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ARTICLE

Effect of Nitrogen Supplementation and *Saccharomyces* Species on Hydrogen Sulfide and Other Volatile Sulfur Compounds in Shiraz Fermentation and Wine

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A Shiraz must with low yeast assimilable nitrogen (YAN) was supplemented with two increasing concentrations of diammonium phosphate (DAP) and fermented with one *Saccharomyces cerevisiae* and one *Saccharomyces bayanus* strain, with maceration on grape skins. Hydrogen sulfide (H_2S) was monitored throughout fermentation, and a total of 16 volatile sulfur compounds (VSCs) were quantified in the finished wines. For the *S. cerevisiae* yeast strain, addition of DAP to a final YAN of 250 or 400 mg/L resulted in an increased formation of H_2S compared to nonsupplemented fermentations (100 mg/L YAN). For this yeast, DAP-supplemented fermentations also showed prolonged formation of H_2S into the later stage of fermentation, which was associated with increased H_2S in the final wines. The *S. bayanus* strain showed a different H_2S production profile, in which production was inversely correlated to initial YAN. No correlation was found between total H_2S produced by either yeast during fermentation and H_2S concentration in the final wines. For both yeasts, DAP supplementation yielded higher concentrations of organic VSCs in the finished wines, including sulfides, disulfides, mercaptans, and mercaptoesters. PCA analysis indicated that nitrogen supplementation before fermentation determined a much clearer distinction between the VSC profiles of the two yeasts compared to nonsupplemented fermentations. These results raise questions concerning the widespread use of DAP in the management of low YAN fermentations with respect to the formation of reductive characters in wine.

KEYWORDS: Hydrogen sulfide; volatile sulfur compounds; DMS; mercaptans; *Saccharomyces cerevisiae*; *Saccharomyces bayanus*; fermentation; nitrogen

INTRODUCTION

Volatile sulfur compounds (VSCs) have a significant influence on the perceived aroma of many foods and beverages (*1*, *2*). In wine, the aroma contribution of VSCs is often considered to be negative, due to their characteristic odors of rotten egg, putrefaction, onion, cabbage, or garlic (*3*). Nevertheless, VSCs are present at low concentrations in the vast majority of wines, and some of these compounds can positively contribute to the aroma complexity of red and white wines (*3*). In some cases VSCs appear to be involved in varietal and aging-related differences between wines (*4*).

Hydrogen sulfide (H_2S) is the most studied VSC in wine, due to its association with "reductive" off-flavors often described as rotten egg and putrefaction (*4*). It is generally agreed that the major portion of H_2S arises as an intermediate in the biosynthesis of sulfur-containing amino acids by yeast during fermentation (*5*, *6*). Yeast strain (*6*−*8*), must turbidity (*9*), availability of fermentation nutrients (*10*, *11*), and presence of metal ions (*12*) can affect the amount of H_2S produced during fermentation. Other low molecular weight VSCs, such as mercaptans, sulfides, and disulfides, have been also identified in wines. Among these, methylmercaptan (MetSH), ethylmercaptan (EtSH), dimethyl sulfide (DMS), and diethyl disulfide (DEDS) have been indicated as potential contributors to wine aroma, due to their low odor thresholds (*3*, *13*−*15*). The odor of these compounds is usually described with attributes such as reductive or putrefaction for the mercaptans, and cabbage, onion, or rubber for sulfides and disulfides (*3*). On the basis of these sensory descriptors, their contribution to wine aroma is generally considered to be negative. However, certain compounds such as DMS can increase the red fruit aroma characteristics of red wine when present in low concentrations (*15*, *16*). Additionally, other VSCs, often referred to as "heavy", are produced during fermentation (*14*). Among these, the amino acid-related thioalcohol 3-(methylthio)-1-propanol (methionol), reported to have a boiled potato odor, is the most abundant VSC in wine (in the mg/L range). Heavy VSCs are generally characterized by odors described as cooked vegetables, boiled potatoes, poultry, and onions, but their contribution to wine aroma has still to be established. Although a large number of studies have investigated the effects of different fermentation conditions on H_2S formation, studies on the factors affecting the

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formation of other VSCs, particularly the low molecular weight ones, are relatively scarce. Particularly, to date, no study has explored the effects of different winemaking practices on the formation of VSCs in red wine.

Various nutrients that are essential to yeast metabolism are often present at suboptimal concentrations in grape must. Among these, yeast assimilable nitrogen (YAN), defined as the nitrogen contained in the ammonia and free α-amino acid (FAN) fractions of juice, provides nitrogen for protein biosynthesis of the cell and is therefore of primary importance for correct functioning of cell metabolism (reviewed in ref *11*). Nitrogen supplementation in the winery, usually in the form of diammonium phosphate (DAP), has long been used for this reason. Previous work has indicated that DAP is a powerful modulator of H_2S and other fermentation-derived volatiles (*6*, *10*, *17*−*19*), but the effects of its addition on other VSCs are still poorly understood.

Choice of yeast strain is also known to play a fundamental role in determining wine aroma composition and characteristics. In regard to VSCs, the ability of strains of *Saccharomyces cerevisiae* to produce different amounts of H_2S is well documented (*7*, *20*), and limited data on other VSCs are also available (*20*). However, in recent years, the use of non-*S. cerevisiae* yeasts has received considerable attention, and strains of *Saccharomyces bayanus*, interspecies hybrids, and mixed starters of *Saccharomyces* and non-*Saccharomyces* strains are now available on the market in the form of active dry yeasts (*21*). The effects of different winemaking practices, including nitrogen supplementation, on the formation of volatile compounds by nonconventional yeast strains have still to be investigated.

In this study, the effects of DAP supplementation on VSCs of experimental *Vitis vinifera* cv. Shiraz wines obtained by fermentation of a low-nitrogen must with winemaking strains of *S. cerevisiae* and *S. bayanus* have been investigated. The aim was to provide a first characterization of the combined effects of yeast selection and nitrogen supplementation on the pool of VSCs in red wine made with maceration on the skins.

MATERIALS AND METHODS

Chemicals. Pure reference compounds (purity > 98%) for EtSH, DMS, diethyl sulfide (DES), dimethyl disulfide (DMDS), DEDS, methyl thioacetate (MTA), ethyl thioacetate (ETA), 2-mercaptoethanol, 2-(methylthio)-1-ethanol, 3-(methylthio)-1-propanol, 4-(methylthio)-1-butanol, benzothiazole, 5-(2-hydroxyethyl)-4-methylthiazole, d_6-dimethyl sulfide, dipropyl disulfide, 3-(methylthio)-1-hexanol, and 4-methylthiazole were supplied by Sigma-Aldrich (Milan, Italy) and Lancaster (Milan, Italy). Anhydrous ethanol (>99%) was obtained from Carlo Erba (Milan, Italy). Sodium hydrosulfide hydrate and carbon disulfide (CS_2) were obtained from Sigma-Aldrich, whereas MetSH was obtained from TCI (Tokyo, Japan).

Winemaking. Shiraz grapes with low YAN were obtained from the Langhorne Creek winemaking district in South Australia during the 2007 vintage. No nutrient supplements had been applied to this vineyard block for 5 years. The grapes were hand-picked and collected in 15 kg plastic bins. Once in the winery, fruit from different bins was pooled together to obtain a homogeneous mass. Individual 30 kg lots were then destemmed and crushed, and the must was collected in 50 L stainless steel fermentation vessels. The analytical parameters of the must were as follows: total soluble solids, 24° Brix; titratable acidity, 7.2 g/L as tartaric acid; pH, 3.3; YAN, 100 mg/L; methionine 1.3 mg/L. Potassium metabisulfite was added at 100 mg/kg to each must approximately 2 h before inoculation. DAP additions were performed according to an experimental design consisting of three YAN concentrations, each one fermented in triplicate, for a total of nine fermentations for each yeast. A control that did not receive any DAP addition represented the lowest nitrogen concentration (100 mg/L YAN), whereas in the two other treatments DAP was added to final YAN concentrations of 250 and 400 mg/L YAN. All DAP additions were performed prior to inoculation. Following DAP additions, the pH of the musts was measured and readjusted to 3.3 by means of 1 N HCl. Then, the samples were inoculated with either of two yeasts, *S. cerevisiae* D 254 (Lallemand) and *S. bayanus* 1176 (Lallemand), at a rate of 1×10^6 cells/mL, following rehydration in water at 40 °C for 30 min. Fermentations were carried out at 22 ± 1.5 °C, with the cap submerged three times per day. Fermentation progress was monitored by enzymatic analysis of the residual sugars. Dominance of the inoculated strain in all treatments was confirmed by transposon PCR analysis (*22*). The wines were left to macerate on grape skins until the slowest treatment reached dryness (residual sugars ≤ 2 g/L), after which the fermented musts were pressed and the wines collected in 20 L stainless steel containers and placed at 4 °C under a headspace of inert gas to accelerate clarification. No malolactic fermentation was carried out. After 4 weeks, 150 mg/L of potassium metabisulfite was added to the wines, which were then filtered through 0.45 μm membranes (Sartorius, Gottingen, Germany) and bottled under ROTE closures. Analysis of the sulfur compounds in the wines was carried out after 3 months of storage at 14 °C.

Grape Must Analyses. Titratable acidity, FAN, and ammonia were measured as previously described (*22*). Ammonia concentration was measured using the Glutamate Dehydrogenase Enzymatic Bioanalysis UV method (Roche, Mannheim, Germany). FAN was determined by using the *o*-phtalaldehyde/*N*-acetyl-L-cysteine spectrophotometric assay procedure. Both ammonia and FAN were analyzed using a Roche Cobas FARA spectrophotometric autoanalyzer (Roche, Basel, Switzerland). YAN was calculated as the sum of ammonia-derived nitrogen and FAN and, therefore, did not include proline. Fermentation progress was monitored by daily analysis of residual sugar by means of an enzymatic kit (*22*).

Analysis of H_2S in Fermenting Samples. The release of H_2S during fermentation was monitored daily using lead acetate selective detector tubes (Komyo, Kitagawa, Japan) as described elsewhere (*23*). The analysis was carried out on 100 mL of fermentor headspace gas. The detector tubes were introduced in the fermentors from a sampling port, and 100 mL of sample was forced into the tube using an AP-20 calibrated air sampling pump (Komyo). The total amount of H_2S released during the whole course of fermentation was estimated by integrating the area under the curves obtained from daily measurements, using Origin 6.0 (Microcal Software Inc., Northampton, MA).

Analysis of VSCs in Wines. H_2S, MetSH, and CS_2 were determined by static headspace analysis with gas chromatography−atomic emission detection (GC-AED), as described elsewhere (*22*). H_2S was obtained by dissolving sodium hydrosulfide hydrate (Sigma-Aldrich) in water at pH 3.2. All of the other VSCs reported in this study were determined by using headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME/GC-MS) method, as described by Fedrizzi et al. (*24*). Individual ethanolic standard solutions for each sulfur compound were prepared, and from these a working solution in ethanol, containing all of the analytes, was made; all of the solutions were stored at −16 °C. The final concentrations of analytes were as follows: EtSH, 2.0 mg/L; DMS, 16.0 mg/L; DES, 1.0 mg/L; DMDS, 4.0 mg/L; DEDS, 4.0 mg/L; MTA, 4.0 mg/L; ETA, 4.0 mg/L; 2-mercaptoethanol, 16.0 mg/L; 2-(methylthio)-1-ethanol, 4.0 mg/L; 3-(methylthio)-1-propanol, 150.0 mg/L; 4-(methylthio)-1-butanol, 8.0 mg/L; benzothiazole, 8.0 mg/L; 5-(2-hydroxyethyl)-4-methylthiazole, 8.0 mg/L. The same procedure was followed to prepare internal standard (IS) solutions at similar concentrations. Calibration curves for each analyte were prepared, using the following as internal standards (ISs): d_6-dimethyl sulfide, dipropyl disulfide, 4-methylthiazole, and 3-(methylthio)-1-hexanol. All compounds were identified by means of coinjection with pure reference compounds or comparison of their retention times and mass spectra with those of reference standards.

Sensory Assessment of the Wines. An informal bench tasting was carried out to provide an indication of the differences in aroma and flavor among the treatments and to assess any variation among fermentation replicates. Ten AWRI tasters with extensive experience in wine sensory assessment, including five members of the AWRI technical quality panel, assessed the six different wines and the three fermentation replicates for this study in constant order. The tasters were asked to write free-choice notes about the wines, also indicating any perceived taints or faults. After the wines were tasted, a discussion was held to give an overall impression of the wines.

Statistical Analysis. Analysis of variance and least significant difference (LSD) test were used to interpret the differences in means at the 95% confidence level. The data were processed using JMP 5.0.1

(SAS, Cary, NC). Principal component analysis (PCA) was carried out using Unscrambler 9.5 (CAMO Technologies Inc., Woodbridge, NJ).

RESULTS

Fermentations of Shiraz musts with low (100 mg/L YAN), medium (250 mg/L YAN), and high nitrogen (400 mg/L YAN) were carried out with *S. cerevisiae* D 254 and *S. bayanus* AWRI 1176 by maceration on grape skins at 22 °C (**Figure 1**). For both yeasts, nitrogen supplementation resulted in increased fermentation rate, with the high YAN musts completing in 10 days, the medium YAN musts in 13−14 days, and the low YAN musts in 20 days. YAN, measured as the sum of FAN and ammonia nitrogen, became undetectable with the method used after 2 days of fermentation for the low YAN musts (approximately 230 g/L residual sugars), after 3 days for the medium YAN musts (approximately 160 g/L residual sugars), and after 4 days for the high YAN musts (100 g/L residual sugars).

The evolution of H_2S during fermentation is shown in **Figure 2** as a function of fermentation progress, expressed as residual sugars. Nitrogen supplementation had a strong impact on the stage of fermentation at which H_2S production started. Generally, H_2S production commenced very early in the nonsupplemented and 250 mg/L YAN fermentations, within the consumption of 20 g/L of sugar, but was delayed up to 150 g/L of residual sugars in the high-nitrogen fermentations. In the case of yeast D 254, a delay in the onset of H_2S formation was observed only in the 400 mg/L YAN fermentation, whereas the control and the 250 mg/L YAN fermentations showed no difference. For both yeasts, in the nonsupplemented fermentations production of H_2S commenced upon depletion of YAN, whereas in the other treatments occurrence of H_2S was already observed before YAN was completely depleted. Nitrogen supplementation also had a strong influence on the stage of fermentation at which cessation of H_2S production occurred. In the case of yeast 1176, production of H_2S became undetectable for all treatments around 100 g/L of residual sugars. Two peaks of production were observed for this yeast in the low-nitrogen fermentations, with the second peak occurring between 100 and 150 g/L of residual sugars. Conversely, for D 254, production of H_2S in the control fermentation stopped around 120 g/L of residual sugars, whereas in the 250 and 400 mg/L YAN treatments it ceased at 50 and 70 mg/L of residual sugars, respectively.

The total H_2S developed during fermentation was affected by both yeast species and nitrogen concentration (**Table 1**). In the case of *S. bayanus* 1176, a remarkable decrease in the total H_2S released occurred when the fermentations were supplemented with DAP. Higher DAP supplementations resulted in the lowest total H_2S concentration recorded in this study, whereas control fermentations gave rise to the highest total H_2S. These results indicate that, for this strain, DAP supplementation was a powerful modulator of H_2S production during fermentation. Conversely, when fermentations were carried out with yeast D 254, the differences in range of H_2S concentrations observed across the various treatments were much smaller. It should be noted that although maximum H_2S production per gram of sugar metabolized by this yeast was observed in the control fermentations (**Figure 2**), the highest total H_2S produced was observed in the 250 mg/L YAN fermentation. **Table 1** also shows the concentration of H_2S in the wines 3 months after bottling, measured with GC-AED. Despite the generally higher H_2S production observed for yeast 1176, no residual H_2S was detected in the wines made with this strain. Conversely, in the case of yeast D 254, the control fermentation showed no detectable H_2S, whereas the nitrogen-supplemented fermentations resulted in wines with residual H_2S, particularly for the 250 mg/L YAN treatment.

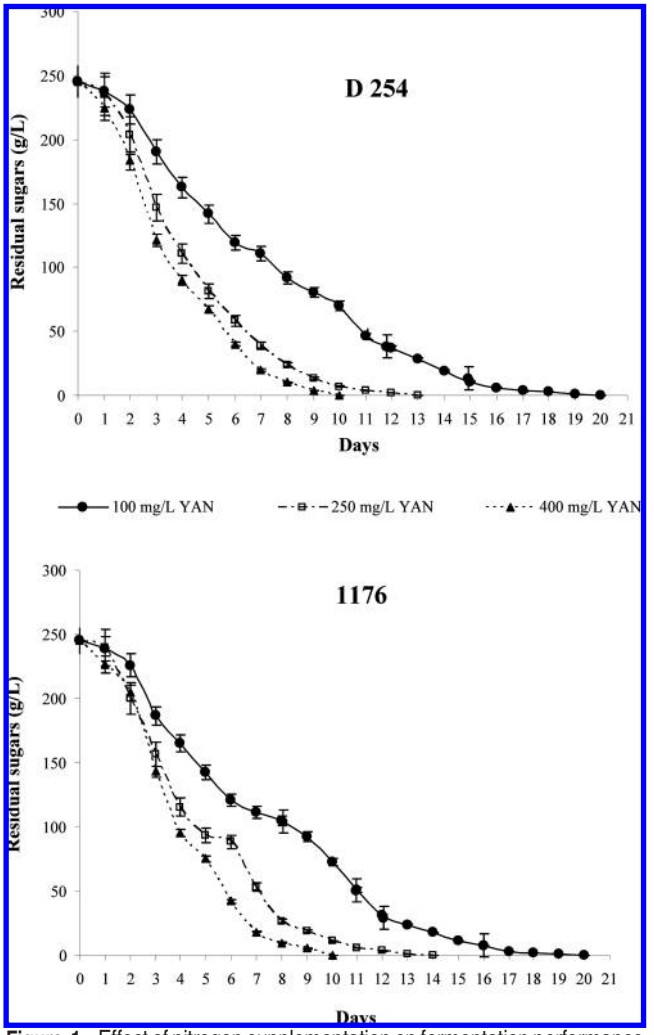

Figure 1. Effect of nitrogen supplementation on fermentation performance of *S. cerevisiae* D 254 and *S. bayanus* 1176 yeasts.

The results of the analysis of the different VSCs are reported in **Table 2**. For both yeasts, sulfides and disulfides increased with nitrogen additions, with yeast D 254 wines showing generally higher concentration values compared to yeast 1176. MetSH was found only in wines from nitrogen-supplemented fermentations, but no difference was observed between the two yeasts for this compound. As for EtSH, nitrogen supplementation stimulated an increase in the concentration of this compound when fermentations were carried out with 1176, whereas no treatment effect was observed for D 254. The two thioesters MTA and ETA showed a general increase in wines obtained from nitrogen-supplemented fermentations. Finally, with the exception of 2-(methylthio)-1-ethanol, which showed a small increase with nitrogen supplementation, no significant difference was observed for the heavy VSCs 2-mercaptoethanol, 3-(methylthio)-1-propanol, 4-(methylthio)-1-butanol, benzothiazole, and 5-(2-hydroxyethyl)-4-methylthiazole with respect to yeast and nitrogen supplementation.

PCA was used to identify the VSCs that best discriminated between the different treatments. The results are given in **Figure 3**. The first principal component (PC1) explained 49% of the total variance and was mainly characterized by MetSH, DES, DEDS, DMS, DMDS, MTA, and ETA, with positive loadings. PC2, which accounted for 16% of the total variance, was characterized by H_2S with negative loadings and by EtSH, 2-mercaptoethanol, and 5-(2-hydroxyethyl)-4-methylthiazole with positive loadings. PC3 accounted for 11% of the total variation and was mainly characterized by 4-(methylthio)-1-butanol with positive loadings

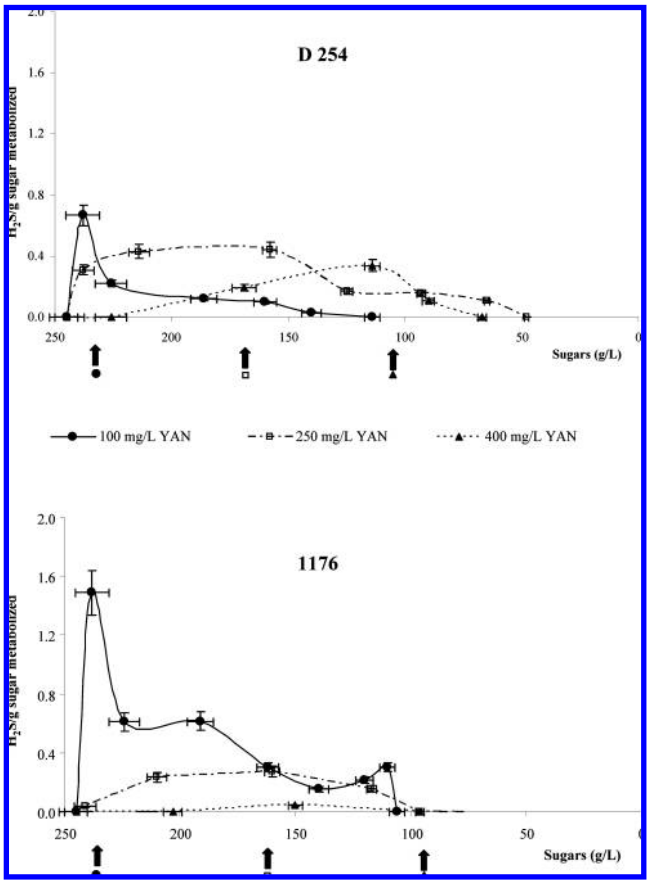

Figure 2. Evolution of H_2S during fermentation with *S. cerevisiae* D 254 and *S. bayanus* 1176 under three different initial nitrogen concentrations. Arrows indicate the stage of fermentation at which YAN became undetectable in each treatment.

Table 1. H_2S Concentration Released during Fermentation and Found in the Bottled Wines[a]

	total H_2S released during fermentation (μg/L must)	fermentation stage when H_2S production ceased (g/L of residual sugars)	finished wines (μg/L wine)
D 254 control	102 c	129 a	nd
D 254 250	284 a	50 c	2.3 a
D 254 400	121 b	70 b	0.5 b
1176 control	326 a	100 a	nd
1176 250	116 b	100 a	nd
1176 400	9 c	100 a	nd

[a] Control, 100 mg/L YAN; 250, same juice as control, but initial YAN increased to 250 mg/L by DAP addition; 400, same juice as control, but initial YAN increased to 400 mg/L by DAP addition. Different letters denote values that are statistically different at $p < 0.05$. nd, not detected.

and for 2-mercaptoethanol and 3-(methylthio)-1-butanol with negative loadings. At low nitrogen concentration, the wines obtained with the two yeasts could not be clearly separated by the two first principal components. As nitrogen was increased by means of DAP addition, a clear distinction between the two yeasts became apparent, with 1176 wines being mainly associated with EtSH, 2-mercaptoethanol, and 2-(methylthio)-1-ethanol. Differences between medium and high initial YAN concentrations for this yeast were, however, moderate. Conversely, in the case of D 254, a further separation was observed between the two nitrogen additions, with wines obtained from an initial YAN of 250 mg/L being strongly characterized by H_2S and wines from the 400 mg/L YAN fermentation being mainly associated with MetSH, DES, DEDS, DMS, DMDS, and the two thioesters.

Table 2. Concentrations (Micrograms per Liter) of VSCs in the Experimental Wines[a]

	D 254			1176		
	control	250	400	control	250	400
CS_2	6.0 b	8.0 a	9.0 a	5.0 a	5.0 a	6.0 a
DES	2.0 b	8.6 a	10.8 a	1.8 c	5.2 b	8.7 a
DEDS	2.6 b	4.5 a	6.1 a	1.9 c	3.3 b	4.3 a
DMS	2.5 b	8.6 a	10.8 a	2.0 c	4.0 b	5.9 a
DMDS	nd c	2.3 b	3.8 a	nd c	0.7 b	1.0 a
MetSH	nd b	0.7 a	0.8 a	nd b	nd b	0.8 a
EtSH	0.8 a	0.8 a	0.9 a	0.7 b	1.1 a	1.0 a
MTA	5.0 b	7.3 ab	8.5 a	3.9 b	4.7 b	6.4 a
ETA	1.1 a	2.2 b	2.8 b	1.2 b	1.9 a	1.8 a
2-mercaptoethanol	38.4 a	38.7 a	39.8 a	40.3 a	39.4 a	41.1 a
2-(methylthio)-1-ethanol	33.7 a	37.7 a	40.3 a	33.2 b	39.3 a	40.8 a
3-(methylthio)-1-propanol	2900 a	3130 a	3003 a	3054 a	2994 a	3005 a
4-(methylthio)-1-butanol	22.4 a	21.7 a	20.7 a	20.1 a	20.8 a	21.8 a
benzothiazole	15.6 a	15.7 a	14.7 a	16.0 a	15.4 a	16.8 a
5-(2-hydroxyethyl)-4-methylthiazole	0.8 a	0.5 a	0.8 a	0.9 a	0.8 a	1.0 a

[a] Control, 100 mg/L YAN; 250, same juice as control, but initial YAN increased to 250 mg/L by DAP addition; 400, same juice as control, but initial YAN increased to 400 mg/L by DAP addition. Different letters denote values that are statistically different at $p < 0.05$. nd, not detected.

Sensory assessment carried out on the experimental wines indicated that the six treatments resulted in wines which were generally different and, in particular, in acetaldehyde type aroma and sulfidic characters. Some differences in fruit and sweaty/cheesy characters were also noted. Of the 10 tasters, 8 commented specifically that the wines were different in sulfide-like characters using descriptors such as hydrogen sulfide-like, onion/rubber, reductive, and dirty. From the discussion, and confirmed by judges' written notes, it was concluded that, in general, high-nitrogen wines for both strains were apparently lowest in sulfide-related aromas. The fermentation replicates were generally all similar.

DISCUSSION

Various biochemical mechanisms can account for the production of H_2S by yeast. However, it is generally accepted that the major portion of the H_2S formed during fermentation derives from the sulfate/sulfite assimilation pathway that leads to the formation of the amino acids cysteine and methionine (*5*, *6*, *10*). Accordingly, H_2S is formed from the reduction of sulfite and is then sequestered by the carbon−nitrogen precursor *O*-acetylhomoserine to form cysteine and methionine. Nitrogen availability regulates the balance between H_2S sequestration and excretion by determining the intracellular concentration of the carbon−nitrogen precursors (*6*). In particular, it has been postulated that, if nitrogen deficiency occurs during the growth phase, the low rate of formation of carbon−nitrogen precursors results in H_2S accumulation in the cell and consequent excretion into the fermentation medium (*6*, *10*). Furthermore, sulfite reductase activity appears to remain stable after nitrogen depletion, leading to prolonged H_2S formation (*25*). The results obtained in the present study are only in partial agreement with these observations. In fact, whereas the data obtained for *S. bayanus* AWRI 1176 strain were consistent with the expected effect of nitrogen supplementation, lowering H_2S formation, increased concentrations of initial nitrogen did not decrease H_2S formation for *S. cerevisiae* D 254, but instead increased total H_2S when DAP had been added to give an initial YAN of 250 mg/L. This is in agreement with the recent observation that a shortage

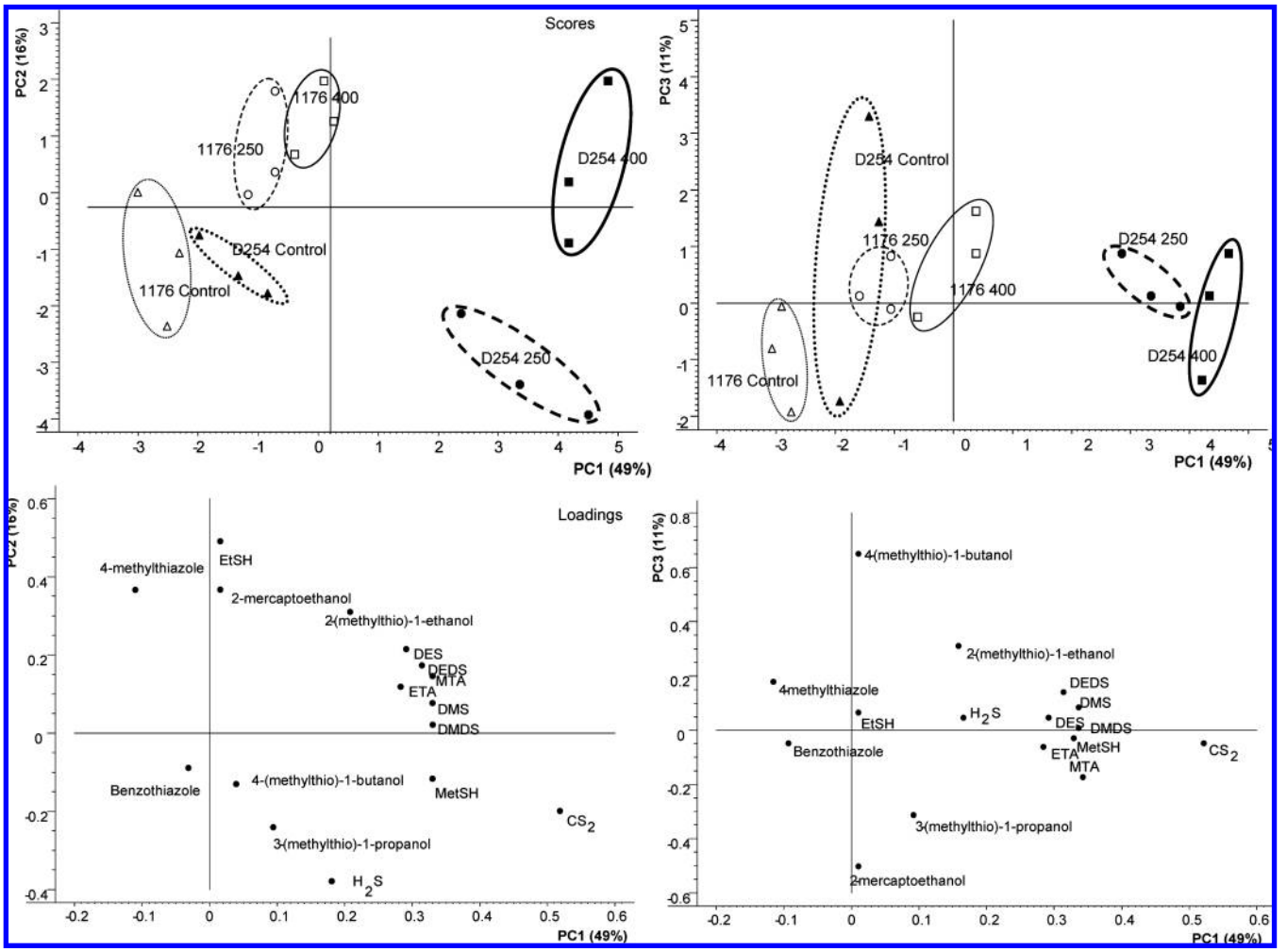

Figure 3. Principal component analysis of volatile sulfur compounds: scores and loadings for the first three principal components.

of *O*-acetylhomoserine is not always responsible for increased production of H_2S (*26*). Other studies have reported a poor correlation between nitrogen availability and formation of H_2S (*26*, *27*). Rauhut et al. (*20*) reported that wines made with certain yeast strains had increased H_2S when DAP was added at 500 mg/L.

Other nutrient deficiencies are known to stimulate H_2S production, such as lack of the vitamins pantothenic acid and pyridoxine (*19*, *28*). According to Wang et al. (*19*), supplementation of nitrogen to model fermentations that were deficient in pantothenic acid can result in increased formation of H_2S. However, the grapes used in our study had a concentration of pantothenic acid of 600 μg/L, which markedly exceeds the minimum pantothenate concentration of 250 μg/L needed to suppress H_2S formation in model media (*19*). Deficiencies of other nutrients or vitamins might, however, might have played a role in nitrogen-unregulated H_2S production by yeast D 254 in the must studied.

Variability in H_2S production between yeast strains has long been known (*6*−*8*), but the biochemical mechanisms regulating H_2S production are poorly understood. Strain differences have been attributed to many factors but are now generally understood to involve genetic mutations in the sulfate assimilation pathway and *S*-amino acid metabolism (*26*). In a recent study it has been shown that several *MET* genes involved in the sulfur reduction pathway, and therefore in the formation of H_2S, are specifically down-regulated under conditions of nitrogen starvation (*29*). This might explain the low H_2S production observed for yeast D 254 at low nitrogen concentrations. Moreover, it was observed that, for yeast D 254, DAP supplementation to 250 mg/L of YAN resulted in a maximum cell density of 7.0×10^7 cells/mL, compared to 4.4×10^7 cells/mL observed in the control. It is therefore possible that the increase in biomass determined by moderate nitrogen supplementation resulted in increased capacity to reduce sulfate to sulfide, leading to a greater liberation of H_2S upon the depletion of nitrogen. These results confirm the complexity of H_2S regulation in yeast fermenting red grapes musts as well as model media and indicate that further research is needed to understand the factors determining the formation of H_2S in wine fermentations.

The concentration of H_2S in the finished wines was affected by both yeast species and nitrogen supplementation. Surprisingly, no correlation was found between total H_2S produced during fermentation and final concentration of H_2S in the wines. H_2S has low solubility and high volatility and can be largely removed by the CO_2 evolving during fermentation (*20*, *30*). However, the final concentration of H_2S appeared to be correlated with the stage of fermentation at which H_2S production ceased. In fact, no residual H_2S was detected in the wines obtained from fermentations in which H_2S production ceased early, around 100 g/L of residual sugars (namely, the low YAN for yeast D 254 and all of the yeast 1176 treatments). Conversely, increasing wine H_2S concentrations were obtained for the treatments yeast D 254 250 and 400 mg/L YAN, in which H_2S production continued until residual sugars had reached approximately 75 and 50 g/L, respectively. According to Henschke and de Kluis (*31*), due to the reduced purging effect of CO_2, which is responsible for the

removal of most of the H_2S produced during fermentation, H_2S formed at the end of fermentation could be of greater importance than that present in the vigorous part of the fermentation. Therefore, fermentations characterized by late formation of H_2S can potentially result in wines with higher residual H_2S concentrations. The results of this study, although still limited, seem to confirm this suggestion, as fermentations showing late H_2S formation were also characterized by higher H_2S concentration in the final wines (**Table 1**).

Several proposals to explain the late formation of H_2S have been made. On the basis of the observation that the active transport of nitrogen sources is progressively inhibited by increasing ethanol concentrations (*32*), it has been suggested that the resulting intracellular depletion of nitrogen stimulates H_2S production. The failure of nitrogen additions late in fermentations to suppress H_2S production is consistent with this proposal (*31*). Alternatively, the degradation of cysteine from either the cytosolic pool or glutathione, which can be induced by nitrogen starvation, represents another mechanism (*33*). Due to the primary importance of H_2S on wine aroma quality (*3*), the relationship between total H_2S produced, timing of production, and concentration of H_2S in finished wines is worthy of further investigation.

In addition to H_2S, a total of 15 VSCs were measured in the wines investigated in this study, with the aim of obtaining a comprehensive evaluation of the effect of the fermentation conditions studied on the volatile sulfur profile of Shiraz wines. Despite the large number of papers describing the effects of fermentation conditions on H_2S formation (*11*), only a limited number of studies have investigated the relationship between fermentation management and formation of VSCs, in particular, mercaptans, sulfides, and disulfides, which can potentially affect wine aroma. In a previous study, we observed no correlation between nitrogen supplementation and final DMS concentration in Shiraz wines (*22*). Conversely, in the current study, for both yeast strains nitrogen supplementation induced a general increase in the concentration of sulfides and disulfides, including DMS. It has been proposed that sulfides and disulfides can be formed by yeast as a result of the catabolism of the sulfur amino acids cysteine and methionine (*34*), but an involvement of sulfur amino acid biosynthetic pathways has also been suggested (*1*). In the case of DMS, there is evidence that formation of this compound by the yeast during grape must fermentation is linked to cysteine, cystine, methionine, or glutathione metabolism (*11*, *14*, *34*). However, a chemical pathway can be also involved in its formation, with *S*-methylmethionine as a possible precursor (*35*). De Boer and Wilson (*36*) proposed that the *S*-methylmethionine synthesized by the yeast can then be chemically transformed into DMS. In beer fermentations it has been shown that yeast can also form DMS through enzymatic reduction of dimethyl sulfoxide (DMSO) (*37*). However, whereas in wort DMSO is formed in large amounts during malt kilning (*37*), its occurrence in grape juice has not yet been demonstrated. As for the other sulfides, the origin of these compounds in wine fermentations remains largely unknown. In a recent study, Buzzini et al. (*38*) suggested that methionine is the essential precursor to DMDS in *Basidiomycetous* yeasts. Finally, both DMDS and DEDS have been identified as products of the reaction between mercaptans in the presence of copper (*39*). This might have also contributed to the higher concentrations observed here, considering the increased formation of EtSH and methanethiol at higher nitrogen treatments.

Two mercaptans, namely, EtSH and MetSH, as well as their corresponding acetate esters, MTA and ETA, were detected and quantified in the experimental wines. MetSH showed a trend similar to that of sulfides; that is, concentrations of this compound increased in conjunction with increased nitrogen supplementation. This seems to confirm the observation, reported by other authors, that sulfides and MetSH derive from interrelated metabolic pathways sharing methionine as common precursor (*14*, *40*). An increase was also found for EtSH with increasing nitrogen in the case of *S. bayanus* AWRI 1176, whereas no nitrogen effect was observed for this compound in the wines produced with *S. cerevisiae* D 254. EtSH can be formed from the reaction of H_2S and acetaldehyde (*14*), which was formed in higher concentrations by the 1176 yeast (data not shown). As for the two mercaptoacetates, these compounds are formed by the yeast through esterification of the corresponding mercaptans (*41*), most likely catalyzed by an alcohol acetyltransferase. The relative proportions observed in this experiment are consistent with those reported by Leppanen et al. (*42*). The generalized increase of MTA and ETA observed here is likely to be due to the increased biosynthesis of acetate esters resulting from nitrogen supplementation, as previously observed in red fermentations (*22*).

Nitrogen supplementation did not cause any variation in the concentration of the high molecular weight sulfur compounds measured in this study, except for a moderate increase of 2-(methylthio)-1-ethanol when fermentations with *S. bayanus* 1176 were supplemented with nitrogen. The concentrations found for this compound in this study are similar to those reported in Merlot (*4*). Grape variety has been shown to be a major source of variation in the concentration of this compound (*9*). Our results suggest the concentration of this compound in wine might depend also on nitrogen availability. Various authors have reported that increased nitrogen availability is negatively correlated with production of 3-(methylthio)-1-propanol by the yeast (*17*, *43*), as this compound derives from methionine via the Ehrlich pathway (*40*). However, it has been also indicated that the presence of high levels of solids can stimulate the production of methionol (*9*), which might have counterbalanced the effects of nitrogen supplementation in the present study. Among the other compounds detected, it has been suggested that homomethionine and cysteine could be the precursors to 4-(methylthio)-1-butanol and 2-mercaptoethanol, respectively (*44*), but variations in nitrogen availability had no effects on these two compounds under our experimental conditions.

The effects of nitrogen supplementation on the VSC composition of the experimental wines are summarized in **Figure 3**. *S. cerevisiae* D 254 showed a strong response to DAP addition, with patterns of VSCs that were also dependent on the initial nitrogen concentration. In particular, the 250 and 400 mg/L YAN wines were characterized, respectively, by increased concentrations of H_2S and of MetSH, sulfides, and disulfides. Conversely, the effects of DAP additions on the pool of VSCs produced by *S. bayanus* 1176 were less pronounced, although increased production of low molecular weight sulfur compounds and of EtSH were generally associated with nitrogen-supplemented fermentations. Interestingly, nitrogen supplementation gave a much clearer distinction between the VSC profiles of the two yeasts compared to nonsupplemented treatments. In a recent study, the highest similarities in fermentation-derived volatiles produced by two *S. cerevisiae* yeasts were found at an initial nitrogen concentration of 250 mg/L YAN, although in that case VSCs were not considered (*18*). All together, these results confirm that individual yeasts respond differently to DAP supplementation, causing significant differences in their characteristic volatile patterns.

The sensory impact of sulfur volatile compounds in wine is documented (*3*). However, there is an extremely wide variation in the odor threshold values reported by different authors. Moreover, certain sulfur compounds are known to contribute positively when they are present in sub- or perithreshold

concentrations, but they can be responsible for off-flavors at higher concentrations (*15*, *16*). For this reason, the use of preference thresholds instead of odor thresholds has been recommended when the potential impact of sulfur compounds on wine aroma is assessed on the basis of compositional data (*4*), although no study has reported these values to date. In general, on the basis of the values recently reviewed (*3*) for thresholds in hydroalcoholic solutions, it appears that H_2S, CS_2, DMS, DEDS, MetSH, EtSH, and methionol were present in the experimental wines in concentrations that suggest a contribution to the aroma of these wines. As for the thioesters MTA and ETA, no threshold values have been reported for these compounds in wine-like matrices. However, the concentrations observed for these two compounds were much lower than the threshold values reported in beer (*14*), suggesting a negligible contribution to the aroma of the experimental wines. However, it has been postulated that MTA and ETA can be hydrolyzed to their corresponding mercaptans during aging (*42*). The changes in thioester concentrations observed in this study in response to nitrogen supplementation might therefore become of sensory relevance with aging.

The results of the informal sensory assessment carried out on the wines, although merely indicative, highlighted the existence of clear differences among the different samples. In particular, the observation, reported by the large majority of the panelists, that odor descriptors such as hydrogen sulfide-like, onion/rubber, and reductive/dirty were present supports the hypothesis that at least some of the VSCs measured played a role in the sensory composition of the wines. However, in a previous study, we observed significant changes in other powerful odorants of Shiraz in response to DAP supplementation (*22*). Therefore, differences in the aroma profiles of the wines could have been the results of complex interactions between VSCs and other aroma compounds that were affected by DAP. Due to the complexity of the topic, these aspects will be addressed in a separate study.

In conclusion, this study has demonstrated that DAP supplementation of a low YAN Shiraz must can affect the pool of VSCs in wine. In the case of H_2S production, different *Saccharomyces* yeasts were shown to respond differently to prefermentation DAP addition. In particular, for one of the two yeast strains tested, DAP addition to achieve a YAN of 250 mg/L resulted in increased production of H_2S compared to nonsupplemented fermentations. For this yeast, DAP-supplemented fermentations were also characterized by prolonged production of H_2S, which was associated with increased H_2S in the final wines. In general, DAP supplementation corresponded to higher concentrations of organic VSCs in the finished wines, irrespective of yeast species. These results confirm the importance of DAP addition to improve fermentation performances but raise concern about the widespread use of DAP to reduce the occurrence of VSCs in wine prepared from low YAN musts.

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