juice free amino acid concentration of all Shiraz/rootstock treatment wines and was the only treatment where a summer application of nitrogen decreased wine colour density and had a significant negative impact on the sensory characteristics of the resulting wine.

Maigre (2002) did not measure colour in the grape juice, but observed that both the anthocyanin concentration and colour density in the wines were influenced by nitrogen to a small degree. In this study, the initial nitrogen status of vines grown with no permanent grass cover (NC) was higher than vines grown with a permanent grass cover (PC). Nitrogen application decreased the anthocyanin concentration and colour density of wines from the NC treatment, but increased both components in the wines from the PC treatment (Maigre 2002). Furthermore, nitrogen application in the vineyard decreased skin anthocyanin concentration and the colour intensity of the juice in a study in which the initial nitrogen status of control vines was adequate to high (Delas 1993). It is not known if these changes would be reflected in the wine. However, it appears that the impact of nitrogen on wine colour might be dependent in part on the initial vine nutrient status. Presumably wine colour might benefit from nitrogen application if the initial vine nitrogen status is low, with a reversal of this effect if the initial vine nitrogen status is adequate to high.

Nitrogen application in the vineyard via its effect on the final berry anthocyanin concentration and fermentation time will play a role in determining the amount of colour extraction achieved. However, while there have been many advances in the area of wine phenolic chemistry, it is not known if nitrogen application in the vineyard has an impact on wine tannins and the development of compounds contributing to the long term colour stability of the wine.

### 3.4 Residual nitrogen and microbial instability

Grapes with a high nitrogen content or excessive addition of DAP during fermentation can lead to significant residual nitrogen, which, for several winemaking processes, such as tirage and MLF, is beneficial. However, residual nitrogen in wine under some circumstances could encourage microbial instability and lead to loss of wine quality. However, note that in addition to the use of DAP, a variety of fermentation conditions, such as choice of yeast strain, temperature, must clarification, aeration, use of yeast foods, yeast ghosts or hulls and yeast lees contact, can to various degrees affect the removal or accumulation of assimilable nitrogen, and consequently affect wine residual nitrogen (reviewed by Henschke and Jiranek 1993, Boulton et al. 1998, Jackson 2000).

Residual nitrogen is also thought to be an important contributor to problems arising from the presence of *Brettanomyces* in wine (P. Godden, pers. comm.). *Brettanomyces bruxellensis*, which is commonly found in red wines globally, has the ability to form volatile phenols from phenolic acids (Heresztyń 1986, Chatonnet et al. 1992), although other volatiles of sensory significance are undoubtedly formed (Coulter et al. 2003). Some of the aroma descriptors associated with wine infected by *Brettanomyces* include ‘band-aid’, ‘burnt plastic’, ‘medical’ and ‘barnyard’. In addition to producing volatile phenols, *Brettanomyces* can produce N-heterocyclic compounds, which elicit an intense off-flavour, often referred to as ‘mousy’ (Herderich et al. 1995, Grbin et al. 1996, Grbin and Henschke 2000). These compounds were found to be
derived from lysine and ornithine metabolism. Under certain conditions, these amino acids were partially denminated and acetylated to form the pyridine compounds, 2-ethyltetrahydropyridine, 2-acetyltetrahydropyridine and 2-acetyl-1-pyrroline. Lactic acid bacteria are also capable of forming 2-ethyltetrahydropyridine, 2-acetyltetrahydroxypridine and 2-acetyl-1-pyrroline from lysine and ornithine (Costello et al. 2001, Costello and Henschke 2002). The proposed pathway for the formation of mousy off-flavour compounds from lysine and ornithine is shown in Figure 13. Although off-flavour compound formation was stimulated by high concentrations of lysine and ornithine, there is no evidence that the formation of mousy off-flavour in wine is triggered by these amino acids. It is more likely that the excessive presence of normally growth-limiting nutrients, such as oxygen and nitrogen, stimulates microbial growth, and hence off-flavour formation.

Brettanomyces, which can utilise a wide range of nitrogen compounds, can readily grow on ammonium salts as a sole source of nitrogen, although complex nitrogen, as provided by yeast extract, greatly stimulates growth (van Zyl 1962, Uscanga et al. 2000). Generally, growth of Brettanomyces is thought to be associated with the indiscriminate use of DAP, but prolonged conservation of wine on yeast lees is also likely to considerably stimulate Brettanomyces growth.

No published data has been found to suggest that a relationship between nitrogen application in the vineyard and the presence of Brettanomyces in wine exists. However, nitrogen application does affect the level of residual nitrogen. Bell et al. (1979) found that the percentage of nitrogen remaining in the wine was, on average, 1.4 times greater in wines from nitrogen-treated vines compared to those from control vines that received no nitrogen. Additionally, the residual proline nitrogen concentration was, on average, 1.8 times higher in wines from nitrogen-treated vines compared to wines from control vines (Ough and Bell 1980). Because proline is not consumed to a large extent during alcoholic fermentation, it is generally the greatest contributor to the total amino acids present in the wine. When proline was the only amino acid present in a mineral medium, Brettanomyces growth was supported (van Zyl 1962), although the minimum amount of oxygen needed to suppress proline assimilation is not known. The minimum nitrogen requirements for Brettanomyces development in wine are still to be defined.

Laboratory fermentation trials with Chardonnay juices supplemented with DAP to give a range of initial YAN values up to 500 mg/L, and fermented with three different yeasts, showed that residual nitrogen was only detected when the initial concentration exceeded 400 mg/L (Jiranek et al. 1995a, Torrea and Vilanova, unpublished data). Surveys of grape juices prepared from different varieties have shown that the mean values rarely exceeded 350 mg N/L for Australian samples (Gockowiak and Henschke 1992, Henschke and Jiranek 1993, Stines et al. 2000), or 400 mg N/L for other countries (Sponholz 1991, Spayd and Andersen-Bagge 1996, Butzke 1998, Shively and Henick-Kling 2001). However, winemaking practices, such as adding excessive amounts of DAP or keeping wine in contact with yeast after the completion of fermentation, during which time yeast efflux various amino acids (Ough et al. 1991, Stuckey et al. 1991, Monteiro and Bisson 1992b, Valero et al. 2003), provide sufficient nutrients to stimulate malolactic fermentation (MLF) (Patynowski et al. 2002), and undoubtedly spoilage microorganisms. The indiscriminate use of DAP could also result in some wines carrying residual nitrogen, but this is not likely to explain the high infection rate of wine worldwide by Brettanomyces.

3.5 Urea and ethyl carbamate
Ethyl carbamate is found naturally in many fermented foods and beverages. This compound has been identified as a mild human carcinogen and is thus an undesirable component of wine (Ough 1991). Generally, only low concentrations (1.2–4.3 µg/L) of ethyl carbamate have been found in table wine produced from grapes (Ough 1991), although up to 100 µg/L has been reported (Ough 1976), with high levels generally being associated with fortified wines, especially, spirits.

The main source of ethyl carbamate in wine is by the chemical reaction of ethanol with urea, although citrulline and carbamyl phosphate can also participate (Ough et al. 1988a) (Figure 14). Net accumulation of urea is influenced by must nitrogen composition. The two major precursor amino acids in grape of ethyl carbamate are arginine and citrulline (Monteiro et al. 1989, Ough 1991). Arginine is present in far larger amounts than citrulline (Huang and Ough 1989), and is therefore likely to be the more important precursor. Urea is formed and excreted as a consequence of arginine degradation by yeast during fermentation, however, strains vary in their ability to excrete urea (Ough et al. 1990). Tegmo-Larsson and Henick-Kling (1991) concluded that yeast metabolism of arginine will only contribute to the total amount of ethyl carbamate precursors if the juice is rich in arginine and total free amino acids. Therefore, nitrogen application in the vineyard, via its effect on juice free amino acid concentration (notably arginine) might influence the ethyl carbamate level found in wine. Nitrogen application resulted in a 3–10-fold increase in wine urea concentrations in comparison to urea levels in wines from untreated control vines (Bertrand et al. 1991). Similarly, Spayd et al. (1994) showed that the highest concentration of urea (2 mg/L) was observed in wines that had received the highest level of applied nitrogen (224 kg N/ha).

Bertrand et al. (1991) also reported a good correlation between juice arginine concentration and urea concentration in wine, but this relationship did not hold for urea concentration and ethyl carbamate. In six out of the eight treatment wines, nitrogen application in the vineyard reduced the ethyl carbamate concentration, despite a consistent increase in the concentration of urea in all treatment wines (Bertrand et al. 1991). However, upon heating at 70°C for 72 hours the ethyl carbamate concentration in wines from nitrogen-treated vines rose by 113% to 214% relative to the concentration in wines from control
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vines. Heating for several hours enhances the formation of ethyl carbamate (Sponholz 1991).

Any factor that influences the precursor levels in the berries has the potential to impact on ethyl carbamate formation in the wine. The proline to arginine ratio plays a significant role in determining the predisposition of a grape variety to produce urea. Ough et al. (1990) observed that Cabernet Sauvignon produced relatively low concentrations of urea. This is not surprising as Cabernet Sauvignon is a high proline accumulator and the amount of available arginine is lower, which reduces the potential for urea production. On the other hand, Sauvignon Blanc, Traminer, Muscat Gordo, Grenache and Pinot tend to have lower ratios of proline to arginine (Huang and Ough 1991, Stines et al. 2000), increasing the potential for ethyl carbamate formation. Nitrogen application in the vineyard generally decreases the proline to arginine ratio, which might have potentially negative consequences with respect to ethyl carbamate formation, particularly for high arginine-accumulating varieties. High levels of nitrogen application also result in higher concentrations of residual arginine in the finished wine. Only trace amounts of arginine remained in finished wine from vines receiving no supplementary nitrogen, but the concentration of residual arginine in the wine increased with increasing rates of nitrogen addition (Bell et al. 1979, Ough and Bell 1980).

Because fermentation conditions can markedly influence the accumulation of urea in wine a strong research focus has been placed on understanding urea metabolism with the view to developing strategies for controlling urea concentration in wine (Ough 1991). Urea is formed by the enzymic cleavage of arginine into urea and ornithine by arginase, the first step of arginine catabolism (Ough et al. 1988b, Monteiro et al. 1989) (Figure 14).

Urea is largely degraded to ammonium and carbon dioxide by urea carboxylase and allophanate hydrolase, however it can also be excreted by some yeast strains. Active and facilitated diffusion transport systems can reabsorb the released urea depending upon the nitrogen status of the ferment (Cooper and Sumrada 1975). Not surprisingly then, the accumulation of urea during fermentation has been found to depend on yeast strain and fermentation conditions. In a comprehensive fermentation study by Ough and colleagues (1991), in which four yeast strains were trialled in eleven grape juices of varying nitrogen content, Epernay 2 and Montrachet consistently produced higher concentrations of urea than Prise de Mousse and Flor, thus altering the ethyl carbamate potential of the wines. Heating wines under standard conditions, which activates the reaction between carbamyl compounds, such as urea, with ethanol, provides a useful indication of the ethyl carbamate potential of wine (Sponholz 1991, Ough 1991). Strain differences in arginine uptake and urea

Figure 13. Proposed pathway for the formation of mousy off-flavour N-heterocycle compounds from lysine and ornithine by Brettanomyces bruxellensis and Lactobacillus hilgardii (adapted from Costello and Henschke 2003 and Grbin 2000).

Figure 14. Formation of urea from arginine and its chemical reaction with ethanol to form ethyl carbamate.
excretion and uptake, especially in relation to nitrogen composition and concentration of the fermenting juice, are likely to be involved (Henschke and Jiranek 1993). The high nitrogen concentration of juice, which typically accompanies a high arginine concentration, can delay arginine uptake and consequently urea excretion and subsequent uptake by some strains. DAP addition, which delays amino acid uptake, including that of arginine, also affects urea removal during fermentation. Curtailing DAP addition is important because high amounts of ammonium limit yeast utilisation of amino acids, such as arginine, which would lead to higher levels of residual arginine, and potentially urea in the wine. Thus, DAP addition in mid-to late-fermentation can exacerbate residual urea accumulation in wine. Factors that stimulate nitrogen uptake, such as exposure to oxygen and increased fermentation temperature, on the other hand, can lead to lower wine residual urea content (Henschke and Ough 1991, Ough et al. 1991). Gene expression studies, such as published by Marks et al. (2003), should help to clarify the apparently complex and conflicting reports on factors affecting urea metabolism.

Bacteria can also contribute to ethyl carbamate formation in wine by forming appropriate precursors (Liu et al. 1994). Depending on the strain, citrulline and carbamyl phosphate can be formed from arginine by the arginine deaminase pathway (Mira de Orduña et al. 2000, Tonon and Lonvaud-Funel 2002). A subsequent reaction with ethanol in a time and temperature dependent manner leads to carbamate formation.

3.6 Biogenic amines
Biogenic amines are another group of nitrogen-containing compounds found in wine. Histamine is one such biogenic amine that is well studied due to its impact on human health. If consumed in sufficient amounts, histamine can cause symptoms such as facialflushing, mild headaches and asthma (Stockley 2004). However, at best only minor amounts of histamine are present in grape must, but other amines, namely the polyamines (e.g. putrescine, spermidine, spermine and cadaverine), are commonly present in the grape must (Radler and Fäth 1991). Histamine is predominantly formed in wine by the bacterial decarboxylation of the amino acid histidine (Radler and Fäth 1991, Sponholz 1991, Stockley 2004). Refer to Lehtonen (1996), Soufleros et al. (1998) and Lonvaud-Funel (2001) for recent reviews on the occurrence and formation of amines in wine.

Nitrogen application in the vineyard increased the concentration of histidine in juice and wine (Bertrand et al. 1991, Spayd et al. 1994, Conradi 2001). Bertrand et al. (1991) showed that the concentration of histidine in wine increased by 1.6- to 3.8-fold upon the application of an excessive level of nitrogen in the vineyard. In the same study, the histamine concentration of Merlot wines from nitrogen-treated vines was on average double the concentration of histamine in wines from untreated vines. Nitrogen had the same effect on the concentration of other amines such as methyamine, ethylamine, tyramine and phenylamine, although the levels were less than that of histamine. Amine concentrations were affected by root-stock, season and nitrogen application and, as a consequence, the concentration of histamine in these wines ranged from 1.86–11.3 mg/L (Bertrand et al. 1991). Stockley (2004) suggests that high microbial spoilage levels in wine might result in higher histamine concentrations. Bertrand et al. (1991) put forward the same argument with regard to the one treatment that had a wine histamine concentration of 11.3 mg/L. This wine had a very high pH of 4.18, which clearly would have been conducive to microbial instability. Bertrand et al. (1991) also reported that putrescine concentrations in wines from nitrogen-treated vines were 1.3 to 3.4 times higher than those of wines from untreated vines. Cadaverine followed a similar trend. However, polyamines, unlike other biogenic amines, such as histamine, have not been reported to have an effect on wine quality.

3.7 Botrytis
The fungus Botrytis cinerea might induce either ‘grey rot’ or ‘noble rot’, with differing effects on wine quality (Ribéreau-Gayon 1988). The noble rot condition is desired for the production of high quality, sweet white wines such as Sauternes. However, it is the undesirable grey rot form that is prevalent in most viticultural areas in the world (Hill 1986).

Botrytis cinerea as grey rot has a negative effect on wine quality via the production of: (a) glucans, which can hinder wine clarification; (b) laccase, an oxidative enzyme that has a significant impact on wine phenols; and (c) wine off-flavours due to products, such as acetic acid, formed by undesirable microorganisms that have gained access to the berry via the entry wound caused by Botrytis infection (Beever and Pak 1988, Ribéreau-Gayon 1988, Godden 2000). The grey rot however, is mainly characterised by degradation of aroma compounds. It has been shown that Botrytis cinerea can metabolise major monoterpenic flavour compounds in the fruit to flavourless polyols. The fruitiness then disappears and unpleasant ‘phewol’ or ‘iodine’ characters can appear (Williams et al. 1986, Ribéreau-Gayon 1988). Not surprisingly, wines made from Botrytis-infected grapes sourced from vines receiving a split application of 50 kg N/ha displayed a reduction in sensory cultivar characteristic and exhibited an ‘off taste’ in comparison to wines made from uninfected grapes (Conradie 2001).

Infection by Botrytis is common in vines with dense canopies (Gubler et al. 1987). Nitrogen application can increase vine vigour (Wolf and Pool 1988, Conradie and Saayman 1989a, Kliewer et al. 1991), which results in increased leaf area and canopy density (Bell and Robson 1999). It is conceivable that increased infection by Botrytis cinerea might be a potential risk upon increasing applications of nitrogen in the vineyard, as the following studies indicate.

The level of tolerance of Botrytis incidence in the Australian wine industry is generally between 3 and 6%. The percentage of berries infected with Botrytis at harvest was, on average, 1.4–1.7 times greater in bunches from vines receiving nitrogen compared to those from vines receiving no nitrogen (Delas 1993). It is unlikely that the
levels of infection recorded in this study would result in the fruit being downgraded as the highest percentage of berries affected was 2.25%. Christensen et al. (1994) reported a 2% increase in the percentage of bunches displaying rot from the plus nitrogen treatment (112 kg N/ha) in comparison to that from the untreated control vines. Whether an incidence level of 7% for the nitrogen-treated bunches was acceptable would be a commercial decision. In contrast, a 9 to 35% increase in the percentage of bunches affected by Botrytis was observed upon addition of 160 kg N/ha (Eynard et al. 2000). In this case, 27 to 58% of the nitrogen-treated bunches were affected by Botrytis, which would not be commercially acceptable.

An increase in susceptibility to Botrytis upon the application of nitrogen in the vineyard also has the potential to significantly reduce the juice YAN concentration. Botrytis-infected berries displayed a 2- to 7-fold reduction in total amino acid concentration when compared to uninfected berries (Rapp and Versini 1996). Making the assumption that infection levels were similar for both cultivars; the magnitude of this effect appeared to be cultivar dependent. Rapp and Versini (1996) showed that in comparison to the arginine concentration in juices from uninfected berries, there was an 11-fold reduction in arginine in the cultivar Castor and a 2.8-fold decrease in arginine in Bacchus when infected by Botrytis cinerea (Rapp and Versini 1996). This has negative implications for the proline to arginine ratio, except that proline as well as the other amino acids were also present in lower concentrations in juices from Botrytis infected berries. Therefore, despite the reduction in juice YAN due to Botrytis infection in both cultivars, the impact may be less for Bacchus, as more proline and less arginine was lost from Botrytis-infected Bacchus juices than infected Castor juices.

The addition of DAP to compensate for YAN losses by infection and to stimulate fermentation, however, carries the risk of increased volatile acidity (Bely et al. 2003). Nitrogen application in the vineyard resulted in a 1.1- to 1.3-fold increase in wine volatile acidity compared to those concentrations in wines from vines receiving no supplementary nitrogen (Spayd et al. 1994, Maigre 2002). Spayd et al. (1994) postulated that this might have been due to Botrytis infection in bunches from vines receiving nitrogen, but did not have the data to make this connection. Fermentation studies, which have examined the formation of volatile acidity or acetic acid in response to nitrogen or vitamins additions to must (Eglinton and Henschke 1993, Bely et al. 2003), suggest that the reduction of these nutrients by Botrytis infection would not explain the increased volatile acidity in wine observed by Spayd et al. (1994) and Maigre (2002).

Given that susceptibility to Botrytis infection may increase upon the application of vineyard nitrogen (even in nitrogen-responsive sites), appropriate chemical and non-chemical (e.g. canopy management) strategies should be put in place to manage this risk.

3.8 Protein and wine haze

Another class of nitrogenous compounds in wines are the proteins. Proteins make a significant contribution to the YNAN concentration in must and wine. They constitute the most common cause of haze or cloudy amorphous precipitates in white wines. Haze is an economically serious processing problem as perceived clarity is an important quality attribute of wine. The identity, origin and factors affecting the formation of the proteins associated with haze have recently been reviewed by Høj et al. (2000). Major proteins synthesised in the berry are the same proteins identified as being responsible for haze formation in white wine, i.e. grape-derived thaumatin-like proteins and chitinases (Waters et al. 1996, 1998). These pathogenesis-related (PR) proteins, which are stable at wine pH and resistant to proteolysis, can, therefore, persist throughout the winemaking process and add to the YNAN content of wine. Aggregation during storage in the bottle can lead to wine haze.

Protein synthesis in berries begins at veraison and the protein fraction continues to accumulate as ripening progresses (Tattersall et al. 1997, Høj et al. 2000, Pocock et al. 2000). Therefore, nitrogen application might be expected to magnify the levels of protein accumulated in the ripening berry, thus increasing the unwanted YNAN component and potentially increasing the risk of haze formation. Only one study reported the impact of nitrogen application in the vineyard on juice soluble protein (Spayd et al. 1994). The soluble protein concentration in wine from the vines receiving nitrogen was up to 4.8-times greater than that in the wine from untreated vines. As a result, the bentonite requirement rose 6-fold. The rate of applied nitrogen in the vineyard should take into account the distinct varietal effect. For example, Sauvignon Blanc berries typically accumulate approximately twice the amount of protein as Sultana berries grown under the same conditions (Pocock et al. 2000). Additionally, 45–54% of the total PR protein content was found in the skins (Pocock et al. 1998). Therefore, applying nitrogen to any high PR protein-accumulating white wine cultivar fermented on skins, and/or transported over long distances under poor conditions, might increase the risk of developing haze in the bottle in comparison to a cultivar that accumulates lower concentrations of PR proteins.

As previously discussed, nitrogen application can increase the susceptibility of grapes to infection by Botrytis cinerea. Botrytis-infected Semillon and Chardonnay grapes from regional vineyards showed a 61–92% reduction in the total concentration of PR proteins in the free run juice compared to uninfected grapes (Girbau et al. 2004). Therefore, Botrytis infection not only reduces YAN, but also appears to reduce a major component of YNAN. Nitrogen application might increase the total PR protein concentration during ripening, but a nitrogen-induced Botrytis infection appears to decrease the PR protein component of YNAN, which might have a positive impact on wine quality by reducing the risk of haze formation in the bottle.

In conclusion, when applying nitrogen in the vineyard the vigneron should take into account the PR protein-accumulating characteristics of the cultivar and put in place strategies that minimise juice skin contact under poor transport conditions.
3.9 Atypical ageing flavour

Ortho-aminoacetoephone (O-AAP) is a nitrogen-containing compound that is responsible for the atypical ageing (UTA) off-flavour observed in some white wines (Rapp et al. 1993). UTA wines lose their varietal character and begin to exhibit atypical aromas/flavours such as ‘acacia blossom’, ‘floor polish’, ‘naphthalene’ (moth balls) and ‘wet towel’ (Sponholz et al. 2000, Winter 2003). These characters are quite distinct from the kerosene-like odour, attributed to 1,1,6-trimethyl-1,2-dihydroxynaphthalene (TDN), that some white wines, notably Riesling, display as they age in bottle (Simpson 1978). The taint was first found in German wines in 1988 and has since been observed in South African, American and other European wines (Christoph et al. 1995, Sponholz et al. 2000, Linsenmeier et al. 2004). To date, there has been no scientific evidence to suggest the occurrence of UTA in Australian white wines (Siebert et al. 2003).

The taint compound, O-AAP, is of interest because it appears that there is a relationship between UTA in wines and the YAN concentration in the must (Sponholz et al. 2000). Wines displaying the UTA character have been associated with grapes grown in conditions that can contribute to low vine nitrogen status, such as warm/dry seasons, the presence of competitive swards, early harvest and high yields (Sponholz et al. 2000, Hoenicke et al. 2001). It has also been postulated that the formation of UTA is the result of the grape berries’ response to growing under conditions of stress, for example, insufficient water and low nitrogen supply in dry seasons (Hoenicke et al. 2002, Linsenmeier et al. 2004).

The precursor of O-AAP is the phytohormone indole-3-acetic acid (IAA), which is formed from tryptophan, an amino acid commonly found in grapes and must (Kliewer 1977b, Sponholz 1991, Henschke and Jiranek 1993). Following fermentation, oxidative degradation of indole-3-acetic acid in the presence of sulfur dioxide produces O-AAP (Hoenicke et al. 2001, Hoenicke et al. 2002). Over six years, Linsenmeier et al. (2004) observed an increase in the must total amino acid concentration from wines receiving supplementary nitrogen in comparison to those from untreated wines. The application of vineyard nitrogen, however, had no significant effect on tryptophan and the bound or free IAA concentrations in the must, although there was a correlation between the total IAA and total amino acid concentration in the must (Linsenmeier et al. 2004). If yeast do have a role to play in the development of UTA in white wines, it might be that nitrogen application in the vineyard has an indirect effect on UTA formation via an impact on yeast metabolism. Comparison of free and conjugated IAA concentrations after fermentation carried out in the presence or absence of DAP showed that the concentration of both the free and conjugated forms of IAA were higher in the DAP supplemented fermentations (Hoenicke et al. 2002). This result suggests that DAP slowed the hydrolysis of conjugated IAA and possibly delayed the disappearance of free IAA, such that the concentration of both forms was highest at the point of sulfur dioxide addition. Whether DAP acted by accelerating fermentation, and therefore altering IAA metabolism or negatively affecting the (enzymatic) release of conjugated IAA release, was not determined.

4.0 Wine sensory characteristics

The volatile compounds that engender wine aroma principally comprise primary and secondary metabolites, which are derived from grape, yeast, malolactic bacteria and wood (usually oak) and modified by ageing, of which more than 680 have been identified (refer to Guth and Sies 2002). Non-volatile compounds, which principally arise from the grape and yeast, and can be modified by wine microorganisms and ageing, contribute to wine appearance, flavour and mouth-feel (Fowles 1992, Herderich and Smith 2005). While instrumental techniques and correlative approaches have advanced greatly in recent years (Hayasaka et al. 2005), the definitive evaluation of wine sensory characteristics is still formal quantitative sensory techniques (Francis et al. 2005). This section will review the effects of nitrogen application in the vineyard and winery on wine sensory attributes.

Nitrogen application in the vineyard has varying effects on the sensory characteristics of the final wine. In a study of 30 Zinfandel wines made from vines subjected to different crop level treatments, Sinton et al. (1978) observed a strong positive correlation between wine taste intensity and must nitrogen content ($R^2 = 0.750$). Wines made from Thompson Seedless vines treated with nitrogen had significantly higher aroma and flavour intensity and overall wine quality when compared to wines from control vines (Bell et al. 1979). However, this same study showed that a point was reached at which higher rates of nitrogen application in the vineyard resulting in a total must nitrogen concentration of 701 mg/L did not further increase sensory quality. Goldspink and Frayne (1997) undertook a descriptive analysis of wines that had been made from fruit sourced from vines receiving no nitrogen or vines receiving 150 g N/vine at different times throughout the season. Sauvignon Blanc wines made from vines that received no nitrogen application scored lower than those wines from vines receiving supplementary nitrogen and attracted comments such as ‘oxidised, thin, sweet, watery and green acid’. Wines from nitrogen-treated vines scored higher and were described as ‘clean, herbaceous, floral, grassy, and aromatic’. Conradie (2001) observed that generally wines from nitrogen-treated Bukettraube vines augmented the detectable cultivar characteristic and achieved
Nitrogen application in the vineyard did not always have a positive effect on wine sensory characteristics. Shiraz wines from the Ramsey rootstock plus summer-applied nitrogen and the Schwarzmann rootstock plus autumn-applied nitrogen displayed a reduction in wine colour density and G-G concentration (Treeby et al. 2000). This resulted in a reduced sensory rating for wine colour, overall wine appeal and final wine score. The Shiraz-Schwarzmann wines from nitrogen-treated vines also attracted a lower rating for palate intensity. Nitrogen application increased the assimilable amino nitrogen concentration in the wines from the other rootstock treatment vines, at both times of nitrogen application, but had no impact on the sensory characteristics of the resulting wines. A likely explanation for these observations is that nitrogen application in the vineyard increased the assimilable amino nitrogen concentration of musts, which shortened fermentation duration and wine contact with skins. Consequently, extraction of pigments and G-G may have been affected by fermentation conditions rather than nitrogen effects on berry related metabolites. Further work is required to differentiate these effects in red grapes.

Several studies did not illustrate a definitive effect of nitrogen application in the vineyard on the final wine sensory characteristics. Triangle taste tests conducted by Webster et al. (1993) showed that aged Riesling wines made from nitrogen-treated (224 kg N/ha) and untreated vines exhibited differences in wine aroma. However, descriptive analysis was not undertaken, and as a result the nature of the differences remains unknown. Similarly Ough et al. (1968b) only observed very small sensory differences between ‘plus nitrogen’ wines and wines from vines receiving no nitrogen, even though the wines that had been made from nitrogen-treated Thompson Seedless vines exhibited higher volatile ester and lower amyl alcohol concentrations. These effects were overshadowed by the more significant rootstock effects. However, the authors did comment that generally ‘plus nitrogen’ wines tended to be of lower quality. In yet another case, Maigre (2002) and Spring (2002) observed that nitrogen application in the vineyard increased must nitrogen concentration, reduced fermentation time and lowered the concentration of other non-nitrogen grape components and aroma descriptors were found. The wine made from low nitrogen juice was rated low in the fruity and floral descriptors and the lowest rating for the undesirable descriptors. The high nitrogen wine received the highest rating for most of the fruity and floral descriptors and the lowest rating for the undesirable descriptors. The high nitrogen wine was given the lowest rating for almost all descriptors except for ‘acetic’ and ‘nail polish remover’.

In summary, nitrogen application in the vineyard, from a sensory perspective, can have an impact on wine quality in some situations. However, it is not surprising that a common trend is difficult to establish, because the nitrogen status of the vines and grape musts in each study varies, and there were differences in winemaking technique employed, particularly between red and white wines. Additionally, although no consistent trends were evident, nitrogen applied in the vineyard can affect the concentration of other non-nitrogen grape components (Table 1), which in turn may contribute to the final quality of the wine. Therefore, nitrogen application in the vineyard triggers a myriad of effects that can interact, resulting in sensorial differences between wines from untreated vines and those from nitrogen-treated vines. Furthermore, the choice of vineyard site, especially with respect to soil type and ‘fertility’ level, amount and pattern of water availability, and many other factors, are likely to produce variable results. Clearly, a better understanding of all the factors linking vineyard nitrogen application, must and wine composition and sensory properties needs to be developed before one can begin to understand fully how nitrogen application in different vineyard sites can affect wine quality.
5.0 Conclusion

Nitrogen application in the vineyard has an impact on the concentration of many grape berry and/or juice components, many of which have the potential to contribute to wine quality. However, despite the number of studies involved, the impact of nitrogen application in the vineyard on the grape juice quality components often resulted in conflicting trends, which do not give a clear view on how nitrogen might affect these components in any given situation. The only consistent effect of nitrogen application on grape juice quality components was an increase in the concentration of the major nitrogenous compounds, such as total amino acids, arginine, proline, ammonium and total nitrogen. The impact of nitrogen application in the vineyard on other grape secondary metabolites was generally small or inconclusive. For example, glycosyl-glucose is considered to be a good measure of potential flavour constituents. However, only one study has been published, and as this study measured wine G-G and did not directly measure berry G-G, the real effects of nitrogen applied in the vineyard remain unknown. Several reports, such as those that measured the fruity thiols, appear to be promising, but cannot yet be relied upon. The lack of knowledge on these important quality constituents signals the need for further research to elucidate their relationship to nitrogen application in the vineyard.

‘Macro’ tuning of berry nitrogen status can be achieved in the vineyard primarily in terms of the amount and composition of the potential juice YAN, given genetic constraints. At low nitrogen sites, this can be achieved by the judicious application of nitrogen supplements such as inorganic and, to a lesser extent, organic nitrogen supplements sold as fertilisers. Maintenance of adequate nitrogen status at moderate nitrogen sites can be achieved in the same way, but nitrogen application should be avoided at sites that are high in nitrogen. Nitrogen application in the vineyard should only be considered when all other factors, such as vine vigour, canopy density, leaf colour and petiole nitrogen analysis, indicate a need for nitrogen.

Leaving aside the effects of nitrogen application in the vineyard on vine growth and productivity, the winemaker should aim to achieve an adequate concentration of juice YAN that will foster an unhampered healthy fermentation, so long as this objective is in step with other fruit quality requirements. Harvest time is commonly dictated by factors other than YAN concentration. Typically, those constituents, such as berry flavour or phenolic ripeness, which cannot be easily changed by winemaking procedures, are now taken into greater consideration.

At the point of harvest, although grape berry composition essentially dictates wine composition, the winemaker has many options available to fine-tune wine style. Choice of fermentation conditions plays a major part in determining style, of which must nitrogen is an important consideration. The potential implications associated with low and high must YAN have been highlighted in this review. In essence, nitrogen, being a growth limiting nutrient in grape must, affects yeast growth and capacity to ferment sugars. Consequently, yeast activity will affect the production of many of the sensorially important meta-

![Figure 15. Descriptive sensory analysis of Chardonnay wines made by fermentation, with Saccharomyces cerevisiae AWRI 796, of a grape juice containing 160 mg N/L (○) or 320 (●) or 480 mg N/L (▲) made by supplementation with ammonium chloride (Torrea and Henschke 2004).]
bolic end products, which in turn can affect wine quality.

**Low must YAN:** has the potential to lead to reduced yeast population and fermentation vigour, increased risk of slow and possibly sluggish/stuck fermentations, increased production of ‘reductive’ volatile thiols (e.g. H₂S, and mercaptans e.g. MeSH, EtSH) and higher alcohols and decreased production of esters and long chain fatty acids.

**High must YAN:** has the potential to lead to increased biomass and high heat output due to high fermentation vigour, and increased formation of ethyl acetate, acetic acid and volatile acidity. Increased concentrations of hae-
causings proteins, urea and ethyl carbamate and biogenic amines (e.g. histamine) are also associated with high YAN musts. There is also an increased risk of microbial insta-
bility, potential taint from Botrytis-infected fruit and possibly atypical ageing character.

Therefore, nitrogen application in the vineyard can be used to manipulate the must YAN to a large degree, but the final ‘micro’ tuning can be more readily and easily achieved in the winery by the use of nitrogen supple-
ments, such as DAP and the choice of fermentation con-
ditions. It has, however, been illustrated that forms of nitrogen other than ammonium, such as the amino acids, are important for the formation of a number of favourable wine components, and might even enhance varietal char-
acter. To this end, various products are currently being developed by commercial yeast suppliers. If not added in the winery, the principal source of amino acids is the grape berry.

Information on the timing and addition rate of DAP to must before and during fermentation has recently improved, but consequences for quality are still to be determined. Furthermore, optimising nitrogen manage-
ment for red wine fermentations is poorly understood and likely to differ from that for white wine fermenta-
tions.

Lastly, uninformd use of DAP bears the risk that a low to moderate must YAN can be instantly converted to a must with a high YAN concentration, to produce a wine of very different, and possibly less preferred, style. This point highlights the need not only to monitor nitrogen in the vineyard especially when located in low ‘fertility’ sites, but to monitor must nitrogen so that appropriate additions can be made when required. It is likely that NIR technol-
ogy will greatly facilitate nitrogen management in the near future and assist in optimising the quality compo-
nents of wine. It can be concluded that optimisation of vineyard and fermentation nitrogen can contribute to quality factors in wine and hence affect its commercial value.

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Implications of nitrogen nutrition


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