AN ABSTRACT OF THE THESIS OF

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Title: Impact of Lees Content, Nitrogen, and Elemental Sulfur on Volatile Sulfur Compound Formation in *Vitis vinifera* L. cv. 'Pinot noir' wine

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James P. Osborne

One of the most common problems in wine production is the formation of hydrogen sulfide (H_2S) and other volatile sulfur compounds (VSCs), which can mask positive aromas at low concentrations and result in unpleasant aromas such as rotten egg, cabbage, garlic, and rubber at higher concentrations. Despite the large body of work researching formation of H_2S during wine fermentation, it remains a frequent issue. Furthermore, much less is known about the formation of these VSCs during post-fermentation aging and impact of factors such as wine lees composition. In particular, little is known about the role of lees composition on the VSC precursor compounds cysteine, methionine, and glutathione. In addition, while the impact of grape must nitrogen content on H_2S formation has been documented, the effect of nitrogen composition on the formation of VSCs is relatively unknown. This study investigated the role of wine lees quantity and

composition on formation of VSCs as well as the impact of nitrogen concentration and composition. The role of elemental sulfur in the formation of VSCs during and after fermentation was also investigated.

The impact of wine lees was investigated by fermenting Pinot noir grapes with either a common Pinot noir yeast, *Saccharomyces cerevisiae* RC212, or a non-H2S producing yeast *S. cerevisiae* P1Y2. After fermentation was complete, wines were settled for 0, 24, or 96 hours, resulting in wines with high, medium, or low amounts of lees. Wines were sampled after 0, 14, 30, 60, 90, 180, and 270 days and assessed for VSCs by GC-PFPD and for free amino acids by HPLC-DAD. Glutathione was also assessed after 0 and 30 days by LC-MS/MS. Results showed an increase in methionine and cysteine between 14 and 30 days, which corresponded to an increase in H_2S at the same time. Concentrations of methionine and cysteine increased during aging and were influenced by yeast strain and level of wine lees. Wines produced using P1Y2 contained higher concentrations of methionine and cysteine than wines produced by RC212 at each level of wine lees; higher lees levels also resulted in higher amounts of cysteine and methionine. Increasing levels of methionine and cysteine did not necessarily result in increased amounts of VSCs in the wines. In general, the concentrations of VSCs in the wines were below sensory thresholds. Hydrogen sulfide decreased after the first 30 days of aging, but other VSCs such as methanethiol and dimethyl sulfide persisted at low levels.

The influence of concentration and composition of yeast assimilable nitrogen (YAN) was also investigated. Fermentations were performed by either a high H_2S producing *Saccharomyces cerevisiae* strain (UCD522) or a non-H2S producing *S. cerevisiae* strain (P1Y2). YAN was adjusted to high (346 mg/L) and low (112 mg/L) treatments containing mostly primary amino acids; a third treatment was prepared where the majority of the 346 mg/L YAN was derived from diammonium phosphate (DAP). Lead acetate tubes were used to monitor H_2S production during fermentation; wines were lead acetate tubes then assessed for additional VSCs by GC-PFPD. YAN concentration and composition affected H_2S production during fermentation. Treatments fermented by UCD522 with high DAP had increased formation of H_2S late in fermentation and significantly more methyl thioacetate in the wine post-fermentation. In addition to YAN, the presence of elemental sulfur is also known to impact VSCs. Pinot noir was fermented with an addition of 0, 5, or 15 mg/g elemental sulfur. Fermentations were conducted by UCD522 or P1Y2 at 27°C and H_2S production was again measured using lead acetate tubes. Wines were assessed for additional VSC by GC-PFPD. Addition of elemental sulfur resulted in H_2S formation during the alcoholic fermentation even with non-H_2S yeast. Elemental sulfur increased production of H_2S late in fermentation and resulted in wines that contained higher concentrations of methyl thioacetate post-fermentation. Wines were analyzed for elemental sulfur by HPLC-DAD; almost no elemental sulfur remained in the wines post-fermentation.

IMPACT OF LEES CONTENT, NITROGEN, AND ELEMENTAL SULFUR ON VOLATILE
SULFUR COMPOUND FORMATION IN *VITIS VINIFERA* L. CV. 'PINOT NOIR' WINE

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CHAPTER ONE

LITERATURE REVIEW

Winemaking

It has often been noted that great wines begin in the vineyard. However, the winemaking process itself begins once the harvested grapes are received at the winery. Here, the fermentative yeast *Saccharomyces cerevisiae* convert the sugar in the grape (glucose and fructose) into alcohol. Because these yeasts are ecologically suited to grow on fruits, no human action beyond collection and storage of fruit is required to start a fermentation. Colloquially known as "brewer's yeast" and literally translated as "sugar-loving," the *Saccharomyces* genus is responsible for the fundamental conversion of simple carbohydrates into carbon dioxide and ethanol in the overwhelming majority of all alcoholic products, from beer and wine to sake and distilled spirits.

While there is some debate over when grapes were first grown by humans with the specific purpose of fermentation, the emergence of winemaking goes back to at least 3500 BCE, the approximate age of an amphora stained with tannins and tartaric acid that was found in the Zargros Mountains of present-day Iran (Haapala, 2004). The Romans were famous for their vineyards and were responsible for introducing grapes to many areas, including Burgundy and Bordeaux, likely the two most famous vine-growing areas on the planet today (Johnson and Robinson, 2007). During the past two millennia, viticulturists have worked to gradually determine

through generations of trial and error, the grape cultivars and winemaking practices that are best fit to each of Europe's wine-producing regions. In step with the development in our understanding of how to grow grapes to produce better wines, is the increased exploration into the factors that impact a wine's quality.

Wine Quality and Aroma

The sensory quality of a wine depends on four senses: sight (appearance), smell (aroma), taste, and touch (Jackson and Lombard, 1993). Of these, wine aroma is perhaps the most complex as more than 800 compounds are known to contribute to it (Mendes-Pinto, 2009). However, the perception of aroma can vary a great amount between people due to genetic and cultural differences (Styger et al., 2011). Aroma is also greatly influenced by visual information, and research has shown that olfactory shifts form a response in regions of the brain that are associated with visual stimulation (Styger et al., 2011). Because of this convolution, it can be difficult to objectively analyze a wine using only one sense. Nevertheless, aroma remains a significant contributor to wine quality.

Wine aroma and flavor can be divided into four main components, with some overlap between them. These consist of: 1) compounds that are found in the grapes (primary varietal aroma, 2) compounds that are grape-derived but whose potency is dependent on processing steps (secondary varietal aroma) 3) aroma produced by the microbial populations of yeast during primary fermentation (fermentative

aroma), and 4) aroma dependent on the aging process (post-fermentative aroma (Rapp and Versini, 1991).

Grape derived aroma compounds are primarily secondary metabolites produced by the grapevine (Jackson, 2008). They include 1) C6 alcohols and aldehydes responsible for herbaceous and green aromas, 2) terpenes, providing citrus and floral aromas, 3) thiols, which can range from passionfruit and box tree to onion and rubber aromas, 4) pyrazines, contributing green pepper and roasted aromas, and 5) norisoprenoids, a powerful contributor to floral and fruity aromas (Jackson, 2008). A select few phenolic esters are also found in grapes, but most of these compounds are produced by yeast (Schreier and Jennings, 1979).

 Fermentative aroma in wine is likewise composed of a wide diversity of chemical compounds. These compounds are produced by yeast metabolism during the fermentation. Perhaps the most common olfactory contributor to fermentative aroma are esters. This group of compounds is formed most often as a result of the amino acid metabolism in the grape. Primary alcohols are produced via the Ehrlich pathway which subsequently react with acids to form esters. However, they can also originate from grape glycosides (Bell and Henschke, 2005; Swiegers et al., 2005). Volatile acids also contribute to fermentative aroma. Acetic acid, with its unmistakable vinegar aroma, is by far the foremost component of this group, but medium-chain fatty acids can also be found in wine at low concentrations (Bardi et al., 1999). Another group of compounds that add complexity to the scent of wine is that of higher alcohols, also referred to as fusel alcohols. The exact role of these

compounds is unclear. They can be produced either from amino acids via the Ehrlich pathway or in an anabolic manner from sugars; the latter of these paths is more common in wine (Bell and Henschke, 2005). Carbonyls such as acetaldehyde, with its nutty or cooked fruit aroma, and diacetyl, characterized by a buttery scent, are also produced by yeast metabolism during fermentation. These compounds are intermediates in the formation of higher alcohols (Bell and Henschke, 2005). The final category of aroma compounds that yeast commonly produce during fermentation are volatile sulfur compounds (VSCs). Hydrogen sulfide (H_2S), characterized by a rotten egg odor, is the most simple, frequent, and reactive of this group of chemicals (Rauhut, 1993).The olfactory descriptors of VSCs are generally negative in the context of wine. Aromas are described as cauliflower, onion, and other vegetables as well as skunk, rubber, and struck matches (Rauhut, 1993).

Volatile Sulfur Compounds

Volatile sulfur compounds are important to wine quality primarily because they can impart negative sensory characteristics at low concentrations. For example, the sensory threshold for H_2S in white wine is just 1.6 ug/L (Siebert et al., 2009). It had previously been reported that the sensory threshold of H_2S in wine was 50-80 ug/L, but this number is likely excessive (Wenzel et al., 1980). There is mounting evidence that H_2S serves a role as an intracellular messenger in many organisms, and its formation is conserved amongst the three kingdoms of life-- prokarya, archaea, and eukarya (Kabil and Banerjee, 2010; Kimura, 2002). Its release is typically catalyzed

by the enzymes cysteine beta-lyase and cysteine gamma-lyase in biological systems (Winter et al., 2014). During winemaking, however, the main reason for hydrogen sulfide formation by yeast is due to cellular demand for methionine and cysteine, end products of the sulfate reduction sequence (SRS) (Rauhut, 1993; Spiropoulos et al., 2000). Additional sulfur-containing compounds necessary for cell function includee S-adenosyl methionine (SAM), which acts as a methyl group donor, and glutathione (GSH), primarily an antioxidant. However, methionine and cysteine are precursors of SAM and GSH, respectively. The production of H_2S can also occur as a result of many other reactions outside of the SRS. These include the degradation of sulfur-containing amino acids and GSH (Hallinan et al., 1999; Jiranek et al., 1995a) as well as the chemical reduction of elemental sulfur (Acree et al., 1972).

Sulfate Reduction Sequence

The initial step of the SRS is to accumulate sulfate, which is present in excess amounts in grape must (Henschke and Jiranek, 1991). Once that has been completed, the energy-intensive reduction of sulfate can begin, as shown in Figure 1.1. Sulfate displaces the pyrophosphate of ATP to form adenosine-5'phosphosulfate (APS), with the assistance of ATP-sulfurylase. APS is then phosphorylated to produce 3'phosphoadenosine-5'phosphosulfate (PAPS) with the help of APS-kinase. NADPH and PAPS-reductase are required to then reduce PAPS to sulfite. Some of this sulfite is excreted into the medium, a portion actively via a sulfite permease, but the remainder by passive diffusion (Stratford and Rose, 1985). The remainder of the
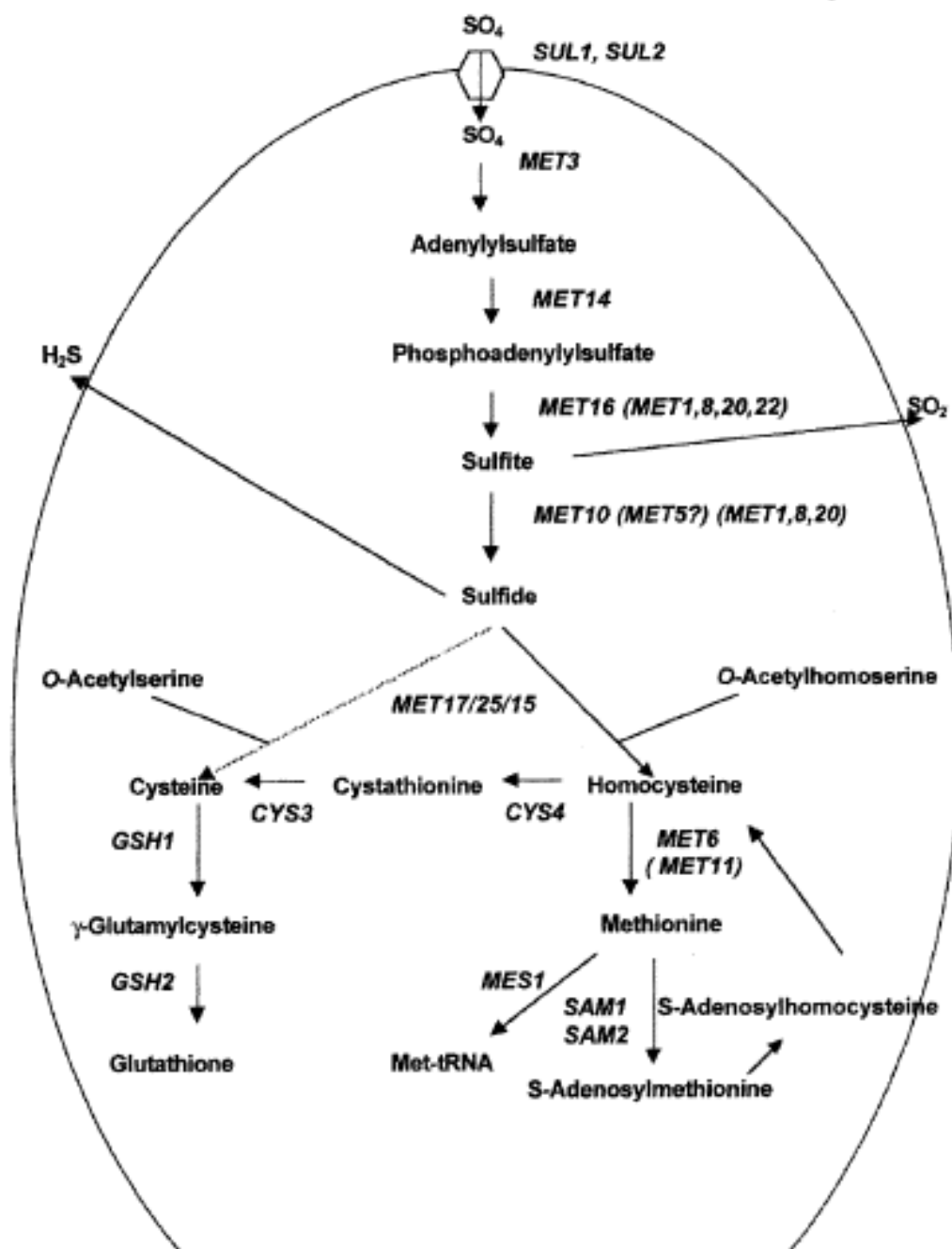
Figure 1.1 Sulfate reduction synthesis (SRS) pathway in *S. cervisiae*. Genes indicated in parenthesis are required for the activity of the enzyme represented on the pathway, but do not encode that activity. Adapted from Spiropoulos et al (2000).

sulfite is reduced to sulfide by one of the two forms of sulfite reductase found in *S. cerevisiae* (Rauhut, 1993). From this point, a number of paths can be followed.

Two distinct pathways utilize sulfide to assemble cysteine. Serine is first acetylated form O-acetylserine via a serine acetyltransferase. Then either cysteine synthase or homocysteine synthase converts it to to sulfhydrylate O-acetylserine. The latter of these enzymes can also form homocysteine, another important compound in cell function. The other method of cysteine assembly begins with beta-cystathione synthase condensing homocysteine and serine into a single molecule. Then, gamma-cystathionase cleaves this compound resulting in cysteine formation. The construction of methionine begins with acetyl-CoA and homoserine acetyltransferase forming O-acetylhomoserine from homoserine. O-acetylhomoserine can either be directly sulfhydralated with a free or bound sulfide, or it can condensed with serine to form cystathionine (aided by gamma-cystathionase) and then split into homocysteine, pyruvate, and ammonia via a beta-cystathionase. Once homocysteine is formed, the sulfur atom is methylated by homocysteine methyltransferase.

Although H_2S is the most commonly produced VSC during fermentation, its high reactivity contributes to the formation of many other volatile sulfur compounds, most of which have low sensory thresholds and negative odor characteristics. Perhaps the most problematic VSCs in winemaking are the mercaptans, methanethiol (or methyl mercaptan, MeSH) and ethanethiol (ethyl mercaptan, EtSH). Methanethiol is characterized by aromas of rotten cabbage, burnt rubber, and

putrefaction, while ethanethiol has a scent of onion, rubber, or fecal (Tsai, 2006). The thioacetic esters of the mercaptans, methyl thioacetate and ethyl thioacetate, can also be formed during fermentation, but are assumed to have sensory thresholds sufficient that they are unlikely to result in negative aromas. However, they can be hydrolyzed to mercaptans during aging under acidic conditions (He et al., 2013).

Disulfides are another group of VSCs that like thioacetates, can also react to form mercaptans during wine storage. Dimethyl sulfide (DMS) is commonly found in fermented beverages and is typified by a corn, asparagus, or molasses odor (Rauhut, 1993). DMS is generally considered a positive at sub-threshold concentrations and may contribute to the aroma of bottle age (Jackson, 2008). It has additionally been shown to enhance fruit character in red wines at low concentrations (De Mora et al., 1987). However, at higher concentrations it can result in negative sensory descriptors in wine such as canned corn (Rauhut, 1993). It has been debated whether DMS production is a result of cysteine degradation or if yeasts can synthesize its precursor S-methyl methionine and thereby directly produce DMS in wine (Rauhut, 1993). Disulfides such as dimethyl disulfide (DMDS), diethyl disulfide (DEDS), and dimethyl trisulfide (DMTS) are also found in wine and generally possess aromatic characters of cooked vegetables and onion (Rauhut, 1993). Additional VSCs found in wine include carbon disulfide (CS_2) and carbonyl sulfide (COS), as well as 3-(methylthio)-1-propanol, 2-mercaptoethanol, and numerous others.

Compound	Threshold value (ppb)			Aroma description
	Wine		12% Ethanol (aq)	
Hydrogen sulfide	0.001-150*		0.8	Rotten egg, decaying seaweed, rubbery
	40-100**			
Methanethiol	1.72-1.82	(red)	0.3	Rotten cabbage, cooked cabbage, burnt rubber, pungent, putrefaction
Ethanethiol	1.1	(white)	0.1	Onion, rubber, fecal, burnt match, earthy, durian
	0.19-0.23	(red)		
Carbon disulfide	30	(white)		Rubber, choking repulsive, cabbage, sulfidy
Dimethyl sulfide	10-160		5-10	Cabbage, asparagus, cooked corn, truffles, vegetal, molasses, black olive
	25	(white)		
	60	(red)		
Diethyl sulfide	0.92-18		6	Garlic, onion, cooked vegetables, rubbery, fecal
	0.92	(white)		
Dimethyl disulfide	20-45		2.5	Cabbage, cook cabbage, onion-like
	29	(white)		
	11.2-23.6	(red)		
Diethyl disulfide	4.3-40		20	Garlic, onion, burnt rubber
	4.3	(white)		
	1.4-2.2	(red)		
Dimethyl trisulfide				Beany
Methyl thioacetate				Sulfurous, rotten vegetables, cheesy, onion, burnt
Ethyl thioacetate				Sulfurous, cheesy, onion, burnt
Methionol	1200-4500			Raw potato, soup-like, meat-like

Table 1.1: Sensory thresholds and aroma descriptors for a variety of VSCs. Adapted from Davis and Qian (2011).

The sensory impacts of VSCs are almost entirely negative to wine quality when present at levels above sensory thresholds. These compounds, in particular H_2S, are one of the most common problems encountered during winemaking, regardless of style, region, or cultivar. Because of this, factors impacting their formation during winemaking have been extensively studied. These studies have tended to focus on H_2S production and have investigated factors such as yeast strain, nitrogen, vitamins, elemental sulfur, and organic sulfur-containing compounds such as glutathione, cysteine, and methionine. What follows is an overview of some of these factors with an emphasis on factors investigated during the current study.

Factors Influencing the Formation of Volatile Sulfur Compounds

Yeast species and strain is perhaps the most important factor impacting the formation of H_2S and other VSCs during alcoholic fermentation. The genome of *S. cerevisiae* varies a wide amount between strains, and the genes that dictate the sulfate reduction sequence and other enzymes that act on sulfur-containing chemicals fluctuate significantly between strains. Some strains, such as *S. cerevisiae* UCD522 (Montrachet), have been frequently reported as high producers of H_2S (Bohlscheid et al., 2007; Linderholm et al., 2010; Wang et al., 2003). Other strains, such as EC1118 (Pris de Mousse), are known for producing low amounts (Mendes-Ferreira et al., 2002). In a survey of 170 yeast strains, it was reported that only seven did not produce any measurable H_2S (Kumar et al., 2010). This work also found that only 12 strains conformed to the conventional assumption of decreases

in H_2S accompanying increasing nitrogen content. In addition 12 strains did not produce H_2S in low nitrogen grape must but did produce it when in a high nitrogen must (Kumar et al., 2010).

Some of the variability in H_2S production between yeast strains is due to genes encoding for sulfite reductase, a key enzyme in the SRS pathway (Linderholm et al., 2006; Rauhut, 1993). Allele differences in the *MET10* gene which encodes for catalytic subunits in sulfite reductase resulted in differences in the amount of H_2S produced by the yeast strain. It was discovered that the *MET10* allele in a vineyard-isolated strain UCD932 prevented the yeast from releasing H_2S during fermentation (Linderholm et al., 2010). When this *MET10-932* allele was swapped into other yeast strains using PCR techniques, it was found that the allele conferred this same quality into those strains, but did not make them auxotrophic for methionine and cysteine. Eventually, after development with backcrossing techniques, non-H_2S-producing yeast strains produced with *MET10-932*, including *S. cerevisiae* P1Y2, were released for use in the wine industry.

Nitrogen

The amount and type of nitrogen present during fermentation can have a significant impact on the alcoholic fermentation and the production of H_2S by *S. cerevisiae*. A number of studies have reported that having inadequate levels of nitrogen during fermentation can lead to H_2S production (Jiranek et al., 1995b; Park et al., 2000; Vos and Gray, 1979). However, others have also seen the opposite trend, and have found

that musts with low levels of nitrogen produce less H_2S than those with higher nitrogen concentrations (Mendes-Ferreira et al., 2009; Ugliano et al., 2011). Although many factors besides nitrogen are at play in these experiments, it undoubtedly still plays an important role in the formation of H_2S.

Grapes are the sole natural source of nitrogen that yeast will utilize during the alcoholic fermentation. Nitrogen in the grape is impacted by a number of different factors including cultivar, rootstock, climate, and soil (Ough, 1968). Vineyard practices such as canopy management, trellis choice, and fertilization will also have a significant impact on grape nitrogen content (Bell and Henschke, 2005; Jackson, 2008). Vineyard fertilization can be performed in several different ways, including the use of compost, inorganic soil amendments, and foliar sprays (Bell and Henschke, 2005). As the rate of nitrogen application increases, grape berry nitrogen continues to increase but at a decreasing rate (Bell, 1994). While adequate nitrogen is necessary for vine health and a strong fermentation, high levels of vine nitrogen should be avoided, since they can result in a shift of vine balance towards excessive vegetative growth at the expense of yield (Kliewer, 1971).

Nitrogen in the grape berry exists in several forms, including proteins and peptides of various lengths, ammonia, and amino acids. However, only two of these forms are able to be utilized by yeast during fermentation; ammonia and amino acids containing a primary amine group (Dukes and Butzke, 1998). These two types of nitrogen are typically assessed and reported as yeast assimilable nitrogen (YAN). Over the course of the growing season, the concentrations of primary amino acids

and ammonia are continuously changing. In general, the ammonium concentration in the berry decreases during ripening, often as a great as a 50% decrease between veraison and harvest (Bell, 1994; Solari et al., 1988). Most primary amino acids in the grape, however, tend to increase during berry ripening (Bell and Henschke, 2005). Oftentimes, the primary amino acid content will plateau in the weeks immediately prior to harvest, and then stabilize or decrease during the final ripening (Hernandez-Orte et al., 1999).

While a grape berry may contain high amounts of amino acids, often a large portion of these amino acids are not assimilable. This is largely due to the fact that for most grape varietals the most abundant amino acid present is proline which is not a primary amino acid (Butzke, 1998). While proline can be utilized by *Saccharomyces cerevisiae* yeast strains under aerobic conditions, when molecular oxygen is sufficient to activate proline oxidase and when catabolite repression of proline permease is inactivated (Lasko and Brandriss, 1981). It is not able to be utilized during anaerobic conditions when yeast have switched to fermentative metabolism.

Many cultivars are considered to be either proline accumulators or arginine accumulators. Arginine tends to begin to accumulate before veraison, while proline does not normally accumulate in berries until after veraison occurs (Stines et al., 2000). However, post-veraison, proline accumulators including Chardonnay and Cabernet Sauvignon experience a large increase in proline that is generally accompanied by a small decrease in arginine (Bell and Henschke, 2005). The fate of the lost arginine is unknown, but it may be shunted to the roots to prepare for the

following season, or may be enzymatically converted to proline (Bell and Henschke, 2005). High arginine accumulators such as Muscat and Gewurztraminer experience increases in arginine during the entirety of ripening (Stines et al., 2000). Pinot noir is typically considered an arginine accumulator, with a mean proline:arginine ratio of less than three (Spayd and Andersen-Bagge, 1996).

A lack of nitrogen during fermentation can result in two major issues. The first is that a stuck or lagging fermentation may occur (Agenbach, 1977). This serious and costly problem is most commonly avoided by use of diammonium phosphate (DAP) to supplement the YAN naturally present in the grapes. While some musts containing as little as 50 mg/L YAN have completed fermentation without a problem, the typical minimum recommendation is 140 mg/L (Bell and Henschke, 2005). Generally, researchers recommend higher levels of YAN from 200-350 mg/L, depending on sugar concentration, to 400-500 mg/L YAN (Bisson and Butzke, 2000; Jiranek et al., 1995a). However, if unused nitrogen remains in the wine after fermentation, it may contribute to both microbial instability and formation of the carcinogen ethyl carbamate (Ough and Amerine, 1988).

The second major issue that nitrogen concentration can cause is the formation of H_2S by *S. cerevisiae* during fermentation. A number of studies have demonstrated that low YAN levels in grape juice/must cause higher production of H_2S (Barbosa et al., 2012; Jiranek et al., 1995a). For example, Jiranek et al. (1995) found that only three amino acids were not able to significantly reduce H_2S during fermentation when added to must. These three were cysteine, proline, and threonine. Other work

has found that non-sulfur-containing amino acids could reduce H_2S production but tended to increase sulfite production (Giudici and Kunkee, 1994). However, other researchers have reported that high YAN levels can also cause increased H_2S production with the composition of YAN playing an important role (Mendes-Ferreira et al., 2009). For example, one study reported that low YAN (66 mg/L) resulted in the lowest amount of H_2S produced, while the highest levels were produced in juice with 267 YAN mg/L (Mendes-Ferreira et. al., 2009). This study concluded that, at least for the yeast strain EC1118, the liberation of H_2S may be impacted more by nitrogen type and/or vitamin deficiency than by nitrogen concentration.

The impact of DAP supplementation to increase YAN has been further reported to cause both increases and decreases in H_2S production by *S. cerevisiae*. For example, Spiropoulos et al. (2000) noted that H_2S production by *S. cerevisiae* increased with a DAP addition when methionine concentration was inadequate but decreased with methionine addition when ammonia concentration was adequate. Methionine is known to suppress the production of hydrogen sulfide as it is involved in the regulation of the sulfate reduction pathway and thereby the production of hydrogen sulfide (Bell and Henschke, 2005). Gobbi et al. (2012) studied Verdicchio must and reported that DAP supplementation at 250 mg/L YAN significantly reduced H_2S formation compared to the control fermentation, while a lower level of YAN from DAP (200 mg/L YAN) did not significantly impact H_2S formation. This study was performed with only one yeast strain, EC1118, and DAP was added incrementally

during days 3-5 of the fermentation. This method of addition is has become a

recommended practice, following the logic that the yeast should be made to utilize

the majority of naturally present nitrogen before receiving supplemental nitrogen.

In support, Mendes-Ferreira et al. (2010) reported that DAP additions 72 hours

after inoculation, rather than at the time of inoculation, decreased total H_2S

production by three different yeast strains. In one final example, Ugliano et. al.

(2010) reported the impact of DAP addition in Syrah grape musts fermented by two

different yeasts. DAP supplementation increased many positive flavor attributes, but

also increased dimethyl sulfide, diethyl sulfide, and the corresponding disulfides.

For one of the yeasts, a moderate DAP addition (to 250 mg/L YAN) resulted in an

increase in H_2S as well. Overall, research findings indicate that too low or too high

amounts of YAN, particularly if YAN is adjusted by DAP, can result in higher H_2S

production. However, many other factors that impact H_2S production make it

difficult to predict what levels of YAN should be so as to minimize the risk of H_2S

production by yeast during alcoholic fermentation.

Vitamins

Aside from nitrogen, another component of grapes can impact H_2S production by

yeast, namely vitamins. While *S. cerevisiae* can synthesize many vitamins from

simpler compounds, a number of them must be present in the fermentation media

for adequate cell growth. Additionally, microbial contamination can result in

decreased levels of vitamins available in the must (Jackson, 2008). Key vitamins

include biotin, thiamin, and pantothenic acid. Biotin, also called Vitamin B7, acts as a cofactor for carboxylase enzymes involved in fatty acid synthesis and gluconeogenesis (Attwood and Wallace, 2002). Its deficiency may result in a diminished synthesis of proteins and can decrease the fermentation rate (Shimada et al., 1978). A slower fermentation could theoretically result in reduced H_2S production, but it may also result in a stuck fermentation. The recommended biotin level in grape must is 3 to 5 ug/L (Monk and Costello, 1984). In a survey of northwest United States vineyards, many samples—in particular white musts-- were found to contain significantly less than 3 ug/L of biotin, and the lone vineyard of Oregon Pinot Noir sampled had an average of 1.14 ug/L (Hagen et al., 2008). However, biotin requirements are strain dependent and for many yeasts 1 ug/L is sufficient (Rauhut, 1993).

Thiamine, or Vitamin B1, is another important vitamin for *S. cerevisiae*. Although the organism can synthesize it, this is not ideal for growth, and requirements total 50-500 ug/L . Genes encoding the overproduction of thiamine in yeast can lead to an increase in H_2S, as it is a sulfur-containing compound (Bartra et al., 2010). Thiamine can also be cleaved by sulfur dioxide, which could further contribute to the release of H_2S (Boulton et al., 1999).

Pantothenic acid, also referred to as Vitamin B5, is a component of coenzyme A (CoA), and is therefore essential for life-sustaining reactions such as lipid synthesis and energy metabolism (Rattray et al., 1975). A deficiency of this vitamin has been repeatedly shown to cause increases in hydrogen sulfide production during

fermentation (Tokuyama et al., 1973; Wang et al., 2003). Additionally, it has been found that in pantothenic-acid deficient media, an increase in YAN increases H_2S, whereas an increase in YAN generally decreases H_2S in musts with adequate levels of pantothenic acid (Wang et al., 2003). Recommended levels of pantothenic acid are at least 150-250 ug/L (Ough and Kunkee, 1968). In a survey that measured pantothenic acid in one Oregon Pinot Noir vineyard the levels were found to be sufficient at 388 ug/L (Hagen et al., 2008).

Sulfite and Sulfate

Sulfates are also an important component of grape berries and are particularly relevant to the anabolism of sulfur-containing amino acids (Rauhut, 1993). The principal inorganic sulfur compound in grapes is sulfate (Henschke and Jiranek, 1991). Sulfates are generally present in excess amounts necessary for the production of methionine and cysteine, averaging 260 mg/L as potassium sulfate in a survey of over 1200 Australian grape juices (Leske et al., 1997). In a survey of German wines, sulfate content ranged from 160-400 mg/L (Lemperle and Lay, 1989).

Sulfur dioxide (SO_2) is another potential contributor to volatile sulfur compound formation in wine. Because it is an intermediate in the sulfate reduction sequence, sulfite can be reduced into sulfide and subsequently released into the wine matrix (Rauhut, 1993). The relationship between sulfite concentration in fermentation

media and subsequent H_2S production has been shown to be linear, but there is a delay in the timing of the H_2S release associated (Stratford and Rose, 1985). Other work has shown that formation of H_2S from sulfite is similar to that from sulphate (Eschenbruch, 1978), but additions of SO_2 to must have repeatedly been shown to increase H_2S formation (Karagiannis and Lanaridis, 1999; Lavigne-Cruege, 1996). Additionally, levels of H_2S have been found to correlate with the timing of SO_2 additions, with the highest levels in wines that were treated immediately after fermentation and the lowest in wines that did not receive SO_2 for several days (Lavigne et al., 2006).

Elemental Sulfur

Grape nutrient content (nitrogen and vitamins) along with the yeast strain that conducts alcoholic fermentation are known to be two of the most important factors impacting H_2S production during winemaking (Rauhut, 1993, Spiropoulos et al., 2000). However, the presence of elemental sulfur during fermentation an additional factor that is known to significantly impact H_2S formation. Elemental sulfur is a frequently used fungicide in the vineyard, where it is used to control the growth of powdery mildew (*Uncinula necator*). It is a particularly useful tool for organic-certified vineyards as elemental sulfur is practically the only control material available for use. There are a number of different types of sulfur used, including colloidal, wettable, dusting, precipitated, and sublimed (Schutz and Kunkee, 1977). In Oregon, wettable sulfur is typically preferred, as early and late season rains are prevalent. In drier regions such as California, dusting sulfur is preferred due to its

affordability. The problem with elemental sulfur use in the vineyard is that if certain levels of sulfur remain on the grapes at harvest then these residues can be reduced to H_2S. Elemental sulfur has also been shown to produce H_2S in a way unrelated to concentration of yeast cells, while H_2S from sulfate behaves in a proportional manner to the fermentative capability (Acree et. al., 1972). This information points to a chemical reduction of elemental sulfur.

The concentration of elemental sulfur required to cause H_2S production as well as how long elemental residues remain on the grapes is still controversial. Early experiments by Rankine (1964) reported that 20 mg/L of elemental sulfur tripled the amount of hydrogen sulfide in wine at the finish of primary fermentation. This finding was consistent across a number of different *S. cerevisiae* strains. Others have reported increased H_2S production when elemental sulfur levels exceeded 10 mg/L (Acree et al.**,** 1972; Schutz and Kunkee, 1977). More recent studies have reported increased H_2S production with levels of elemental sulfur as low as 1 ug/g (Kwasniewski et al., 2014). With regards to the type of sulfur used, Schutz and Kunkee (1977) found that colloidal sulfur produced more than double the amount of the other four types of sulfur used. In addition, Kwasniewski et. al. (2014) reported that the type of elemental sulfur applied in the vineyard impacted how long the residue persisted on the grapes which in turn impacted how much H_2S was produced during fermentation.

This persistence of elemental sulfur in the vineyard is also not fully understood. The few studies that have investigated how long elemental sulfur persists report

conflicting information (Kwasniewski et. al., 2014). Nearly all studies have reported levels significantly lower than the 20 mg/L elemental sulfur used in Rankine's experiments. However, in Germany, levels as high as 8 mg/L were found on grapes despite discontinuation of sulfur spray 7 weeks prior to harvest (Wenzel et. al., 1980). A study on the application of dusting sulfur in California showed that sulfur applications six weeks prior to harvest resulted in sulfur levels between 1-3 ug/g by the time of harvest (Thomas et al., 1993a). More recently, Kwasniewski et al. (2014) reported that ceasing elemental sulfur application > 35 days prior to harvest resulted in elemental sulfur residues of < 10 mg/L. However, sulfur residues > 1 ug/g correlated with increased H_2S production during fermentation and that these levels were found on fruit sprayed within 56 days of harvest.

Other work has investigated the effect of elemental sulfur in fermentations of synthetic media and Thompson Seedless concentrate. This study concluded that 3.4 mg/L additions of dusting sulfur did not exert any significant effects on the production of hydrogen sulfide (Thomas et al., 1993b). The authors proposed and reported that often during fermentation there were two distinct peaks of H_2S produced. One larger peak early during growth phase, and another smaller peak towards the end of fermentation (Thomas et al., 1993b). Based on these results, the authors hypothesized that the first H_2S peak depended mainly on yeast strain, while the second peak was related to the chemistry and conditions of the fermentation media. The second peak, though its cause is unknown, may be more problematic as

it could contribute to residual H_2S in the wine at the end of fermentation and lead to the formation of other VSCs during wine aging.

Formation of Volatile Sulfur Compounds Post-Fermentation

Unlike the formation of H_2S during fermentation, the formation of VSCs in wine aging has not been thoroughly elucidated. Despite the lack of research into this area, some causes for VSC formation during aging have been established, particularly with regard to MeSH. It has been previously shown that methionine can be metabolized into MeSH by yeast, but the conditions leading to this reaction have not been investigated (de Mora et al., 1986). Work in model fermentation media has shown that the formation of MeSH can be dependent on pH, suggesting that non-enzymatic pathways may be a significant contributor (Wainwright et al., 1972). Exposure to sunlight can also catalyze the formation of MeSH and is a frequent cause of sulfur off-odors in sparkling wines (Jackson, 2008; Maujean, 1984). Methionine can also be metabolized during malolactic fermentation (MLF) by certain strains of *O. oeni* to form MeSH, DMDS, and other sulfur compounds (Pripis-Nicolau et al., 2004).

Both enzymatic and chemical degradation of cysteine is also believed to contribute to the formation of VSCs, particularly H_2S, late in fermentation and during aging (Ugliano et al., 2009; Jackson, 2008). Many studies have reported the role of cysteine in causing increased formation of H_2S during fermentation (Acree et al., 1972;

Giudici and Kunkee, 1994; Moreira et al., 2002). However, these studies have not demonstrated the same relationship during wine aging. Additionally, substantial differences in enzymatic degradation of cysteine to form H_2S is present among genetic variants of *S. cerevisiae* (Winter et al., 2014). Yeast strains that are high producers of H_2S from cysteine tend to have mutations in mitochondrial iron-sulfur homeostasis genes (Winter et al, 2014). It is believed that an excess of cysteine is necessary for this phenotype to result (Linderholm et al., 2008). Regardless, more research is needed to understand the mechanisms of cysteine degradation in a wine environment as both methionine and cysteine could potentially play an important role in the formation of VSCs during wine aging.

An additional sulfur containing compound that may play a role in VSC formation during wine aging is glutathione. Glutathione is perhaps the most common sulfur-containing compound in yeast and many other organisms (Penninckx, 2002). Glutathione (GSH) is a tripeptide consisting of glutamic acid, glycine, and cysteine. It is an important component of yeast and other higher organisms, acting as an internal antioxidant. It can account for one percent of the yeast dry weight, roughly a 10mM concentration (Elskens et al., 1991; Penninckx, 2002). When glutathione is oxidized, it becomes glutathione disulfide (GSSG), alternatively known as L (-) glutathione, which consists of two GSH molecules connected by a disulfide bridge (Ugliano et. al., 2011). Despite GSH being a significant source of S-containing compounds, its role in the formation of VSCs during the aging of wine of the lees is relatively unknown. However, GSH is known to protect certain sulfur containing

compounds such as volatile thiols from oxidation. Compounds such as 3-mercaptonexan-1-ol (3MH), 3-mercaptohexylacetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP) are important to white wine aroma, particularly that of Sauvignon Blanc, and provide passionfruit and box tree aromas (Ugliano, 2013). It has been shown that 3MH existed in significantly higher concentrations in wines treated with GSH (Dubordieu et al., 2006). This may be due to GSH's inhibition of the formation of the extremely reactive ortho-quinone molecules (Kritzinger et al., 2013).

However, in addition to protecting positive thiols such as 3MH, GSH can also shield negative sulfur compounds such as H_2S and MeSH. In a study investigating all three of these compounds, it was found that GSH was able to slow degradation of 3MH, but that H_2S accumulated to higher concentration in the GSH treatment (Ugliano et. al., 2006). This aligns with the expected chemistry of GSH acting as an internal antioxidant. Formation of H_2S has been correlated with GSH activity in various studies. When GSH was specifically inhibited, it was found that H_2S concentration was consequently decreased (Kritzinger et al., 2013). Additionally, *S. cerevisiae* have been shown to utilize GSH as a nitrogen source when in circumstances of nitrogen starvation; this may be a primary cause of H_2S release in low nutrient musts (Hallinan et. al., 1999). Whether the yeast remaining at the completion of fermentation continue this tendency during aging has not been clarified.

Concentrations of GSH tend to increase during the second half of fermentation in white musts, and have been positively correlated with total YAN as well as primary

amino acids (Park et. al., 2000). However, GSH concentrations tend to decrease during the aging of wine, as the compound both oxidizes to GSSG and other compounds, and some work has found no detectable GSH after only 6 months in bottle (Di Lecce et al., 2013) . Very little is known about GSH in red wine. Because red wine contains a much greater concentration of antioxidant phenolic compounds, oxidative browning is not a frequent concern for young red wines; this may explain the lack of research in this area.

One of the factors that may impact the concentrations of sulfur containing compounds such as cysteine and glutathione during aging is the amount and composition of the wine lees during aging. After a red wine has finished fermentation and has been pressed off the skins a certain amount of solids remain in the wine and these are referred to as lees. Aging on the lees has been reported to be a contributing factor to the formation of VSCs during wine aging (Rauhut, 1993) but the relationship between the formation of VSCs and the composition of the wine lees has not been established. Wine lees are primarily composed of *S. cerevisiae* cells (living and dead) but also may include some tartaric acid and inorganic matter (Pérez-Serradilla and de Castro, 2008). Lees can help to remove a number of undesirable compounds such as mycotoxins from spoilage fungi and synthetic fungicides (Perez-Serradilla and Luque de Castro, 2008). In addition, some VSCs can be removed by aging on the lees (Palacios et al., 1997; Vasserot et al., 2003). Two proposed mechanisms explain the removal of VSCs by lees. First is the cation-mediated reaction of VSCs (Lavigne, 1996). Another explanation is the formation of

disulfide bridges between VSCs and cysteine molecules embedded in yeast mannoproteins (Palacios et al., 1997).

While lees can aid in the removal of VSCs, lees content and must turbidity have also been shown to contribute to the formation of VSCs both during and after fermentation. This may be dependent on grape varietal, as one study found that H_2S production increased with increasing turbidity in fermentation of Batiki must, but was unchanged in the fermentation of Muscat (Karagiannis and Lanaridis, 1999). However, the yeast that conducted the fermentation in this study was unknown as fermentations were performed by yeast naturally present in the ferments. As yeast strain is known to impact $H2S$ production the differences in H2S production may have been caused by different yeast strains performing the fermentations rather than the different grape varietals. Other studies have reported that addition of sediment to a synthetic fermentation medium increased H_2S formation across various cultivars (Eschenbruch et al., 1978). Vos and Gray (1979) also reported that the addition of bentonite, a protein fining and settling aid, was an effective method of decreasing H_2S production. Finally, research in sparkling wine has found correlations between the storage temperature, amount of time aging on the lees, and production of VSCs, particularly DMS (Fedrizzi et al., 2011).

The causes of H_2S and VSC formation during and after alcoholic fermentation are numerous. Despite the large number of studies that have investigated the formation of H_2S, information specifically related to the causes of VSC formation in particular *Vitis vinifera* cultivars, such as Pinot noir, is scarce. Yet, wine industry professionals

continue to struggle with the formation of these compounds during winemaking. In particular, Oregon winemakers have reported issues with VSC formation during aging of wines on the lees. Therefore, the objective of this study was to investigate the role of lees quantity and composition on the formation of VSCs during wine aging. The impact of yeast strain and lees quantity on the concentration of potential VSC precursors cysteine, methionine, and glutathione will be investigated. In addition, the impact of YAN concentration and composition (ammonia nitrogen versus amino acid nitrogen) and elemental sulfur on the production of H_2S and formation of VSCs post-fermentation will be explored.

CHAPTER 2

IMPACT OF LEES CONTENT ON VOLATILE SULFUR COMPOUND FORMATION

ABSTRACT

Development of volatile sulfur compounds (VSCs) post-fermentation can be a

significant issue during both red and white winemaking. Unfortunately, our

understanding of contributing factors or conditions that impact VSCs is limited, due

in part to the complexity of their formation. This study focused on the development

of VSCs in Pinot noir during post-fermentation aging and the impact of wine lees

levels and composition. Pinot noir wine was produced from grapes harvested from

the Oregon State University vineyard. Fermentations were performed using two

different *Saccharomyces cerevisiae* strains: RC212, a commonly used strain used for

Pinot noir, or P1Y2, a non-H_2S producing strain. After pressing, wines were settled

for 0, 24, or 96 hours before being dispensed into 12-L carboys. This resulted in

wines with high, medium, or low amounts of lees. Wines were stored at 13°C and

sampled after 14, 30, 60, 90, 180, and 270 days. Samples were analyzed for VSCs by

GC-PFPD and for amino acids by HPLC-DAD. Concentrations of methionine and

cysteine increased during aging and were influenced by yeast strain and level of

wine lees. Wines produced using P1Y2 contained higher concentrations of

methionine and cysteine than wines produced by RC212 at each level of wine lees.

Higher lees levels also resulted in higher amounts of cysteine and methionine in the

wines. Increasing levels of methionine and cysteine did not necessarily result in

increased amounts of VSCs in the wines. In general, the concentrations of VSCs in the wines were below sensory thresholds. Dimethyl sulfide increased for all treatments during aging and was significantly higher in wines with heavy lees. Concentrations of methyl thioacetate and methionol were higher in wines produced by RC212 and changed little over time. Hydrogen sulfide increased in most wines between 2 and 4 weeks aging and decreased thereafter, corresponding with a simultaneous increase in sulfur-containing amino acids.

INTRODUCTION

In the process of making wine, volatile sulfur compounds (VSCs) may be generated that negatively impact wine aroma. These compounds are particularly problematic because of their low detection threshold and the difficulty of removing them from wine (Rauhut, 1993). Labeled with such descriptors as rotten egg, onion, cabbage, and rubber, these compounds provoke a severely negative reaction when present in high levels. Included are H_2S, methanethiol (MeSH), ethanethiol (EtSH), dimethyl sulfide (DMS), and many others. The production of H_2S during fermentation has been extensively studied (Rankine, 1963; Acree et al., 1972; Jiranek and Henschke, 1991; Guidici and Kunkee, 1994; Edwards and Bohlscheid, 2007; Spiropoulos et al., 2000; Kumar et al., 2010). Previous work has identified many causes of H_2S formation such as, free amino acid content (Jiranek and Henschke, 1991; Guidici and Kunkee, 1994; Jiranek et al., 1995), pantothenic acid (Tokuyama et al., 1973; Edwards and Bohlscheid, 2007), yeast strain (Spiropoulos et al., 2000; Kumar et al.,

2010), and fermentation conditions (Rankine, 1963; Karagiannis and Lanaridis, 1999). However, research on the formation of H_2S and other VSCs post-fermentation is limited despite the fact that even the formation VSCs post-fermentation is typically more problematic for wine quality (Rauhut, 1993; Thomas et al., 1993; Moreira et al., 2002).

In addition to the formation of H_2S during fermentation, additional VSCs can be generated post-fermentation. These compounds include thiols, such as MeSH and EtSH; sulfides, DMS; disulfides, such as dimethyl disulfide (DMDS), diethyl disulfide (DEDS); trisulfides, such as dimethyl trisulfide (DMTS); thioesters, such as methyl thioacetate, ethyl thioacetate, and sometimes, ethyl 3-methylthiopropanoate and 3-methylthiopropyl acetate. Development of these compounds occurs post-fermentation, typically as wine ages on the wine lees, a technique often employed during both red and white wine production to enhance mouthfeel (Escot et al., 2001; Perez-Serradilla and Luque de Castro, 2008).

One potential cause of VSC formation post-fermentation is the aging of wine on lees. Aging of wine on its lees, primarily yeast sediment deposit, is a common practice in many regions and in various styles of winemaking (Boulton et al., 1996). Studies have found that lees is capable of both adsorbing and releasing various chemical compounds (Perez-Serradilla and Luque de Castro, 2008; Vasserot et al., 2003; Palacios et al., 1997). Other work has found that the inclusion of additional sediment or lees promotes H_2S formation (Tamayo et al., 1999; Karagiannis and Lanaridis, 1999). Anecdotally, Oregon winemakers have noted formation of sulfur off-odors

early during barrel aging on wine lees. When wine was racked into a different barrel, the remaining lees has been identified as the source of these off-odors. These observations suggest that the composition of the wine lees was giving rise to the formation of the VSCs. Compositionally, the presence of compounds such as cysteine, methionine, and glutathione, known precursors for formation of VSCs (Schutz and Kunkee, 1977; Moreira et al., 2002), could impact the formation of VSCs but this relationship has not been reported.

The sulfur-containing amino acids cysteine and methionine have been shown to impact H_2S formation by chemical or enzymatic means during alcoholic fermentation (Winter et al., 2014; Rauhut, 1996; de Mora et al., 1986; Moreira et al., 2002). Metabolism of methionine can result in the formation of MeSH, but the conditions promoting this reaction are unknown (de Mora et al., 1986). However, methionine is known to act as an inhibitor of H_2S production during fermentation, as it is the end product of the sulfate reduction sequence (SRS), and therefore exerts a repressive effect on this pathway (Giudici and Kunkee, 1994, Mendes-Ferreira et al., 2002). Cysteine, on the other hand, has been found to promote H_2S formation during fermentation, despite it also being another end product of the SRS. Several studies have demonstrated that cysteine increases H_2S formation (Giudici and Kunkee. 1994; Moreira et al., 2002; Wainwright, 1971). It is believed that cysteine degradation is a primary cause of H_2S formation post-fermentation, but this has not been substantially proven (Tokuyama, 1973; Jackson, 2008). In addition, glutathione (GSH), a tripeptide containing cysteine, has also been proposed as a

possible precursor to H$_2$S and other VSCs. Making up more than 1% of dry yeast

(Pennickx, 2002), this compound is an important antioxidant in the cell (Kritzinger

et al., 2013). It has been shown to protect H$_2$S from oxidation (Ugliano et al., 2006),

and the formation of H$_2$S is decreased when GSH synthesis is specifically inhibited

(Kritzinger et al., 2013).

The formation of VSCs post-fermentation can cause significant wine quality issues

but the underlying causes for this issue are not well understood. Based on

winemaker reports of early formation of VSCs post-fermentation being related to

the wine lees this study investigated the role of wine lees quantity and composition

on formation of VSCs. In particular, the concentration of potential VSC precursors

cysteine, methionine, and glutathione were measured as Pinot noir wines were aged

on various amounts of wine lees.

MATERIALS and METHODS

Grapes

Pinot noir grapes were harvested from Oregon State University's Woodhall

Vineyard (Alpine, Oregon, USA) on September 27[th,] 2013. Grapes were immediately

destemmed upon arrival to the OSU research winery with a Velo DPC 40

destemmer/crusher (Altivole, Italy).   The crusher was not employed in fruit

processing. The grapes were pooled and divided into six 100 liter tanks, each

containing approximately 60 liters of grape must. After destemming, sealed lids were placed on the tanks and a pre-fermentation maceration (cold soak) was performed for four days at a temperature of 8°C. Samples were taken from each tank to assess basic juice parameters at the end of the cold soak. Measurement of pH was performed with an OrionStar A111 pH meter (Thermo Scientific, Waltham, MA, USA). °Brix were measured with an Anton Paar DMA 35N density meter (Graz, Austria). Titratable acidity was determined by titration with 0.1 N sodium hydroxide solution. Yeast assimilable nitrogen (YAN) was quantified as the sum of primary amino acids and ammonia in units of mg/L N. Primary amino acids were measured according to Dukes and Butzke (1998) with a test kit (Gusmer Enterprises Inc., Fresno, CA, USA) and ammonia was measured by enzymatic test kit (R-Biopharm, Darmstadt, Germany). A Genesys 10UV spectrophotometer (Thermo Scientific, Waltham, MA, USA) was utilized for both YAN and ammonia analysis.

Alcoholic Fermentation

Three tanks of grape must was inoculated with either *Saccharomyces cerevisae* RC212 (Lallemand, Montreal, Canada), a strain commonly used in Willamette Valley Pinot noir production, or P1Y2 (Phyterra, Napa, CA, USA), a strain developed to prevent production of hydrogen sulfide during fermentation. Yeast were hydrated and inoculated at a rate of 0.25 g/L according to manufacturer recommendations. Tanks were placed into a temperature-controlled room at 27 °C (80 °F). Cap punch downs were performed uniformly twice daily and temperature and °Brix were

measured with an Anton-Paar DMA 35N Density Meter (Graz, Austria). Separate

punch-down tools were used for each treatment and punch down tools were

sanitized with alcohol (70% v/v) after use. Completion of alcoholic fermentation

(reducing sugar concentration below 0.2g/100mL) was confirmed by testing with

Bayer Clinitest tablets (Morristown, NJ, USA). Once fermentations had reached

dryness, wines were pressed with a Willmes model 6048 pneumatic bladder press

(Lorsch, Germany). The press was held at 1 bar of pressure for 2 minutes, then the

press door was opened to in order to mix the skins by hand before pressing at 1.5

bar for 2 additional minutes.

Settling, Storage Conditions, and Sampling

The three tanks of wine for each yeast strain treatment were pressed and pooled in

a large 500 L stainless steel tank. An addition of 50 mg/L sulfur dioxide (from

potassium bisulfite) was made at this point. The wine was mixed and then three 12L

carboys were filled with wine for each treatment. This resulted in wine with no

settling of any lees and represented the heavy lees treatment. The remaining wine

was placed in a cold room at 4° C for 24 hrs. After this time period wine was racked

from the top of the tank (no stirring) and used to fill another three 12L carboys per

yeast strain treatment. This represented the moderate lees treatment. The

remaining wine was placed back in the cold room for an additional 72 hrs. At this

point wine was again racked off the top of the tanks (no stirring) and used to fill

three 12L carboys per yeast treatment. This represented the light lees treatment. All

carboys were fully topped so there was no headspace, and samples were taken for turbidity, amino acid, and VSC analyses at filling. Wines were assessed for turbidity in units of NTU by a 2100AN Turbidimeter (Hach Company, Loveland, CO, USA). Wines were stored in a temperature-controlled room at 13° C with air locks fitted on all carboys. Wines were assessed monthly for free sulfur dioxide (SO_2) by aeration-oxidation ((Buechsenstein and Ough, 1978). Additional SO_2 was added from a 10% potassium bisulfite solution to maintain a level of 30 mg/L free SO_2.

Each carboy was sampled after 2 weeks, 1 month, 2, months, 3 months, 6 months, and 9 months of storage. Large stirbars (VWR, Radnor, PA, USA) were sanitized and added to each of the 18 carboys during storage. Wines were stirred for 5 minutes at low rpm (approximately 300 rpm) using a stirplate prior to sampling. Two 15 ml sample volumes were removed from the center of each carboy using a sterile 25 mL glass pipette. Nitrogen gas was flushed into the carboys after sampling. Samples were assessed immediately for VSCs or frozen at -80° C for additional analysis.

Volatile Sulfur Analysis

Analysis of volatile sulfur compounds was performed by gas chromatography as detailed in previous work (Fang and Qian, 2005). This work was completed by Qin Zhou, a doctoral candidate in Dr. Qian's laboratory. In brief, sulfur analyses were made on a Varian CP-3800 gas chromatography equipped with a pulsed-flame photometric detector (PFPD) (Varian, Walnut Creek, CA, USA) operating in sulfur

mode. Two milliliters of samples were placed in a 20 mL autosampler vial, and then diluted with eight milliliters of Milli-Q water. An aliquot 100μL of the internal standard solution (500ppb EMS and 2ppb Isopropyl DS) and 50μL 5% acetaldehyde (w/v) were added to each vial. Duplicate analysis was performed for each wine sample.

An automatic headspace sampling system (CombiPAL autosampler, CTC Analytics AG, Zwingen, Switzerland) equipped with an 85μm Carboxen-PDMS SPME fiber (SUPELCO, Bellefonte, PA, USA) was used for extraction of organic sulfur compounds. Before use, the fiber was pre-conditioned in a split/splitless GC injector port at 300°C for 1 hour. After extraction, the SPME fiber was directly injected into the GC injection port with the splitless mode at 300 °C and kept for 7 min. The separation was performed using a DB-FFAP capillary column (30 m × 0.32 mm I.D., 1 m film thickness, from Agilent, Santa Clara, CA, USA). The oven temperature was programmed as follows: 35 °C (initial hold 3 min), ramp at 10 °C/min to 150 °C (hold for 5 min), and then ramp at 20 °C/min to 220 °C (final hold 3 min). The carrier gas was nitrogen with a constant flow rate of 2 mL/min. The temperature of the detector was 300 °C, and the detector was supplied with 14 mL/min hydrogen, 17 mL/min air 1, and 10 mL/min air 2. The detector voltage was 500 V, the gate delay for sulfur compounds was 6 ms, and the gate width is 20 ms. All sulfur compounds were identified by comparing their retention times with those of the pure standards. The sulfur responses of specific compounds were calculated by the square root of peak area.

Amino Acid Analysis

Free amino acids were analyzed by high performance liquid chromatography

(HPLC) using a Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA, USA) equipped

with HP ChemStation software and photodiode array detector (DAD). Free amino

acid analysis was performed according to Henderson et al. (2006) with some

modifications. In brief, samples of grape juice or wine were centrifuged in 1.5ml

microcentrifuge tubes (VWR, Radnor, PA, USA) for 10 minutes at 6000 rpm with an

Allegra X-22 centrifuge (Beckman Coulter, Brea, CA). Juice samples were then

filtered through 0.45 um syringe filters (Pall Corporation, Port Washington, NY,

USA). Before injection, inline derivitizations with o-phthaldehyde (OPA) and 9-

fluorenylmethyl chloroformate (FMOC) were performed to react primary and

secondary amino acids into fluorescent products, respectively.

The instrument was fitted with a Zorbax Eclipse AAA analytical column (150 mm x

4.6 mm, 5 um, Agilent Technologies, Santa Clara, CA, USA) and guard column (12.5

mm x 4.6 mm, 5 um, Agilent). Mobile phase A was a 40 mM sodium phosphate

solution, adjusted to pH 7.8 with 6 N sodium hydroxide solution. Sodium phosphate

stock solution was made with Milli-Q water (Millipore, Bedford, MA, USA) and

monobasic sodium phosphate (Avantor Performance Materials, Center Valley, PA,

USA). Mobile phase B was acetonitrile, methanol, and water (45:45:10 v/v/v).

Mobile phase B solvents were from EMD Millipore (Billerica, MA, USA). Gradients of

mobile phase A and mobile phase B were applied as follows: 0% B (2.0 ml/min) from 0 to 1.9 min, 0-57% B linear (2.0 ml/min) from 1.9 to 18.1 min, 57 to 100% B linear (2.0 ml/min) from 18.1 to 18.6 min, static at 100% B (2.0 ml/min) from 18.6 to 22.3 min, 100-0% B (2.0 ml/min) from 22.3 to 23.2 min, and static at 0% B (2.0 ml/min from 23.2 to 26 min to re-equilibrate the column to initial conditions. Identification and quantification of amino acids was determined from calibration curves using amino acid standards. Amino acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). OPA and FMOC were obtained from Agilent Technologies Inc. (Santa Clara, CA, USA). Analytes were identified based on elution order and retention time.

Glutathione Analysis

Glutathione was quantified by Dr. Jaewoo Choi at Oregon State University Central Labs using LC-MS/MS. The technique was adapted from a method for analysis of GSH in blood (Moore et al., 2013). Samples were centrifuged for 10 minutes at 6000 rpm and supernatant was decanted into a fresh 1.5 ml microcentrifuge tube. 100 μL of each sample was collected and added to 180 μL of precipitating solution (24 mM NEM +20 mM EDTA) in a blood Eppendorf tube. Then, 20 μL of internal standard (100 μM of GSH $^{13}C_2$, ^{15}N) was added before vortexing for 3 seconds. Next, 20 μL of 20% SSA was added before vortexing vigorously for 10 seconds. Finally, samples were incubated for 20 min at room temperature (20 ^{o}C). Following this

derivatization, samples were centrifuged for 5 min at 14,000 rpm at room temperature. Supernatant (approximately 150 µL) was transferred to mass spectrometry vials for either immediate LC-MS/MS analysis or storage at -80 °C.

LC-MS/MS was performed on an Applied Biosystems 4000 QTRAP hybrid linear ion trap-triple quadrupole instrument (AB Sciex, Concord, ON, Canada) operated at a source temperature of 400 °C with ion spray voltage of 5000 kV in positive ion mode. Instrument parameters for GSH and GSSG were as follows: declustering potential (40 for GSH, 60 for GSSG), collision energy (30), entrance potential (8), curtain gas (30) and collision cell exit potential (3).

A Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD, USA), consisting of two LC-20AD pumps, a DQU-20A$_5$ degasser, and an SIL-HTC autosampler were used for all chromatography. Chromatographic separations of GSH-NEM and GSSG were achieved on a Hypercarb column (Thermo Scientific, 2.1 mm x 100 mm x 5 um) at room temperature. Mobile phase A was water containing 0.1 % formic acid, and mobile phase B was 100% acetonitrile containing 0.1 % formic acid. The run consisted of isocratic delivery of 5% phase B for 0.3 min, linear gradient of 5-40% phase B over 3.3 min, a second linear gradient of 40-95% phase B over 0.9 min, isocratic delivery of 95% phase B for 1.5 min, and final wash with 100% phase B over 2 min. Parent → product ion transitions for SRM were developed using standards. SRM transitions used for quantitation included: 433.3 → 304.3 for GSH-NEM, 436.3 → 307.3 for GSH-^{13}C$_2$,^{15}N-NEM, and 613.4→ 355.2 for GSSG.

Statistical Analysis

Statistical tests consisted of analysis of variance (ANOVA) tests performed with R Studio (Boston, MA, USA). Tukey's HSD multiple comparison was performed to test least squares means of treatment effects at the $p < 0.05$ significance level.

RESULTS

Turbidity and YAN

Initial values for Pinot noir juice chemistry were TA 0.56 ± 0.02g/100 ml, pH of 3.51 ± 0.21, °Brix of 23.3 ± 0.5, and YAN of 150.9 ± 7.5 mg/L N (28.2 ± 7.5 mg/L N from ammonia, 122.7 ± 5.2 mg/L N from primary amino acids). Fermentations proceeded rapidly for all treatments with all ferments reaching dryness (< 0.5 g/L reducing sugar) 7-8 days after yeast inoculation. After the wines were pressed basic wine chemistry was as follows: for RC212-fermented wines, pH 3.66 ± 0.01, TA 0.67 ± 0.04 g/100ml, and ethanol was 12.6 ± 0.2% ethanol (v/v). For P1Y2-fermented wines, the pH was 3.70 ± 0.02, TA was 0.64 ± 0.01 g/100ml, and ethanol was 13.2 ± 0.1% (v/v).

By varying the length of time the wine was settled before racking the amount of lees present in the wines could be altered (Table 2.1). When no settling occurred the wine was extremely turbid and the nepholometer turbidity unit (NTU) reading was

beyond the range of the instrument (>10,000 NTU). After settling for 24 hours, the

NTU value of the wines decreased compared to the no setting with P1Y2 wines

having greater turbidity (Table 1). After an additional 48 hrs of settling there was

little change in turbidity for wine produced by P1Y2 while RC212 produced wines

decreased by more than 200 NTU (Table 2.1).

Table 2.1. Turbidity (NTU) of Pinot noir wines made with two different *S. cerevisiae* commercial yeast after set periods of settling at 4°C. n=3

	0 hrs settling	24 hrs settling	96 hrs settling
RC212	>10,000	1044	804
P1Y2	>10,000	662	653

Pinot noir grape must and wines produced were analyzed for free amino nitrogen

(FAN) content. At each relative lees level (low, medium, high) wines produced by

P1Y2 contained higher primary amino acids than wines fermented by RC212 (Table

2.2). Additionally, primary amino acid concentrations were in wines containing a

high amount of lees compared to medium or low lees levels. These trends continued

throughout 180 days of aging on the lees.

Table 2.2: Concentration of free amino nitrogen (mg/L) in Pinot noir wine fermented by *S. cerevisiae* RC212 or *S. cerevisiae* P1Y2 and aged for various lengths of time at 13°C on heavy, medium, or light lees. n=3

Treatment		14 days	30 days	60 days	90 days	180 days
RC212	Low	55.2±2.3	51.3±0.5	50.0±2.2	48.4±0.6	53.0±1.8
	Medium	59.1±2.4	51.2±1.3	48.9±1.8	49.5±0.7	51.8±1.3
	High	65.1±1.7	54.6±2.5	50.9±2.1	61.6±2.6	64.2±0.6

P1Y2	Low	65.2±2.6	59.3±0.8	59.6±2.0	52.7±0.8	57.1±3.9
	Medium	64.3±0.9	62.0±0.9	63.0±3.9	56.4±0.7	61.9±2.9
	High	74.4±0.5	65.3±1.1	72.6±3.4	67.4±0.8	74.1±1.3

Amino Acids

Free amino acids were quantified by HPLC after wines were pressed and during 270 days aging at 13°C. During the first 30 days of aging on the lees the concentrations of most amino acids increased with the exception of arginine, leucine, and isoleucine (See appendix A). Aside from an initial decrease in methionine and cysteine concentrations after fermentation, the concentrations of methionine and cysteine increased as storage time increased (Figures 2.1 and 2.2). For example, in wines with high lees and fermented with RC212 the methionine concentration was initially 1.8 mg/L but rose to over 4.5 mg/L during storage on the lees. High lees treatments contained significantly more methionine than other treatments after 60 days of aging, but not before then. After 180 days, there was no significant difference in methionine between treatments, but after 270 days of aging, medium and low lees treatments again contained significantly less methionine than high lees treatments (Figure 2.1). Similarly, high lees treatments contained significantly more cysteine than other treatment after 60 days of aging but not previous to that sampling point (Figure 2.2). After 180 days of aging, high lees treatments contained significantly more cysteine than low lees, but there was no significant difference between high lees and medium lees. After 270 days aging high lees treatments contained significantly more cysteine than medium or low lees treatments (Figure 2.2).

Yeast strain also impacted the amount of methionine and cysteine in the wines. At each lees level methionine concentration in P1Y2 wines was significantly greater than in RC212 wines during the entire aging process. The cysteine concentration was significantly greater in P1Y2 wines than RC212 wines after 14, 30, and 90 days, but not after 60 and 270 days of aging. Overall, after 270 days storage wine fermented by RC212 and aged on light lees contained the lowest levels of methionine (2.6 mg/L) and cysteine (0.6 mg/L) while wines fermented by P1Y2 and aged on heavy lees contained the highest amount of methionine (5.9 mg/L) and cysteine (1.3 mg/L).
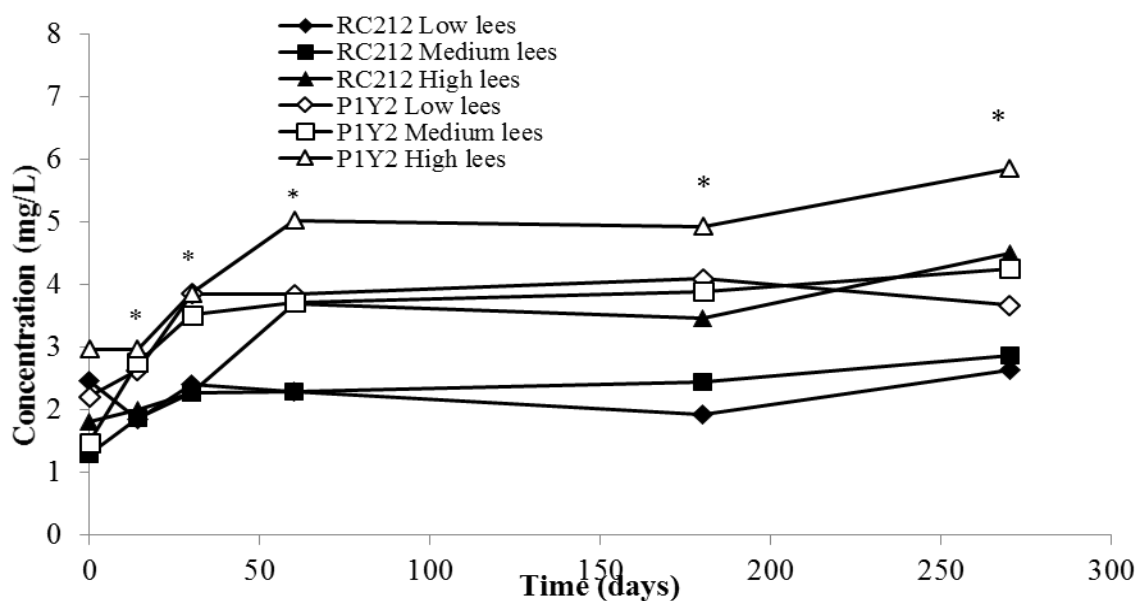
Figure 2.1: Concentration of methionine in Pinot noir wines fermented by *S. cerevisiae* strain RC212 (closed symbols) or strain P1Y2 (open symbols) and aged on light, medium, or heavy lees at 13°C. * indicates significant differences at $p \leq 0.05$, n=3.
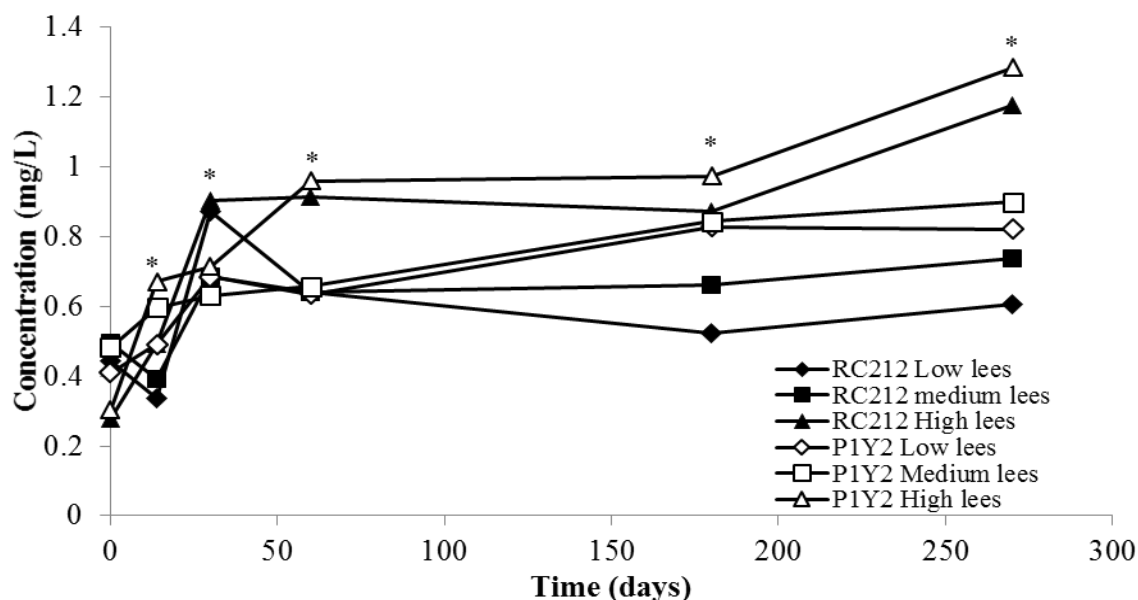
Figure 2.2: Concentration of cysteine in Pinot noir wines fermented by *S. cerevisiae* strain RC212 (closed symbols) or strain P1Y2 (open symbols) and aged on light, medium, or heavy lees at 13°C. * indicates significant differences at $p \leq 0.05$, n=3.

Glutathione

Glutathione (GSH) and its oxidized form, glutathione disulfide (GSSG), were assessed after wines were pressed and after 30 days of aging. No significant difference in GSH was noted between wines after pressing (Table 2.3) with the unsettled wine (high lees) containing close to 100 ug/L (Table 2.3). However, for both yeasts, the concentration of GSH was significantly lower after settling as wine with low lees contained approximately 20 ug/L lower GSH concentrations (Table 2.3). After 30 days of aging GSH concentrations did not significantly change in wines aged on light lees but increased significantly in heavy lees treatments. Furthermore, GSH concentrations were significantly higher in wines produced by RC212 and aged on

high lees than those produced by P1Y2 (Table 2.3). Glutathione disulfide (GSSG), the

oxidized form of GSH, was also quantified in the same samples (Table 2.4).

Concentrations of GSSG were lower than those measured for GSH. At settling,

significantly more GSSG was found in RC212 high lees wines than in any other

treatment, and after 30 days of aging, this trend remained true.

Table 2.3: Concentration of GSH (ug/L) in Pinot noir wines containing high or low
lees after 0 or after 30 days storage at 13°C.

Treatment		Day 0	Day 30
RC212	Low	69.5±0.1[ac]	98.5±6.2[a]
	High	100.1±8.5[b]	563.0±49.0[b]
P1Y2	Low	71.8±2.2[a]	77.4±16.4[a]
	High	93.5±8.7[bc]	218.4±44.2[c]

*Values represent the means of three replicate ferments. Mean values with different
superscript letters within a column were significantly different at $p \leq 0.05$

Table 2.4: Concentration of GSSG (ug/L) in Pinot noir wines containing high or low
lees after 0 or after 30 days storage at 13°C

Treatment		Day 0	Day 30
RC212	Low	22.7±7.1[a]	14.5±1.3[a]
	High	60.9±18.5[b]	50.3±8.8[b]
P1Y2	Low	5.4±1.8[a]	12.3±0.9[a]
	High	15.2±2.5[a]	10.0±1.1[a]

*Values represent the means of three replicate ferments. Mean values with different
superscript letters within a column were significantly different at $p \leq 0.05$

Volatile Sulfur Compounds

Volatile sulfur compounds (VSCs) were assessed in the wines after pressing (Day 0) and after aging of the wine on low, medium, or high lees for up to 270 days. H_2S was present in all wines after pressing with the highest amount being measure in wines produced by RC212 (Table 2.5). Settling the wines reduced the amount of H_2S in the case of RC212 produced wines. For RC212 the highest amount of H_2S measured during the aging process was after 14 days aging on high lees (Table 2.5). For all other treatments the highest concentrations of H_2S were measured after 30 days aging. After 270 days aging on the lees there was no detectable H_2S in any of the wines (Table 2.5).

Concentrations of methanethiol (MeSH) were very similar between all the wine treatments (Table 2.6). Wines produced by P1Y2 and aged on high lees contained significantly higher MeSH than P1Y2 wines aged on light or medium lees after 14 days aging but the results were reversed after 90 days aging (Table 2.6). Small differences in dimethyl sulfide (DMS) were noted between treatments at each time point with levels typically being significantly higher in the high lees treatments for wine produced by either RC212 or P1Y2 (Table 2.8).

While only minor differences in MeSH and DMS were noted for wines produced by RC212 or P1Y2, larger differences were noted for methyl thioacetate (MeSOAc). MeSOAc concentrations were significantly higher in wines produced by RC212 than in wines produced by P1Y2 (Table 2.8). These differences were present at every

time point independent of the lees level. This trend was also apparent for methionol

(Table 2.9) where except for samples measured after 60 days aging the wines

fermented by RC212 contained significantly higher methionol than wines produced

by P1Y2. Again, very little differences were noted between methionol

concentrations at the three different lees levels (Table 2.9).

Table 2.5: Concentration of H_2S (ug/L) in Pinot noir wine fermented by *S. cerevisiae* strain RC212 or strain P1Y2 during aging at 13°C on heavy, medium, or low lees

Treatment		0 days[1]	14 days	30 days	60 days	90 days	180 days	270 days
RC212	Low	2.66	2.15±0.32[ab]*	5.63±0.37[ab]	2.68±0.44[a]	2.24±0.68[a]	1.20±0.18[a]	ND
	Medium	2.33	3.64±0.68[b]	4.23±0.10[b]	2.91±0.41[a]	2.45±0.45[a]	0.95±0.11[a]	ND
	High	7.06	7.46±0.95[c]	4.92±0.89[ab]	1.87±0.25[a]	1.51±0.14[a]	1.35±0.64[a]	ND
P1Y2	Low	1.94	1.88±0.21[a]	6.83±1.14[a]	3.75±0.53[a]	2.98±0.54[a]	1.31±0.22[a]	ND
	Medium	2.47	2.38±0.20[b]	3.52±0.72[b]	2.17±0.33[a]	2.01±0.19[a]	1.47±0.24[a]	ND
	High	2.61	2.74±0.12[b]	4.89±0.23[ab]	2.15±0.27[a]	1.78±0.23[a]	1.30±0.07[a]	ND

*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$
[1]Values from day 0 represent a single sample from a pooled tank of wine for each treatment
ND = not detectable

Table 2.6: Concentration of MeSH (ug/L) in Pinot noir wine fermented by *S. cerevisiae* strain RC212 or strain P1Y2 during aging at 13°C on heavy, medium, or low lees

Treatment		0 days[1]	14 days	30 days	60 days	90 days	180 days	270 days
RC212	Low	1.49	1.63±0.05[ab]*	1.54±0.06[a]	1.45±0.12[a]	1.93±0.14[ab]	1.69±0.18[a]	1.40±0.16[a]
	Medium	1.10	1.62±0.04[ab]	1.56±0.10[a]	1.19±0.20[a]	1.32±0.22[ab]	0.55±0.06[a]	ND
	High	1.42	1.70±0.01[ab]	1.55±0.14[a]	1.64±0.13[a]	1.44±0.20[ab]	0.75±0.11[a]	0.72±0.10[a]
P1Y2	Low	1.00	1.38±0.19[a]	1.33±0.22[a]	2.45±0.45[a]	2.04±0.33[ab]	1.78±0.11[a]	1.15±0.15[a]
	Medium	1.00	1.33±0.13[a]	1.28±0.10[a]	1.68±0.30[a]	2.15±0.18[a]	1.84±0.05[a]	1.81±0.22[a]
	High	0.84	1.89±0.26[b]	1.39±0.08[a]	1.08±0.08[a]	1.22±0.13[b]	0.85±0.12[a]	0.67±0.14[a]

*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$
[1]Values from day 0 represent a single sample from a pooled tank of wine for each treatment
ND = not detectable

Table 2.7: Concentration of DMS (ug/L) in Pinot noir wine fermented by *S. cerevisiae* strain RC212 or strain P1Y2 during aging at 13°C on heavy, medium, or low lees

Treatment		0 days[1]	14 days	30 days	60 days	90 days	180 days	270 days
RC212	Low	1.89	2.07±0.10[a*]	1.79±0.08[a]	3.17±0.37[a]	3.20±0.28[a]	2.66±0.12[a]	5.53±0.22[b]
	Medium	1.88	2.01±0.13[a]	1.79±0.13[a]	2.95±0.59[a]	2.96±0.37[a]	2.49±0.20[a]	5.70±0.53[ab]
	High	1.82	2.70±0.14[b]	2.71±0.18[b]	4.62±0.12[bc]	4.51±0.18[b]	3.77±0.24[b]	7.10±0.86[a]
P1Y2	Low	1.59	1.94±0.22[a]	1.91±0.16[a]	3.75±0.34[ab]	3.25±0.11[a]	2.76±0.07[a]	5.98±0.24[a]
	Medium	1.10	2.02±0.15[a]	1.81±0.14[a]	3.69±0.22[ab]	3.26±0.19[a]	2.94±0.25[a]	6.03±0.56[a]
	High	1.97	2.95±0.08[b]	2.60±0.05[b]	4.77±0.10[c]	4.65±0.21[b]	3.69±0.02[b]	6.95±0.36[a]

*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$
[1]Values from day 0 represent a single sample from a pooled tank of wine for each treatment

Table 2.8: Concentration of MeSOAc (ug/L) in Pinot noir wine fermented by *S. cerevisiae* strain RC212 or strain P1Y2 during aging at 13°C on heavy, medium, or low lees

Treatment		0 days[1]	14 days	30 days	60 days	90 days	180 days	270 days
RC212	Low	3.61	4.09±0.10[b*]	3.23±0.07[bc]	4.12±0.17[bc]	4.06±0.17[b]	3.66±0.03[c]	3.24±0.07[bc]
	Medium	3.35	3.75±0.66[b]	3.36±0.13[c]	4.69±0.07[c]	3.97±0.07[b]	3.66±0.09[c]	3.61±0.15[c]
	High	3.25	4.54±0.09[b]	3.10±0.08[b]	3.73±0.11[b]	3.48±0.11[b]	2.98±0.06[b]	2.37±0.07[b]
P1Y2	Low	ND	0.67±0.01[a]	0.58±0.03[a]	0.66±0.04[a]	0.94±0.10[a]	0.66±0.05[a]	0.35±0.03[a]
	Medium	ND	0.44±0.15[a]	0.58±0.09[a]	0.55±0.09[a]	0.52±0.05[a]	0.74±0.07[a]	0.33±0.02[a]
	High	0.40	0.83±0.05[a]	0.56±0.04[a]	0.56±0.10[a]	0.49±0.08[a]	0.64±0.08[a]	0.35±0.03[a]

*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$
[1]Values from day 0 represent a single sample from a pooled tank of wine for each treatment
ND = not detectable

Table 2.9: Concentration of Methionol (mg/L) in Pinot noir wine fermented by *S. cerevisiae* strain RC212 or strain P1Y2 during aging at 13°C on heavy, medium, or low lees

Treatment		0 days[1]	14 days	30 days	60 days	90 days	180 days	270 days
RC212	Low	3.30	3.06±0.21[b*]	2.84±0.26[d]	2.80±0.08[c]	2.69±0.04[b]	2.26±0.16[b]	2.26±0.20[b]
	Medium	3.11	2.93±0.12[b]	2.80±0.12[cd]	2.79±0.10[c]	2.76±0.09[b]	2.35±0.14[b]	2.06±0.07[b]
	High	3.58	2.99±0.07[b]	2.93±0.14[d]	2.91±0.14[bc]	2.87±0.11[b]	2.37±0.04[b]	2.11±0.08[b]
P1Y2	Low	1.74	1.94±0.16[a]	1.64±0.05[ab]	1.59±0.11[ab]	1.49±0.08[a]	1.36±0.00[a]	1.34±0.01[a]
	Medium	1.85	1.67±0.05[a]	1.76±0.19[ac]	1.58±0.07[a]	1.45±0.05[a]	1.31±0.08[a]	1.35±0.00[a]
	High	2.54	1.73±0.04[a]	1.71±0.29[b]	1.65±0.12[a]	1.56±0.05[a]	1.31±0.07[a]	1.32±0.09[a]

*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$

[1]Values from day 0 represent a single sample from a pooled tank of wine for each treatment

DISCUSSION

Wine lees levels and yeast strain impacted the amount of free amino nitrogen (FAN) in the wine during aging as well as the concentration of individual amino acids. While having sufficient free amino nitrogen available for primary fermentation is crucial, nitrogen not metabolized by *S. cerevisiae* could potentially be available for spoilage microorganisms as proposed by Bisson and Butzke (2000). In the present study up to 75 mg/L of FAN was present in some of the wines at the completion of alcoholic fermentation. This was despite the fact that the original grape must only contained 122.7 mg/L FAN. In support, Childs et al. (2015) reported high residual FAN in wines made from synthetic grape juice with starting YANs of either 150 or 250 mg/L. Childs et al. (2015) also noted that the higher residual nitrogen concentrations did not influence the growth of the important spoilage yeast *Brettanomyces bruxellensis* and that YAN concentrations as low as 6 mg/L were sufficient for *B. bruxellensis* to attain populations $> 1 \times 10^7$ cfu/mL. These YAN levels were far exceeded in the present study which also demonstrate that while increasing settling times may minimally decrease the amount of nitrogen in the wine the levels in the wine will still be sufficient for growth of *B. bruxellensis*. The yeast strain used also affected the FAN content of the wines, with P1Y2 wines

containing higher concentrations of amino acids throughout aging. While confounded by yeasts' growth patterns and resulting concentrations of dead cells at the end of fermentation, this variation is well-documented (Martinez-Rodriguez et al., 2002, Fiechter et al., 2011).

Concentrations of FAN decreased in all treatments between 14 days and 30 days of aging, and increased in all treatments between 90 and 180 days of aging. This is in contrast to other findings which observed a gradual increase of nitrogen in a number of strains between 30 and 270 days of aging (Leroy et al., 1990; Martínez-Rodríguez et al., 2002). However, changes during aging in total free amino acid content can also be dependent on yeast strain (Fiechter et al., 2011). Additionally, most other work in this area is performed on sparkling wine, which undergoes different processing steps including secondary bottle fermentation.

Early during aging on heavy lees wines fermented by RC212 contained significantly higher level of H_2S. This decreased between 14 and 30 days of aging while all other treatments increased in H_2S concentration. The changes in H_2S concentration in wine during aging could be due to a number of factors. H_2S is highly reactive, easily oxidizable, and typically lost during fermentation due to the sparging effect of yeast produced CO_2 (Jackson, 2008; Zeeman et al., 1982). During aging H_2S can be oxidized or react to form additional VSC compounds. In the present study there was a

decrease in H_2S over time as by 270 days of aging there was no detectable H_2S in any of the treatment wines. Some of this loss may have been due to oxidation as the headspace in each carboy increased after every sampling and removal of 30 mLs of wine. However, carboys were sparged with nitrogen gas at sampling and 30 mg/L free SO_2 levels were maintained in the wines during the 270 days of aging.

H_2S has also been reported to form during lees aging of wine (Rauhut, 1993) although the exact mechanisms involved are not well understood. One potential mechanism is the degradation of the sulfur containing amino acids cysteine and methionine. This may occur enzymatically through the action of *S. cerevisiae* (Winter et al., 2014) or chemically (Acree et al., 1972). In the present study there was no clear relationship between a decrease in cysteine and methionine concentrations and concentrations of H_2S in the wines. In fact, while H_2S concentrations tended to increase between days 14 and 30 of wine aging, methionine and cysteine concentrations increased during this same time period rather than decreasing as might be expected if their degradation was responsible for the H_2S. However, the concentrations of H_2S were below 10 ug/L, while combined concentrations of cysteine and methionine were between 2 and 5 mg/L, three orders of magnitude greater. This finding suggests that there is an equilibrium between sulfur-containing amino acids and the proportion that is degraded to H_2S. However, as this

correlation was only observed between 14 and 30 days of aging, additional explanations are needed beyond a pure chemical equilibrium. One possibility is that yeast enzymatic activity by cysteine beta-lyase, cysteine gamma-lyase (Winter et al., 2014), or other unknown enzymes is heightened during this period. The reactivity of H_2S and changing dissolved oxygen conditions over the aging process may also play a role.

A possible explanation for the lack of H_2S is that the wine contained high concentration of SO_2 to prevent microbial activity. The presence of SO_2 would have inhibited any yeast growth and activity and thus preventing degradation of cysteine or methionine by the yeast produced enzymes cysteine beta-lyase and cysteine gamma-lyase implicated in the release of H_2S from cysteine degradation (Winter et al., 2014). Additional studies should include the presence of live *S. cerevisiae* during early lees aging to determine if elevated S-containing amino acid concentrations can result in increased H_2S formation via enzymatic degradation.

Aside from H_2S, a number of other VSCs were measured during wine aging on the lees. Methanethiol (MeSH) was found at levels above the sensory detection threshold of 1.8 ug/L (Davis and Qian, 2011) in three of the treatments: RC212 light lees, P1Y2 light lees, and P1Y2 medium lees. The highest concentrations recorded for these treatments were either after 60 or 90 days of aging. As with H_2S, changes in MeSH concentrations did not

correlate with a decrease in the S-containing amino acid thought to be responsible for its formation (de Mora et al., 1986, Rauhut, 1993). However, increases in methionine levels observed during the first 14 days of aging supports the hypothesis that MeSH may be produced from methionine metabolism by yeast. Though yeast populations were not quantified, methionine increases were observed during this time, as aforementioned. Although not statistically significant, MeSH concentrations tended to be lower in wines with higher lees which may be in part a result of the ability of lees to adsorb this compound (Palacios et al., 1997).

Dimethyl sulfide (DMS) was also found in all wines, although never above the sensory threshold of 60 ug/L (de Mora et al., 1986). While not in high concentrations, strong trends were observed regarding the formation of DMS during the aging process. After the first month of aging, DMS concentration was consistently greater in high lees treatments, significantly so in most cases throughout the aging process. Additionally, significant increases in DMS were observed in all treatments between 30 and 60 days of aging and between 180 and 270 days of aging. However, all treatments decreased slightly in DMS concentration between 90 and 180 days. It has been debated whether or not DMS increases over time (de Mora et al., 1986, Marais, 1979; Spedding and Raut, 1982) and it is reported that the vintage and grape varietal carry a more significant impact. However, in the present

study increases in DMS occurred during the 9 month aging period studied although the concentrations were still very low.

While the presence of cysteine and methionine did not correlate with formation of VSCs in the present study, they were impacted by lees level and the yeast that performed the fermentation. Aside from the initial 14 days of aging, these S-containing amino acids increased during aging. This is likely due to yeast autolysis and release of amino acids into the wine (Alexandre and Guilloux-Benatier, 2006; Alexandre et al., 2001; Charpentier and Freyssinet, 1989). The fact that there were differences between lees levels may indicate that there was higher yeast cell mass in the heavy lees and therefore more potentially releasable amino acids, or that the heavy lees resulted in conditions in the wine more conducive to yeast autolysis. Given that yeast autolysis rate is more driven by temperature, pH, and ethanol (Alexandre and Guilloux-Benatie, 2006), it is unlikely that the presence of higher lees would impact yeast autolysis rates.

In addition to lees level, yeast strain also impacted the concentration of methionine and cysteine. At the same lees levels, wines fermented by P1Y2 contained higher amounts of the S-containing amino acids throughout aging. The reason for this is unknown. If the amount of S-containing amino acids released into the wine during aging is reflective of their intracellular concentration then it might be expected that P1Y2 yeast would contain

lower amounts of S-containing amino acids. This is because P1Y2 was bred and selected as a non-H_2S producing yeast due to allele differences in the *MET10* gene which encodes for catalytic subunits in the enzyme sulfite reductase (Linderholm et al., 2010). Disruption of the sulfite reductase activity would be expected to impact formation of cysteine and methionine. However, Linderholm et al. (2010) also reported that strains with the allele difference were not auxotrophic for cysteine or methionine. Additional studies using a larger range or yeast strains should be conducted to better understand the potential impact of yeast strain on the concentration of S-containing amino acids in wine.

An additional potential source of VSCs in wine is the tripeptide glutathione (GSH). GSH content has been reported to influence the formation of VSCs in wine (Ugliano et al., 2013, Winter et al., 2014). However, while the results obtained in this work demonstrate that aging in the presence of a high amount of lees significantly increases the concentration of GSH in wine, there was no subsequent effect on VSCs in these wines. It is interesting to note the impact of yeast strain on GSH content after 30 days of aging, as RC212 high lees wines contained more than double that of P1Y2 high lees wines. The impact of yeast on GSH has been previously reported (Lavigne et al., 2007; Rauhut, 2009) and is thought to be due to release of yeast-metabolized GSH near the end of alcoholic fermentation (Lavigne et al.,

2007; Rauhut, 2009). The concentrations of GSH measured in the present study were relatively low compared to the few reports of GSH in red wine and very low compared to the concentrations found in many white wines which can exceed 10 mg/L (Janeš et al., 2010; Park et al., 2000).

Inverse to GSH levels, GSSG decreased during the first 30 days of aging, providing further support for the wine environment becoming more reductive than oxidative during this initial aging period. Although not suitable as a direct comparison to dissolved oxygen, changes in ratio of GSH to GSSG may indicate the oxidative state of a wine, as most GSH-related reactions involve either molecular oxygen or oxidases (Wu, 2014). If GSSG is the only product GSH is oxidized to in these reactions, one may expect the sum of GSH and GSSG to then remain constant. However, this is evidence of GSH complexing other sulfur compounds in thermophilic bacteria (Rohwerder, 2003), but this has not been shown in *S. cerevisiae*. The degradation of GSH into its constituent amino acids or the bonding of these constituent amino acids together into GSH could occur during the early part of aging when yeasts could still be in active metabolism.

CONCLUSIONS

Yeast strain and lees content, as influenced by settling time, had a significant impact on the total free amino acids content during aging, as well as the individual sulfur-containing amino acids. Both methionine and cysteine increased initially before plateauing during the remainder of the experiment. An increase in H_2S occurred in most treatments between 14 and 30 days of aging, and this increase was simultaneous with those in methionine and cysteine. Although the H_2S observed in this experiment was not sufficient to result in sensory defects, it may be formed by the same mechanism as wines that exhibit this behavior in a more reductive situation. Future work should investigate the roles of enzymatic and chemical degradation of sulfur-containing amino acids, respectively, and should attempt to characterize what if any enzymes may be responsible. Additionally, future work should involve larger volumes of wine that form an environment with minimal oxygen ingress, influencing the reduction-oxidation state of the wine.

CHAPTER 3

IMPACT OF NITROGEN AND ELEMENTAL SULFUR ON VOLATILE SULFUR
COMPOUND FORMATION IN PINOT NOIR WINE

ABSTRACT

The influence of yeast assimilable nitrogen (YAN) content and elemental sulfur (S^0)

on the formation of volatile sulfur compounds (VSCs) during and after fermentation

were investigated. A synthetic grape juice was utilized where the concentration and

composition of YAN was adjusted. High (346 mg/L) and low (112 mg/L) YAN

treatments were prepared where nitrogen was derived from primary amino acids,

while a third treatment was prepared where the majority of the 346 mg/L YAN was

derived from di-ammonium phosphate (DAP). Fermentations were performed by

either a high H2S producing Saccharomyces cerevisiae strain (UCD522) or a non-

H2S producing S. cerevisiae strain (P1Y2). H2S production was monitored with lead

acetate tubes during fermentation and wines were assessed for additional VSCs by

GC-PFPD. Variation in YAN concentration and composition impacted H2S

production during fermentation and concentrations of VSCs in the wine. In

particular, treatments fermented by UCD522 with high DAP had increased

formation of H2S late in fermentation and significantly more methyl thioacetate in

the wine post-fermentation. The role of elemental sulfur in the formation of H2S and

other VSCs post-fermentation was also investigated. Pinot noir fermentations were

performed where an addition of 0, 5, or 15 mg/g elemental sulfur was made.

Fermentations were conducted by UCD522 or P1Y2 at 27°C and H2S production

measured using lead acetate tubes. Wines were assessed for additional VSC by GC-

PFPD. Addition of S^0 resulted in H_2S formation during the alcoholic fermentation

independent of which yeast strain was used. H_2S production was higher in

fermentations performed by UCD522 with increasing amounts of S^0 resulting in

increased production of H_2S. In addition, higher S^0 additions also resulted in higher

H_2S production late in fermentation (<1 ^{o}Brix). Higher S^0 also resulted in wines that

contained higher concentrations of methyl thioacetate post-fermentation. Very low

levels of S^0 remained in the wines post-fermentation.

INTRODUCTION

Hydrogen sulfide (H_2S) and other volatile sulfur compounds (VSCs) produced

during the winemaking process can be extremely detrimental to the quality of a

wine due to their offensive aromas, described as rotten egg, onion, cabbage, and

rubber (Rauhut, 1993). Although a number of studies have investigated the

formation of VSCs ((Rankine, 1963; Acree et al., 1972; Guidici and Kunkee, 1994;

Jiraneck et al., 1995; Spiropoulos et al., 2000; Kumar et al., 2010) formation of these

compounds cause problems during winemaking and the underlying causes are still

not completely elucidated. Of all of the VSCs, H_2S is the most widely studied. H_2S

may be produced by the fermentative yeast *Saccharomyces cerevisiae* as part of the

formation of sulfur-containing amino acids methionine and cysteine (Rauhut, 1993,

Spiropoulos et. al., 2000) via the sulfate reduction sequence (SRS). H_2S can also be

formed by the non-enzymatic reduction of elemental sulfur (S^0) (Schutz and Kunkee,

1977). Yeast strains vary greatly in their production of H_2S with some yeast such as

S. cerevisiae UCD522 (Montrachet), reported as high producers of H_2S (Linderholm

et al., 2010; Bohlscheid et al., 2007; Wang et al., 2003) while other strains such as

EC1118 are noted as low producers (Mendes-Ferreira et al., 2002). Recently strains

have been developed that purportedly produce no H_2S due to allele differences in

the *MET10* gene which encodes for catalytic subunits in sulfite reductase, a key

enzyme in the SRS.

Aside from yeast strain a number of factors such as, nitrogen content, elemental

sulfur residues, vitamins, turbidity, and excessive amounts of sulfite (Spiropoulos et

al., 2000; Jiranek et al., 1995; Schutz and Kunkee et al, 1977; Tokuyama et al., 1973;

Karagiannis and Lanaridis, 1999; Lavigne-Cruege, 2006) can impact its formation.

One of the most important of these is nitrogen content. The nitrogen content of a

grape must or juice is typically expressed as yeast assimilable nitrogen (YAN). YAN

is a measurement of nitrogen that will be available for the yeast during fermentation

and excludes the amino acid proline. YAN is composed of ammonia and amino acids

containing a primary amine group. Sufficient YAN is required during fermentation

so that the yeast, *Saccharomyces cerevisiae*, can successfully complete the

fermentation (Jiranek and Henschke, 1991). While musts with as low as 50 mg/L

YAN have been able to successfully complete fermentation, the risk of a stuck

fermentation is substantial with extremely low YAN values, and the recommended

minimum value is 140 mg/L YAN (Bisson and Butzke, 2000). Others have

prescribed 400 mg/L YAN as a benchmark (Jiranek and Henschke, 1991). Generally,

most supplementation is done with diammonium phosphate (DAP) because of its

low cost and ease of use. However, many winemakers also use yeast hulls and other organic forms of nitrogen for supplementation.

YAN composition and content has been shown to influence the production of H_2S during fermentation. Jiranek et al. (1995) found that most amino acids, with the exception of threonine, proline, and cysteine, were successful at reducing H_2S formation. A number of researchers have also reported that additions of DAP can reduce H_2S production, often in a linear manner dependent on total YAN concentrations (Jiranek et al., 1995; Tamayo et al., 1999; Park et al., 2000; Rauhut et al., 1996). However, others have found that moderate DAP supplementation increases H_2S formation compared to control fermentations (Ugliano et al., 2011; Mendes-Ferreira et al., 2009). Additions of DAP have also been shown to increase methanethiol (MeSH) and ethanethiol (EtSH) content in wines (Ugliano et al., 2010). While the conventional belief is that increases in YAN lead to decreases in H_2S, this is not an absolute rule, and other factors must be considered as well. For example, it has been suggested that the type of nitrogen (ammonia versus amino acid nitrogen) as well as concentration can impact the formation of H_2S produced during fermentation (Ugliano et al., 2011; 2009). In addition, while the impact of YAN on H_2S has received considerable study, little is known about the impact of YAN on the formation of other VSCs or on possible VSC precursors such as cysteine, methionine, and glutathione.

Aside from formation by yeast, H_2S can also be formed by the reduction of S^0 during fermentation. The reductive environment established during the fermentation is

sufficient for this non-enzymatic conversion to occur (Schutz and Kunkee, 1977). S^0

is frequently used as a fungicide to inhibit the growth of powdery mildew, one of the

most common problems in viticulture. Early studies reported large concentrations

of H_2S being produced in fermentations with levels of 10-20 ug/g of S^0 as well as an

observed lack of connection between fermentative capacity and H_2S production

(Rankine, 1963; Acree et al., 1972; Schutz and Kunkee, 1977). Additionally, research

showed that the type of S^0 present, whether wettable, dusting, or colloidal,

significantly impacted the resulting H_2S production (Schutz and Kunkee, 1977).

Typically, the application of S^0 in the vineyard is halted well before harvest so as to

minimize the risk of residual S^0 being present on the grapes at harvest. However,

there is debate over how long this period of time should be based on how long S^0

persist on the grapes as well as what concentrations of S^0 would increase

production of H_2S during fermentation. For example, Thomas et al. (1993) observed

that application of dusting sulfur up to but not after veraison resulted in 1-3 ug/g S^0

on the fruit at harvest. Thomas et al. (1993b) noted this concentration did not cause

a significantly increase in H_2S production during fermentation. Others however have

noted that concentrations as low as 1 ug/g may cause H_2S problems (Kwasniewski

et al., 2014). In addition, the role of S^0 in the formation of other VSCs, particularly

their formation post-fermentation, has not been well studied. For example, it is not

known if having high levels of S^0 on fruit at the beginning of fermentation will result

in residual S^0 being present in the wine at the end of fermentation. This may be

important to the formation of VSCs post-fermentation as in non-wine systems

reactions between S^0 and glutathione (Rohwerder, 2003) or cysteine (Schutz and

Kunkee, 1977) have generated H$_2$S. Whether this reaction occurs under wine conditions is unknown.

Because of the importance of YAN concentration and composition and the potential role of elemental sulfur in the production of H$_2$S and other VSCs, the objective of this study was to:

1) Investigate the impact of YAN concentration and composition on the formation of H$_2$S and other VSCs

2) Study the impact of increasing amounts of elemental sulfur on the formation of H$_2$S and other VSCs during fermentation of Pinot noir grapes and separate yeast-produced H$_2$S formation from H$_2$S formed from the reduction of elemental sulfur by the use of a non-H$_2$S producing yeast.

MATERIALS and METHODS

Synthetic Grape Juice Experiment

A synthetic grape juice (SGJ) was prepared to assess the impacts of nitrogen supplementation on H$_2$S production during fermentation. The SGJ composition was a modified version of the media outlined Wang et al. (2003) and contained amino acid concentrations adjusted to mimic the composition of Pinot noir grape juice as determined by Lee and Schreiner (2010). See appendix B for the complete composition. Aside from the adjustment of amino acid content, the SGJ was modified to contain 50 ug/L pantothenic acid. This concentration is considered a low to

moderate level based on the reported concentrations in grapes (Hagen et al., 2008).

The YAN content of the juice was manipulated by altering the amino acid and

ammonia content of the juices. Three juices were prepared with three different YAN

compositions. Low amino acid juice contained a total of 112 mg/L YAN where 81

mg/L YAN was derived from primary amino acids while the remaining 31 mg/L was

derived from ammonia (added as diammonium phosphate (DAP)). The high amino

acid juice contained 346 mg/L YAN where primary amino acids provided 315 mg/L

while ammonia again provided 31 mg/L YAN. In the high DAP juice the YAN content

was 350 mg/L but the majority of the YAN was provided by ammonia (269 mg/L).

Aside from nitrogen composition and concentration, all other components were

identical between the three synthetic juices. The basic parameters of the juices were

23 °Brix, pH 3.40, and 0.65 g/100 mL titratable acid. pH was measured using an

OrionStar A111 pH meter (Thermo Scientific, Waltham, MA, USA). °Brix was

measured with an Anton Paar DMA 35N density meter (Graz, Austria). Titratable

acidity was determined by titration with 0.1 N sodium hydroxide solution. Yeast

assimilable nitrogen (YAN) was quantified as the sum of primary amino acids and

ammonia in units of mg/L N. Primary amino acids were measured according to

Dukes and Butzke (1998) with a test kit (Gusmer Enterprises Inc., Fresno, CA, USA)

and ammonia was measured by enzymatic test kit (R-Biopharm, Darmstadt,

Germany). A Genesys 10UV spectrophotometer (Thermo Scientific, Waltham, MA,

USA) was utilized for both YAN and ammonia analysis.

Fermentation

After preparation, 1.8 L of SGJ was sterile filtered (0.45 um polyethersulfone sterile filter (G.W. Kent, Ypsilanti, MI, USA)) into autoclaved 3 L Erlenmeyer flasks (VWR, Radnor, PA, USA). Yeast for inoculation were prepared by inoculating yeast peptone dextrose broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, pH 4.5) with either active dry yeast granules of *S. cerevisiae* UCD522 (Lallemand, Montreal, Canada) or P1Y2 (Phyterra, Napa, CA, USA). After incubation for 48 hours at 25 °C the broth was centrifuged at 5000 rpm for 10 minutes, supernatant was decanted an the cell pellet re-suspended in 0.1% peptone solution. This process was repeated and the re-suspended cell pellet was used to inoculate the SGJ at approximately 10^6 cfu/mL. Fermentations were conducted in a temperature-controlled room at 25 °C (77 °F). All treatments were performed in triplicate except for P1Y2 DAP, which was in duplicate. Samples were taken with sterile glass pipettes twice per week during the fermentation for Brix analysis and yeast viable cell counts. Yeast viable cells were quantified by plating on YPD agar (pH 4.5) after appropriate dilutions in sterile 0.1% w/v peptone. Plates were incubated for 48 hours at 25 °C in an aerobic environment prior to colony enumeration.

Hydrogen Sulfide Detection and Yeast Enumeration

To measure H_2S produced during fermentation the Erlenmeyer flasks were fitted with one-hole rubber stoppers fitted with lead acetate hydrogen sulfide detection tubes (Gastec, Ayase, Japan). Both 4M and 4H tubes were used. Tubes were monitored daily and replaced with fresh tubes when needed. H_2S concentration was

determined by relating the distance of color change on the detection tube to that observed for calibration standards (Ugliano and Henschke 2010). Calibration curves were prepared by addition of a known amount of sodium sulfide nonahydrate (Sigma-Aldrich, St. Louis, MO, USA) to an acidic environment. The hydrogen sulfide gas generated was passed through the detection tube with the aid of Alka-Seltzer tablets (Bayer, Morristown, NJ, USA) and measured in millimeters with a ruler.

Elemental Sulfur and Pinot noir Experiment

Pinot noir grapes were harvested from Oregon State University's Woodhall Vineyard (Alpine, Oregon, USA) on September 27th, 2014. After arrival at the OSU research winery, grapes were kept in a temperature-controlled room at 4 °C until destemming with a Velo DPC 40 destemmer/crusher (Altivole, Italy). After destemming, 1.5 kg of grapes was distributed into 4L microfermentors equipped with plungers for cap management as described by Takush and Osborne (2012). The microfermenters were modified so that the original fermentation lock was replaced with a fermentation lock where fermentation gases were forced to pass through an H_2S detection tube (Gastec, 4H tubes). Basic grape parameters were 22.5°Brix, pH 3.20, 0.84 g/100 mL titratable acid and 80 mg/L YAN. Analysis of pH, TA, °Brix, and YAN was performed as previously detailed. Because YAN levels were so low (54 mg/L N) an addition of the yeast nutrient Fermaid K (Lallemand, Montreal, Canada) (0.125 g/L) and DAP (0.25 g/L) was made to raise the YAN content to approximately 150 mg/L. Elemental colloidal sulfur (S^0) (Sigma-Aldrich, St. Louis,

MO, USA) was added in different amounts to each treatment. In one set of fermenters no S^0 was added, to another set of fermenters 5 μg/g of S^0 was added, while to a third set of fermenters 15 μg/g of S^0 was added. The original residual S^0 content of the Pinot noir grapes was measured utilizing the method outlined byp0 Kwasniewski et al (2011) and no S^0 was detected on the grapes.

Fermentation

Two different yeast strains were used. In one set of fermenters (0, 5, 15 μg/g S^0) the non-H_2S producing yeast strain *S. cerevisiae* P1Y2 was inoculated while in another set of fermenters *S. cerevisiae* UCD522 was inoculated. Yeast were hydrated according to manufacturer's specifications prior to inoculation at a rate of 0.25 g/L. All treatments were performed in triplicate. Fermentations were conducted in a temperature-controlled room at 27 °C (81 °F). °Brix was monitored twice per day with an Anton Paar DMA 35N density meter (Graz, Austria) while H_2S production was assessed using lead acetate tubes as previously detailed. Tubes were monitored daily and replaced with fresh tubes when needed. Completion of fermentation was confirmed with Bayer Clinitest tablets (Bayer, Morristown, NJ, USA). Once all fermentations had reached dryness, wines were pressed using a small custom basket press modified with a pressure gauge to apply constant pressure at 15 psi for 1 minute. Replicates were pressed into 500 mL Schott bottles (VWR, Radnor, PA, USA) and samples taken for analysis.

Wines were inoculated for malolactic fermentation (MLF) with *Oenococcus oeni* strain PN4 (Lallemand, Saint-Simon, France) according to manufacturer's specifications. Wines were stored at a temperature of 21 °C (70 °F) until MLF was complete in all wines. Malic acid concentrations were monitored using an enzymatic assay (Vintessential Laboratories, Dromana, Victoria, Australia). When MLF was complete (< 50 mg/L malic acid), an addition of 50 mg/L SO_2 (from potassium bisulfite) was made to each replicate. When all replicates had finished MLF, wines were stored at 13 °C. During MLF and storage the Schott bottles were fitted with a 4L lead acetate hydrogen sulfide detection tube to measure any additional H_2S released.

Volatile Sulfur Analysis

Analysis of volatile sulfur compounds was performed by gas chromatography as detailed in previous work (Fang and Qian 2005). This work was completed by Qin Zhou, a doctoral candidate in Dr. Qian's laboratory. In brief, sulfur analyses were made on a Varian CP-3800 gas chromatography equipped with a pulsed-flame photometric detector (PFPD) (Varian, Walnut Creek, CA, USA) operating in sulfur mode. Two milliliters of samples were placed in a 20 mL autosampler vial, and then diluted with eight milliliters of Milli-Q water. An aliquot 100μL of the internal standard solution (500ppb EMS and 2ppb Isopropyl DS) and 50μL 5% acetaldehyde (w/v) were added to each vial. Duplicate analysis was performed for each wine sample.

After extraction, the SPME fiber was directly injected into the GC injection port with the splitless mode at 300 ◦C and kept for 7 min. The separation was performed using a DB-FFAP capillary column (30 m × 0.32 mm I.D., 1 m film thickness, from Agilent, Santa Clara, CA, USA). The oven temperature was programmed as follows: 35 ◦C (initial hold 3 min), ramp at 10 ◦C/min to 150 ◦C (hold for 5 min), and then ramp at 20 ◦C/min to 220 ◦C (final hold 3 min). The carrier gas was nitrogen with a constant flow rate of 2 mL/min. The temperature of the detector was 300 ◦C, and the detector was supplied with 14 mL/min hydrogen, 17 mL/min air 1, and 10 mL/min air 2. The detector voltage was 500 V, the gate delay for sulfur compounds was 6 ms, and the gate width is 20 ms. All sulfur compounds were identified by comparing their retention times with those of the pure standards. The sulfur responses of specific compounds were calculated by the square root of peak area.

Amino Acid Analysis

Free amino acids were analyzed by high performance liquid chromatography (HPLC) using a Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA, USA) equipped with HP ChemStation software and photodiode array detector (DAD). Free amino acid analysis was performed according to Henderson et al. (2006) with some modifications. In brief, samples of grape juice or wine were centrifuged in 1.5ml microcentrifuge tubes (VWR, Radnor, PA, USA) for 10 minutes at 6000 rpm with an Allegra X-22 centrifuge (Beckman Coulter, Brea, CA, USA). Juice samples were then filtered through 0.45 um syringe filters (Pall Corporation, Port Washington, NY,

USA). Before injection, inline derivitizations with o-phthaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) were performed to react primary and secondary amino acids into fluorescent products, respectively.

The instrument was fitted with a Zorbax Eclipse AAA analytical column (150 mm x 4.6 mm, 5 um, Agilent Technologies, Santa Clara, CA, USA) and guard column (12.5 mm x 4.6 mm, 5 um, Agilent). Mobile phase A was a 40 mM sodium phosphate solution, adjusted to pH 7.8 with 6 N sodium hydroxide solution. Sodium phosphate stock solution was made with Milli-Q water (Millipore, Bedford, MA, USA) and monobasic sodium phosphate (Avantor Performance Materials, Center Valley, PA, USA). Mobile phase B was acetonitrile, methanol, and water (45:45:10 v/v/v). Mobile phase B solvents were from EMD Millipore (Billerica, MA, USA). Gradients of mobile phase A and mobile phase B were applied as follows: 0% B (2.0 ml/min) from 0 to 1.9 min, 0-57% B linear (2.0 ml/min) from 1.9 to 18.1 min, 57 to 100% B linear (2.0 ml/min) from 18.1 to 18.6 min, static at 100% B (2.0 ml/min) from 18.6 to 22.3 min, 100-0% B (2.0 ml/min) from 22.3 to 23.2 min, and static at 0% B (2.0 ml/min from 23.2 to 26 min to reequilibrate the column to initial conditions. Identification and quantification of amino acids was determined from calibration curves using amino acid standards (Sigma-Aldrich). OPA and FMOC were obtained from Agilent Technologies Inc. (Santa Clara, CA, USA). Analytes were identified based on elution order and retention time.

Glutathione Analysis

Glutathione was quantified by Dr. Jaewoo Choi at Oregon State University Central Labs using LC-MS/MS. The technique was adapted from a method for analysis of GSH in blood (Moore et. al. 2013). Samples were centrifuged for 10 minutes at 6000 rpm and supernatant was decanted into a fresh 1.5 ml microcentrifuge tube. 100 μL of each sample was collected and added to 180 μL of precipitating solution (24 mM NEM +20 mM EDTA) in a blood Eppendorf tube. Then, 20 μL of internal standard (100 μM of GSH $^{13}C_2$, ^{15}N) was added before vortexing for 3 seconds. Next, 20 μL of 20% SSA was added before vortexing vigorously for 10 seconds. Finally, samples were incubated for 20 min at room temperature (20 ^{o}C). Following this derivatization, samples were centrifuged for 5 min at 14,000 rpm at room temperature. Supernatant (approximately 150 μL) was transferred to mass spectrometry vials for either immediate LC-MS/MS analysis or storage at -80 ^{o}C.

LC-MS/MS was performed on an Applied Biosystems 4000 QTRAP hybrid linear ion trap-triple quadrupole instrument (AB Sciex, Concord, ON, Canada) operated at a source temperature of 400 °C with ion spray voltage of 5000 kV in positive ion mode. Instrument parameters for GSH and GSSG were as follows: declustering potential (40 for GSH, 60 for GSSG), collision energy (30), entrance potential (8), curtain gas (30) and collision cell exit potential (3).

A Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD), consisting of two LC-20AD pumps, a DQU-20A$_5$ degasser, and an SIL-HTC autosampler were used for all chromatography. Chromatographic separations of GSH-NEM and GSSG were

achieved on a Hypercarb column (Thermo Scientific, 2.1 mm x 100 mm x 5 um) at

room temperature. Mobile phase A was water containing 0.1 % formic acid, and

mobile phase B was 100% acetonitrile containing 0.1 % formic acid. The run

consisted of isocratic delivery of 5% phase B for 0.3 min, linear gradient of 5-40%

phase B over 3.3 min, a second linear gradient of 40-95% phase B over 0.9 min,

isocratic delivery of 95% phase B for 1.5 min, and final wash with 100% phase B

over 2 min. Parent → product ion transitions for SRM were developed using

standards. SRM transitions used for quantitation included: 433.3 → 304.3 for GSH-

NEM, 436.3 → 307.3 for GSH-^{13}C$_2$,^{15}N-NEM, and 613.4→ 355.2 for GSSG.

Wine Elemental Sulfur Analysis

HPLC analysis was conducted to measure elemental sulfur remaining in the wine

after fermentation. A Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA, USA)

equipped with HP ChemStation software and photodiode array detector (DAD) was

used. This method was based on the technique outlined by Kamyshny et. al. (2009).

Wine samples of 25 ml were mixed with 2 ml of dichloromethane in a 50 ml

separatory funnel and mixed for 1 minute. 20 ul of extract was injected onto a

LiChroSpher reverse-phase C18 analytical column (250 mm x 4 mm, 5 um, Merck,

Darmstadt, Germany) and guard column (12.5 mm x 4 mm, 5 um, Merck). HPLC

grade methanol (EMD Millipore) was the sole mobile phase used. Run time was 20

minutes; wavelengths detected were 230 and 265 nm. Dilutions of a model wine

solution (13% ethanol (v/v), 6 g/L tartaric acid) containing colloidal sulfur (Sigma-Aldrich) were used to generate calibration curves.

Statistical Analysis

Statistical tests consisted of analysis of variance (ANOVA) tests performed with R Studio (Boston, MA, USA). Tukey's HSD multiple comparison was performed to test least squares means of treatment effects at the $p < 0.05$ significance level.

RESULTS

Synthetic Grape Juice Experiment

All fermentations proceeded rapidly in the synthetic grape juice (SGJ) at first but struggled to complete (Figure 3.1). This resulted in all wines containing residual sugar 38 days after yeast inoculation. Yeast strain and nitrogen treatment did not impact the speed of fermentation or whether the fermentation completed. Populations of *S. cerevisiae* P1Y2 and UCD522 both peaked at approximately 1×10^8 cfu/ml two days after inoculation (Figure 3.2). Treatments P1Y2 high amino acids and P1Y2 low amino acids maintained the highest viable yeast populations as fermentation proceeded, with approximately 2.8×10^6 cfu/ml after 38 days of fermentation, while treatments UCD522 low amino acids and UCD522 DAP possessed the lowest viable yeast populations after 38 days, with approximately 1×10^5 cfu/ml (Figure 3.2).

During the course of the fermentations, H_2S production was measured using H_2S detection tubes. Total H_2S produced during fermentation is shown in Figure 3.3. No H_2S was produced by yeast strain P1Y2 during fermentation in any of the treatments and so Figure 3.3 illustrates production by yeast strain UCD522. Because of higher than anticipated production of H_2S early in fermentation, H_2S production during a 24 hr period early during fermentation (between days 2-3) was not recorded as the lead acetate detection tubes were not replaced. Interpretation of data from this time period is therefore limited.
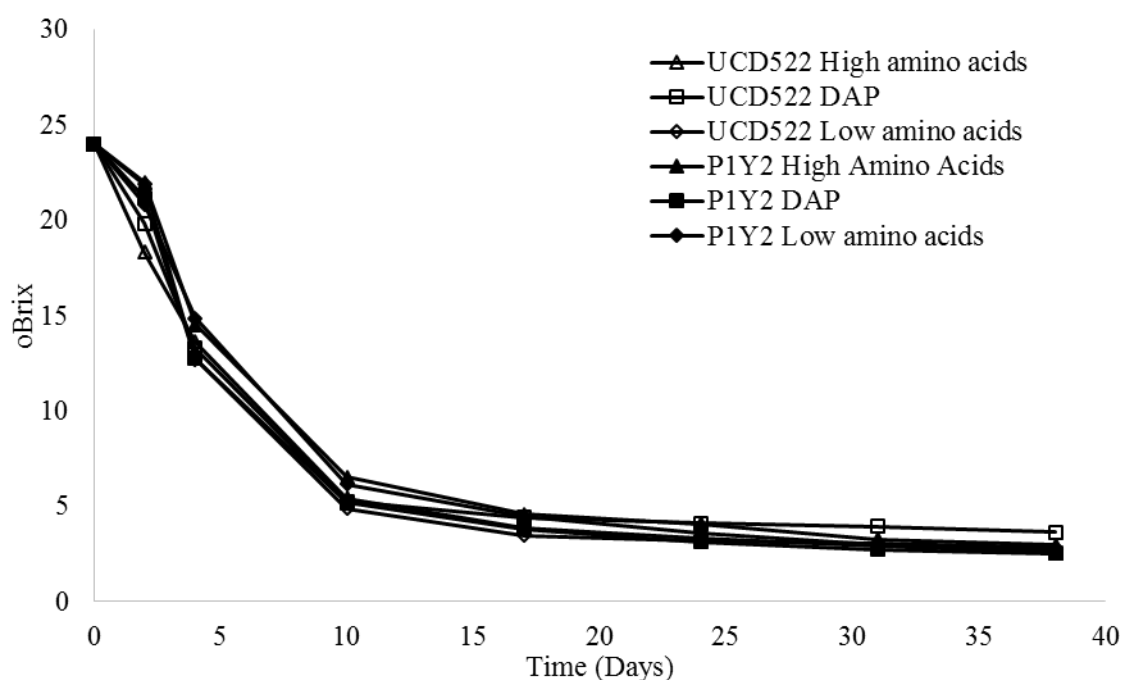
Figure 3.1: Change in Brix during fermentation by *S. cerevisiae* strain UCD522 (open symbols) or strain P1Y2 (closed symbols) of synthetic grape juice containing low amino acids, high amino acids, or low amino acids and diammonium phosphate (DAP).
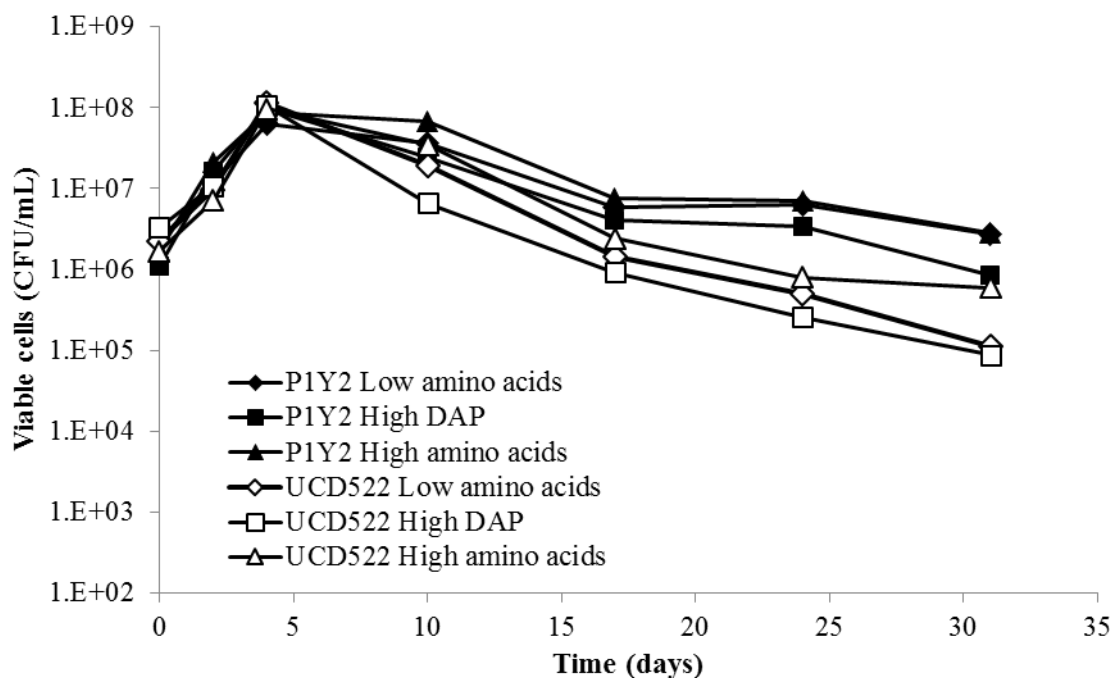
Figure 3.2: Growth of *S.cerevisiae* strain P1Y2 (open symbols) or UCD522 (closed symbols) in synthetic grape juice containing different concentrations of amino acids and/or diammonium phosphate (DAP).

The highest total production of H_2S occurred in fermentations of synthetic juice containing high amino acids (347 mg/L YAN) or high DAP (350 mg/L YAN). Fermentation of the low YAN juice (low amino acids) resulted in a lower amount of total H_2S being produced. However, because of the large standard deviation between replicates, there were no significant differences
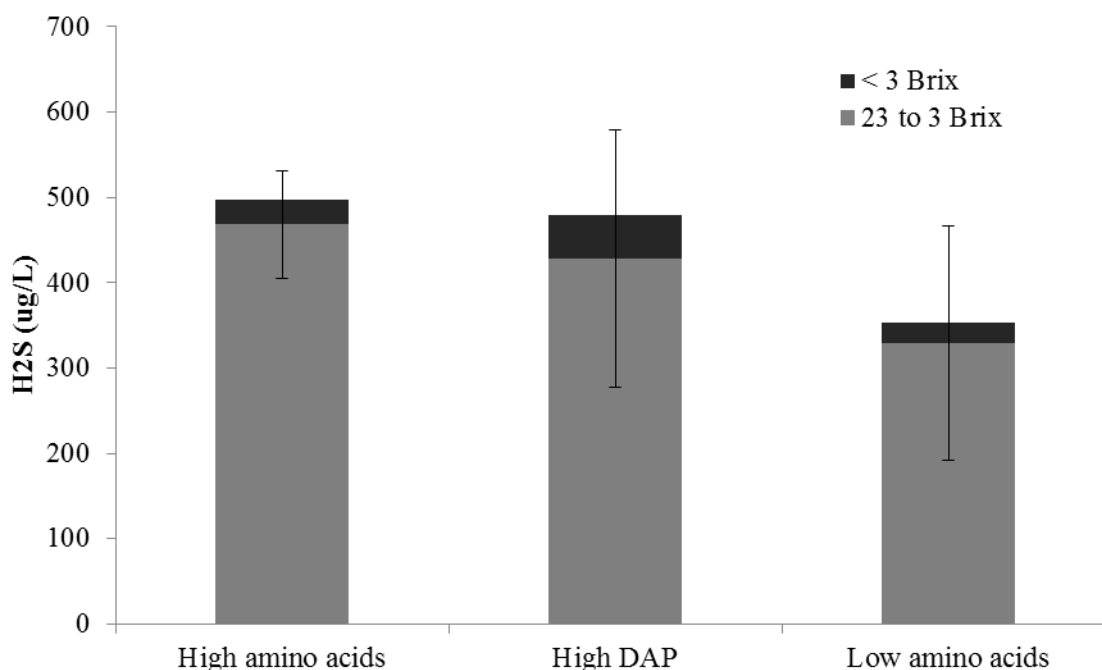
Figure 3.3: Total H$_2$S production by *S. cerevisiae* strain UCD522 during fermentation of synthetic grape juice containing different concentrations of amino acids and/or diammonium phosphate (DAP). Values represent the means of three replicate ferments. Mean values with different superscript letters were significantly different at *p*≤0.05

The concentration of amino acids in wines produced from SGJ containing different

YAN concentration and composition was quantified by HPLC. Concentrations of all

the amino acids are shown in appendix C. Differences were noted for the sulfur-

containing amino acids methionine and cysteine. Concentrations of methionine

were highest in wine made from low amino acid SGJ and fermented by P1Y2 (4.46

mg/L). This was significantly more than wines produced by UCD522 from high

amino acids SGJ which contained the lowest concentration of methionine (Table

3.1). Cysteine was not detected in wines produced by UCD522 from high amino

acids SGJ and DAP SGJ (Table 3.3). Large variation between replicates for wines

produced by UCD522 from low amino acid SGJ was also noted. Aside from the fact

that no cysteine was measured in the UCD522 high amino acids and DAP wines, no

other significant differences in cysteine concentrations were observed (Table 3.1).

Table 3.1: Concentrations of sulfur-containing amino acids (mg/L) in wine produced
from synthetic grape juice containing different concentrations of amino acids
and/or diammonium phosphate (DAP) and fermented by either *S. cerevisiae* strain
P1Y2 or UCD522.

Treatment		Methionine	Cysteine
UCD522	Low aa	3.81±1.71*[ab]	1.40±1.43[a]
	High aa	1.76±0.78[b]	ND
	DAP	2.43±0.92[ab]	ND
P1Y2	Low aa	4.46±0.20[a]	0.97±0.04[a]
	High aa	3.44±0.30[ab]	0.85±0.20[a]
	DAP	4.39±0.49[ab]	1.12±0.23[a]

Low aa= Low amino acids (112 mg/L YAN)
High aa= High amino acids (347 mg/L YAN)
DAP = Low amino acids supplemented with DAP (350 mg/L YAN)
*Values represent the means of three replicate ferments. Mean values with different
superscript letters within a column were significantly different at $p \leq 0.05$
ND = not detectable

Glutathione (GSH) concentrations were measured 38 days after yeast inoculation.

Wines produced by UCD522 contained higher GSH concentrations than wines

fermented by P1Y2 no matter what the nitrogen composition (Table 3.2). Wine

fermented by UCD522 from SGJ containing high DAP contained the greatest amount

of GSH (2535 ug/L) while P1Y2 fermented wine made from low amino acid SGJ

contained the lowest amount of GSH. UCD522 low amino acids and UCD522 DAP

contained the highest amount of GSSG but the concentrations in all wines was low

compared to GSH concentrations (Table 3.2).

Table 3.2: GSH and GSSG concentrations (ug/L) in wine produced from synthetic grape juice containing different concentrations of amino acids and/or diammonium phosphate (DAP) and fermented by either *S. cerevisiae* strain P1Y2 or UCD522.

Treatment		GSH	GSSG
UCD522	Low aa	2119.7±496.7*[a]	9.3±2.1[b]
	High aa	1815.9±258.8[a]	3.3±2.9[a]
	DAP	2535.5±160.2[a]	8.7±2.5[b]
P1Y2	Low aa	1117.8±338.8[b]	7.5±1.1[bc]
	High aa	850.2±121.0[b]	1.0±0.8[a]
	DAP	1060.1±87.8[b]	3.0±1.1[ac]

Low = Low amino acids (112 mg/L YAN)
High = High amino acids (347 mg/L YAN)
DAP = Low amino acids supplemented with DAP (350 mg/L YAN)
*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$

Volatile Sulfur Compounds

The concentrations of volatile sulfur compounds were analyzed in synthetic wines in samples that were taken 51 days after inoculation. Although numerous VSCs were quantified in the synthetic wines, dimethyl sulfide (DMS) and methionol were absent from these wines. H_2S was only present in detectable levels in UCD522 fermented wines produced from high amino acid SGJ or DAP amended SGJ (Table 3.3) and large variability between replicates was noted. However, MeSH was measured in all wines fermented by UCD522 and P1Y2 fermented wines made from SGJ containing low or high amino acids but not from DAP-supplemented SGJ (Table 3.3). Levels of MeSH in UCD522 fermented wines were significantly higher than levels in P1Y2-fermented wines.

Concentrations of MeSOAc were not detected in any P1Y2 wines, but were present at levels close to 12 ug/L in UCD522-fermented wines. UCD522-fermented wines

from SGJ supplemented with DAP contained significantly more MeSOAc than did UCD522-fermented wines from SGJ containing high amino acids (Table 3.3). Ethyl thioacetate (EtSOAc) was likewise present in UCD522-fermented wines but not in P1Y2-fermented wines (Table 3.3). Similar to MeSOAc, EtSOAc was significantly higher in UCD522 low amino acids and UCD522 DAP compared to UCD522 high amino acids (Table 3.3). All wines also contained CS_2 and DMDS. Although the trend found was for wines fermented by UCD522 to have higher amounts of these two VSCs, there was no statistically significant differences between treatments for CS_2 concentrations. DMDS was only significantly higher in UCD522 wine from low amino acid SGJ when compared P1Y2-fermented wine made from low or high amino acid SGJ (Table 3.3).

Table 3.3: Concentration of volatile sulfur compounds in wine produced from synthetic grape juice containing different concentrations of amino acids and/or diammonium phosphate (DAP) and fermented by either *S. cerevisiae* strain P1Y2 or UCD522.

Treatment		H_2S	MeSH	MeSOAc	EtSOAc	CS_2	DMDS
UCD522	Low aa	N.D.*[a]	4.63±0.80[bc]	9.79±1.74[bc]	4.62±0.41[b]	5.25±1.16[a]	2.83±0.30[b]
	High aa	1.04±1.81[a]	4.43±0.74[bc]	8.21±0.71[c]	3.19±0.14[c]	5.57±1.27[a]	1.46±0.46[ab]
	DAP	4.04±6.99[a]	6.42±0.59[c]	11.44±0.34[b]	5.31±0.96[b]	3.27±1.00[a]	2.05±1.28[ab]
P1Y2	Low aa	N.D.[a]	1.74±3.01[ab]	N.D.[a]	N.D.[a]	2.72±1.27[a]	0.42±0.53[a]
	High aa	N.D.[a]	1.27±1.80[a]	N.D.[a]	N.D.[a]	3.18±0.78[a]	0.44±0.76[a]
	DAP	N.D.[a]	N.D.[ab]	N.D.[a]	N.D.[a]	3.43±0.76[a]	0.66±0.32[ab]

Low aa= Low amino acids (112 mg/L YAN)
High aa= High amino acids (347 mg/L YAN)
DAP = Low amino acids supplemented with DAP (350 mg/L YAN)
ND: Below the detection limit
*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$

Elemental Sulfur and Pinot noir Fermentation

Fermentations proceeded quickly in all treatments and were below 0 °Brix after

168 hours. UCD522 fermentations, particularly those with 15 ug/g of elemental

sulfur added, fermented slightly slower between 60h and 100h (Figure 3.4A) H_2S

production peaked during the first 48-72 hrs after inoculation with the highest

rate of production occurring in fermentations containing $15\mu g/g$ S^0 conducted by

UCD522 (Figure 3.4B). As expected, wines fermented by UCD522 released H_2S at a

higher rate during fermentation than the non-H_2S producing strain P1Y2.

However, H_2S was still produced in fermentations conducted by P1Y2 when S^0 was

added to the fermentations (Figure 3B). Over the course of the entire fermentation,

wines fermented by UCD522 with addition of 15 ug/g S^0 produced more than

triple the amount of H_2S than any other treatment, totaling nearly 2800 ug/L

(Figure 3.5). Wines fermented by P1Y2 with addition of 15 ug/g S^0 produced

significantly more H_2S than other P1Y2 treatments and totaled more than 600

ug/L (Figure 3.5). Additionally, both high sulfur treatments released H_2S at the end

of fermentation. For example, after fermentations had reached 0 °Brix UCD522 and

P1Y2 fermented wines with 15 ug/g S^0 added produced 222 and 136 ug/L of H_2S,

respectively (Figure 3.5).
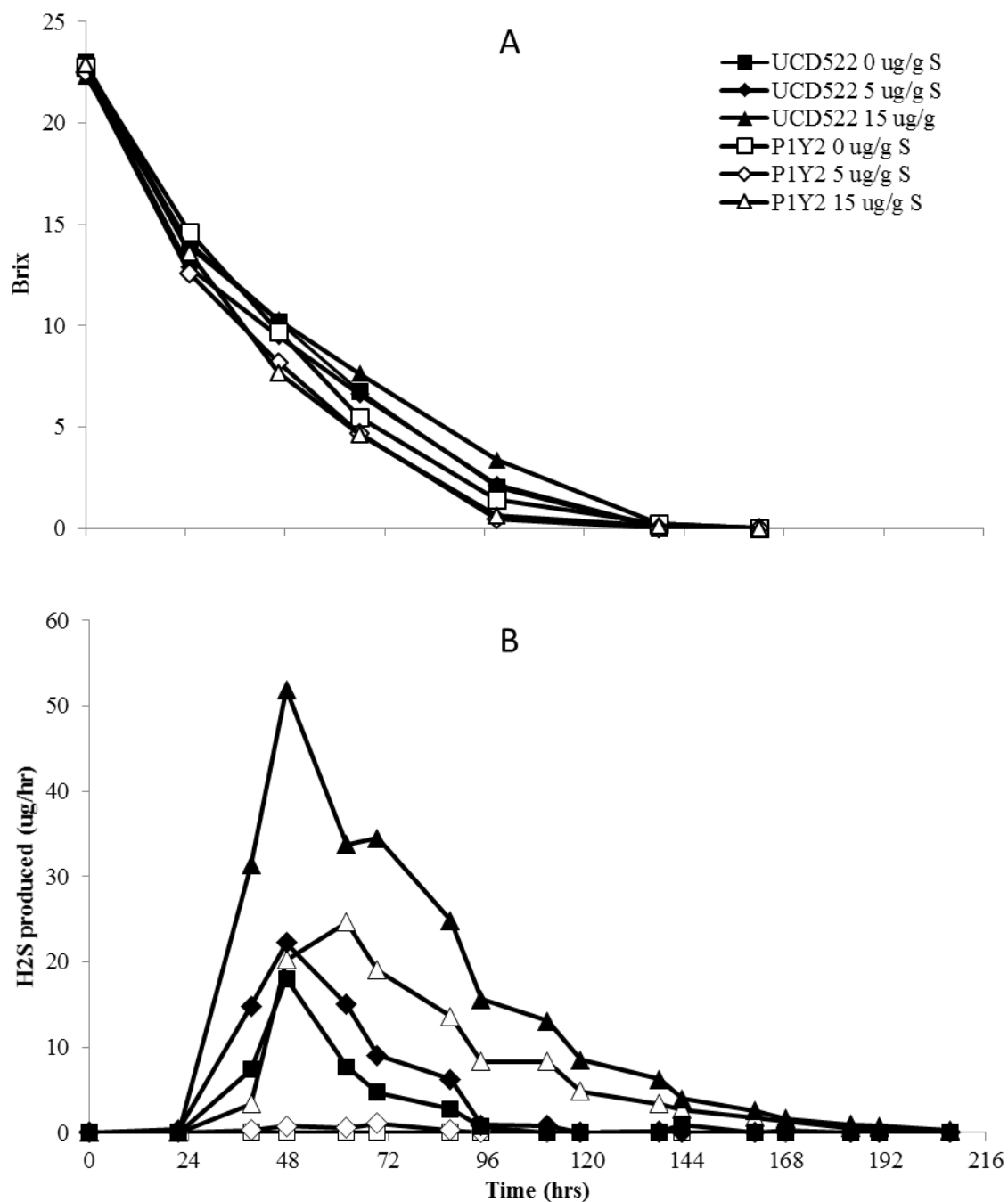
Figure 3.4: Change in Brix (A) and production per hour of H_2S (B) by *S. cerevisiae* strain UCD522 (closed symbols) or strain P1Y2 (open symbols) during fermentation of Pinot noir grapes with the addition of 0, 5, or 15 μg/g elemental sulfur.
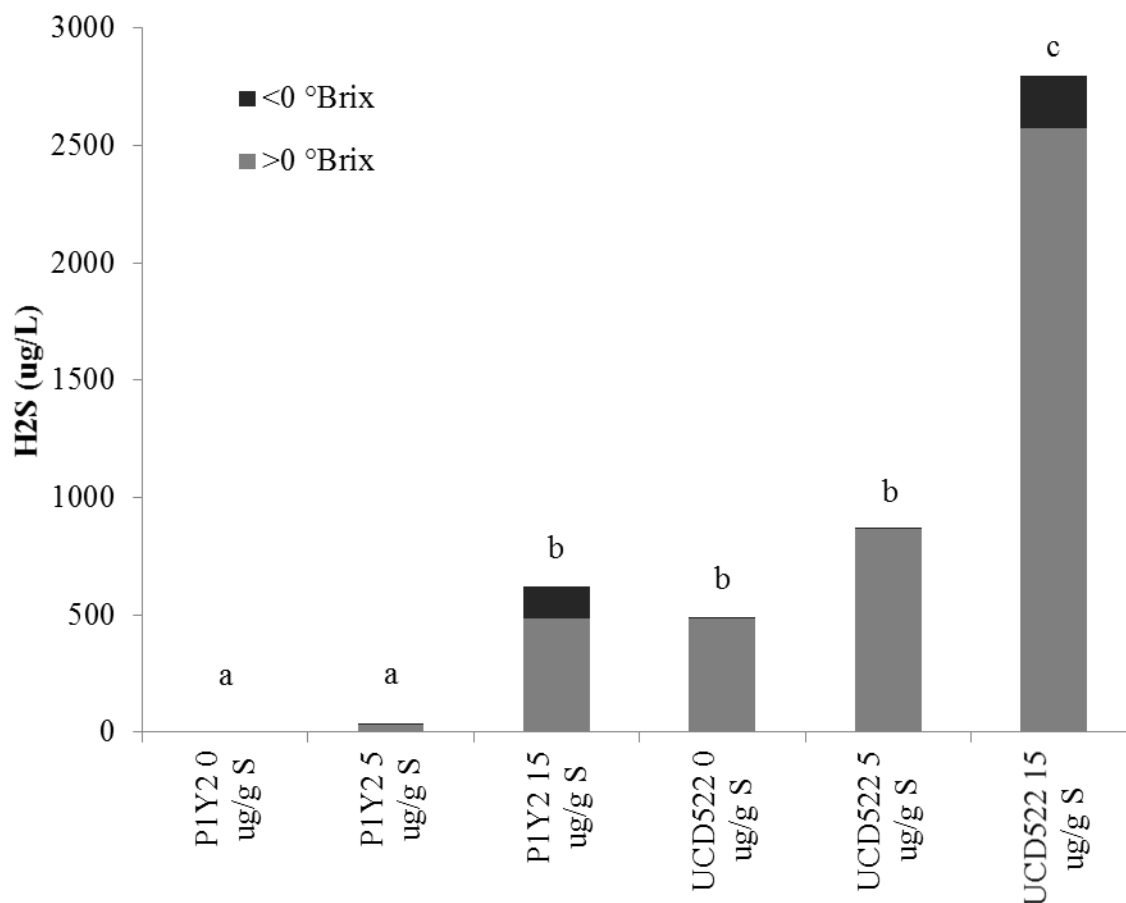
Figure 3.5: Total H₂S produced by *S. cerevisiae* strain UCD522 or strain P1Y2 during fermentation of Pinot noir grapes with the addition of 0, 5, or 15 ug/g elemental sulfur. Columns with different letters indicate values significantly different at $p \leq 0.05$, n=3

Concentrations of primary amino acids were greater in wines that had no colloidal sulfur added, but not by a significant amount. Wines fermented with P1Y2 yeast also contained more primary amino acids at the end of fermentation than did those fermented with UCD522 (Table 3.4).

Table 3.4: Primary amino acid concentration (mg/L N) in Pinot noir wine fermented by *S. cerevisiae* strain UCD522 or strain P1Y2 with the addition of 0, 5, or 15 ug/g elemental sulfur

Treatment		Primary amino acids
UCD522	0 ug/g S^0	$104.4 \pm 17.2 *a$
	5 ug/g S^0	94.9 ± 7.3^a
	15 ug/g S^0	89.6 ± 7.8^a
P1Y2	0 ug/g S^0	121.0 ± 8.3^b
	5 ug/g S^0	94.4 ± 8.6^a
	15 ug/g S^0	108.8 ± 2.1^c

*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$

The amino acid content of the Pinot noir wines fermented with different levels of S^0 was measured after pressing and after 30 days aging on the lees at 13°C. Wines fermented by P1Y2 with 0 ug/g S^0 contained the highest methionine concentrations (Table 3.5) followed by wines fermented by UCD522 that also contained no elemental sulfur (Table 3.5). Cysteine concentrations were similar for wines produced with varying S^0 (Table 3.6). While cysteine levels increased very little after 30 days aging (Table 3.6), methionine levels increased (Table 3.5).

Wines were also analyzed for elemental sulfur post-fermentation by HPLC (Figure 3.7). Only one treatment, P1Y2-fermented wines with 15 ug/g of S^0 added, contained any detectable elemental sulfur. Large variation between the replicates was noted (Table 3.7).

Table 3.5: Concentration of methionine (mg/L) in Pinot noir wine fermented by *S. cerevisiae* strain UCD522 or strain P1Y2 with the addition of 0, 5, or 15 ug/g elemental sulfur. Analysis occurred after pressing (0 days) and after 30 days storage at 13°C

Treatment		Methionine (0 days)	Methionine (30 days)
UCD522	0 ug/g S^0	2.55±1.22*b	8.67±2.17ab
	5 ug/g S^0	1.92±0.86b	6.69±1.03ab
	15 ug/g S^0	1.20±0.16b	5.35±0.84ab
P1Y2	0 ug/g S^0	6.72±2.72a	10.89±3.18a
	5 ug/g S^0	1.90±0.26b	3.15±0.90b
	15 ug/g S^0	2.67±0.79b	3.15±0.91ab

*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$

Table 3.6: Concentration of cysteine (mg/L) in Pinot noir wine fermented by *S. cerevisiae* strain UCD522 or strain P1Y2 with the addition of 0, 5, or 15 ug/g elemental sulfur. Analysis occurred after pressing (0 days) and after 30 days storage at 13°C

Treatment		Cysteine (0 days)	Cysteine (30 days)
UCD522	0 ug/g S^0	2.02±0.19*b	2.41±0.48a
	5 ug/g S^0	1.98±0.41b	2.41±0.26a
	15 ug/g S^0	2.15±0.13ab	2.33±0.66a
P1Y2	0 ug/g S^0	2.69±0.83ab	3.32±0.54a
	5 ug/g S^0	3.34±0.12a	3.18±0.10a
	15 ug/g S^0	2.01±0.58b	2.99±0.17a

*Values represent the means of three replicate ferments. Mean values with different superscript letters within a column were significantly different at $p \leq 0.05$

Table 3.7: Concentration of elemental sulfur (ug/g) in wine produced from Pinot noir grapes that had an addition of 0, 5, or 15 ug/g elemental sulfur prior to fermentation with either *S. cerevisiae* strain P1Y2 or UCD522.

Yeast	0 ug/g S^0	5 ug/g S^0	15 ug/g S^0
P1Y2	ND	ND	0.26±0.38*
UCD522	ND	ND	ND

ND: Below the detection limit
*Values represent the means of three replicate ferments.

Only two VSCs were detected in the Pinot noir wines, MeSOAc and CS_2.

Concentrations of CS_2 were low in all wines (Table 3.8). Concentrations of MeSOAc

were significantly higher in wines with S^0 added (Table 3.8), with the highest

concentrations measured in wines where 15 ug/g S^0 was added. Also, wines made

from grapes with 15 ug/g S^0 added contained significantly more MeSOAc if

fermented by P1Y2 rather than UCD522 (Table 3.8).

Table 3.8: Concentrations of volatile sulfur compounds (ug/L) in Pinot noir wine
fermented by *S. cerevisiae* strain UCD522 or strain P1Y2 with the addition of 0, 5,
or 15 ug/g elemental sulfur

Treatment		MeSOAc	CS_2
UCD522	0 ug/g S^0	N.D.*[a]	0.087 ± 0.004[bc]
	5 ug/g S^0	2.06 ± 1.02[b]	0.060 ± 0.003[ab]
	15 ug/g S^0	$8.02\pm.15$[c]	0.079 ± 0.018[b]
P1Y2	0 ug/g S^0	N.D.[a]	0.044 ± 0.011[a]
	5 ug/g S^0	2.31 ± 0.17[b]	0.077 ± 0.006[b]
	15 ug/g S^0	11.89 ± 0.93[d]	0.109 ± 0.013[c]

*Values represent the means of three replicate ferments. Mean values with
different superscript letters within a column were significantly different at $p\leq0.05$

DISCUSSION

Synthetic grape juice experiment

The concentration and composition of YAN in grape juice has been shown to

impact yeast fermentation and H_2S production (Jiranek et al., 1995). In the present

study, synthetic grape juices with varying amounts and types of YAN were

fermented by known low and high H_2S-producing yeast strains.
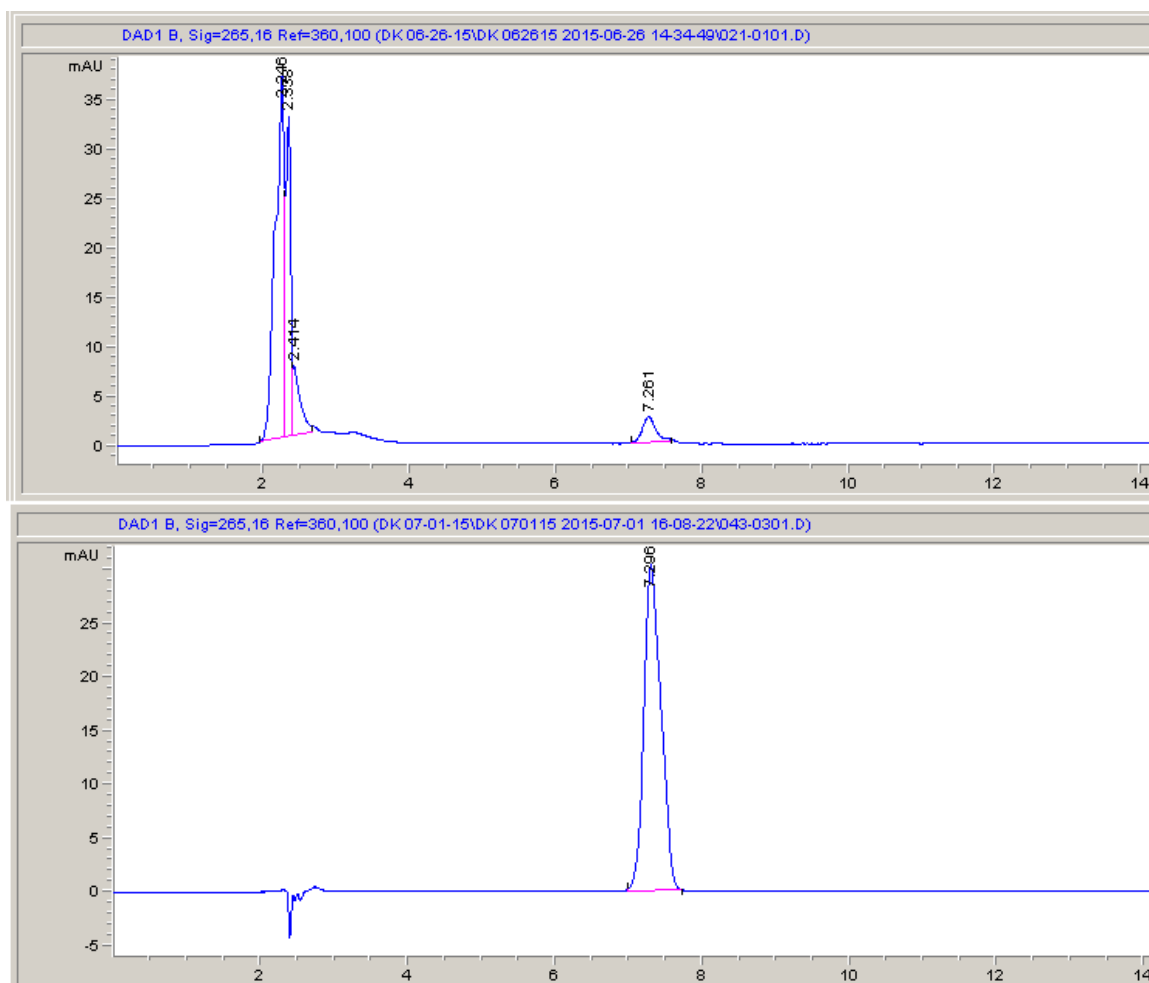
Figure 3.6: HPLC chromatogram at 265 nm of (A) Pinot noir wine produced from grapes containing 15 ug/g elemental sulfur and fermented by *S. cerevisiae* P1Y2 and (B) a 8 ug/g elemental sulfur standard solution.

Unexpectedly, there was little difference in the time taken for fermentation between ferments containing high or low YAN. In contrast, Bell and Henscke (2005) reported that increases in juice nitrogen concentration have a subsequent increase on the fermentation rate. However, there is still some dispute over how much YAN is required for *S. cerevisiae* to complete fermentation with Bisson and

Butzke (2000) recommending 200 mg/L YAN is required for a 21 °Brix grape must

with an additional 25 mg/L YAN for every °Brix increase. In contrast, Wang et al.

(2003) and Ugliano et al. (2009) reported that YAN levels as low as 100 mg/L were

sufficient to ferment 25 °Brix grape musts to dryness. In the present study, all

treatments regardless of YAN content failed to ferment to dryness suggesting

factors other than YAN were responsible for the sluggish end to fermentation. In

this experiment conducted in synthetic grape juice, neither yeast strain nor

nitrogen was found to significantly impact the fermentation rate. This was not the

expected result, as many studies have found that increases in nitrogen

concentration have a subsequent increase on the fermentation rate (Bell and

Henscke, 2005). Yeast strains also vary in their efficiency and utilization of

nitrogen (Jiranek et al., 1995) and so it was not surprising that one strain, P1Y2,

achieved higher cell populations during fermentation.

No H_2S was measured during fermentations conducted by P1Y2 reinforcing its

manufacturer's claims that it is a "no-H_2S" producing yeast strain. In contrast, large

amounts of H2S were produced in all fermentations conducted by UCD 522

regardless of the YAN concentration or composition. Although low YAN is often

noted to be a major cause of H_2S production by yeast during fermentation (Jiranek

et al., 1995), it has also been noted by others that high H_2S production can still

occur under high YAN conditions (Ugliano et al., 2009; Wang et al., 2003). The H_2S

production by UCD 522 was more likely driven by the fact that this yeast is a

known high H₂S producer ((Linderholm et al., 2010; Bohlscheid et al., 2007; Wang et al., 2003) and also by the fact that low pantothenic acid concentration was present in the SGJ. According to early work by Ough and Kunkee (1968), the recommended amount of pantothenic acid to prevent H_2S formation is 150-250 ug/L. As low levels of pantothenic acid tend to promote H_2S formation in high-nitrogen musts (Wang et al., 2003), the low pantothenic acid concentrations used in this experiment may explain why the low amino acids treatment produced the least amount of H_2S.

Because of large variability between replicates there was not a significant difference in H_2S produced during fermentation of SGJ with varying YAN concentration and composition. Recent work by Ugliano et al. (2009) reported that high DAP supplementation during fermentation yielded higher H_2S production for one yeast strain but not for another. This study also noted that ferments supplemented with high amounts of DAP had late formation of H_2S, a finding also noted in the present study. As has been noted by others (Ugliano et al. 2011; 2009) total H_2S production during fermentation did not correlate with the final H_2S concentration in the wine as very low concentrations of H_2S were measured in the wine by gas chromatography. This is likely due to the low solubility and high volatility of H_2S and the sparging effect of CO_2 produced by the yeast during fermentation (Rauhut, 1996). This is also why late formation of H_2S can be

problematic; the sparging effect of CO_2 would be reduced near the end of fermentation resulting in wines with potentially higher residual H_2S.

While only low concentrations of H_2S were present in the final wines, significant concentrations of methanethiol (MeSH) and methyl thioacetate (MeSOAc) were found in all wines fermented by UCD 522. Concentrations of more than double that of the reported sensory threshold for MeSH were measured. In P1Y2 wines, a small amount of MeSH was measured in wines fermented from low or high amino acid SGJ. The formation of MeSH is related to that of H_2S, but this has not been specifically demonstrated (Rauhut, 1993). These findings suggest that there are multiple pathways for MeSH production as MeSH concentrations were measured in wines fermented by P1Y2 where no H_2S was produced during fermentation. It is believed that thioacetates are formed by a reaction of mercaptans and acetyl-coA, but this has not been proven in a wine matrix (Rauhut, 1993). Although thioacetates have high sensory thresholds, 40 and 300 ppb for ethyl and methyl thioacetates in a beer matrix, respectively, these compounds can be hydrolyzed to form mercaptans during aging in a reductive environment (Rauhut, 1993; Leppanen, 1980; Rauhut, 1996), which have extremely low sensory thresholds. Disulfides can also be reduced during aging to form mercaptans, and although concentrations of dimethyl disulfide (DMDS) were less than half of MeSH in this experiment, this could be an additional contributor to sulfur off-flavor during aging.

Concentrations of methyl thioacetate (MeSOAc) were significantly higher in wines made from DAP-supplemented SGJ and fermented by UCD522 than in UCD522-fermented wines from high amino acids SGJ. This finding is in support of Ugliano et al. (2009) where higher MeSOAc was reported in high DAP-supplemented treatments. Ugliano et al. (2009) also reported that this effect was yeast strain dependent, as was seen in the present study. Thioacetates such as MeSOAc are thought to be formed indirectly from H_2S (Rauhut. 1996) and the findings of the present study support this assertion, as no MeSOAc was present in P1Y2 wines where no H_2S was produced during fermentation, but MeSOAc was present in the final wine in high amounts in UCD522-fermented wines where large amounts of H_2S were produced. If thioacetates are formed as a reaction of acetyl-CoA and H_2S, then pantothenic acid concentrations may also influence its production, as pantothenic acid is the foundation for coenzyme A (Tokuyama et al., 1973).

Similarly, concentrations of ethyl thioacetate (EtSOAc) were significantly higher in wines made from DAP-supplemented SGJ and fermented by UCD522 than in UCD522-fermented wines from high amino acids SGJ. Previous research has suggested that MeSOAc may be proportional to H_2S produced early in fermentation, while EtSOAc is proportional to H_2S produced late in fermentation (Rauhut, 1996; 1999). The data obtained in this experiment suggest that the relationship is more complicated and is not controlled by linear first-order

kinetics. Because ethanethiol (EtSH) was not measured, it cannot be speculated whether EtSOAc may have been produced from EtSH.

The concentrations of sulfur-containing amino acids were also influenced by nitrogen supplementation. For both yeasts used, the low amino acids treatment had the highest concentration of methionine, followed by DAP, and finally high amino acids. While these differences were not significant, they reveal an interesting trend. P1Y2 fermentations also contained more methionine than UCD522, but not significantly. This is in accordance with the findings in Chapter 2. Cysteine was not found in UCD522 DAP or UCD522 high amino acids treatment wines. This could indicate either a degradation of cysteine or a possible reactions of cysteine to form other compounds including possible formation of glutathione (GSH) by yeast late in the fermentation. In support, glutathione (GSH) levels were higher in UCD522 fermentations than in P1Y2 fermentations. This is also similar to the findings of Chapter 2. However, the concentrations were much higher than those previously reported in Chapter 2. The fact that the fermentation became sluggish and very slow near the end of fermentation may have resulted in higher GSH concentrations. This is because GSH tends to be at a maximum during the middle and late stages of fermentation (di Lecce et al. 2013). The extremely low levels of GSSH detected (<10 ug/L for all treatments) is likely also a result primarily of the remaining fermentative activity maintaining a reductive environment.

Elemental sulfur experiment

In addition to YAN composition, the presence of elemental sulfur (S^0) during fermentation is known to impact H_2S formation during winemaking (Acree et al., 1972). In the present study various amounts of S^0 were added to Pinot noir grapes and fermented by a low/no or high H_2S producing yeast strain. All fermentations proceeded rapidly and no differences in time to complete fermentation were noted. H_2S formation did not occur until sugar levels were rapidly decreasing and no H_2S was produced during the first 24 hours of fermentation despite a reduction of at least 7 °Brix in all treatments. This finding is in contrast to the findings of Acree et al. (1972) and may be due to the rapid fermentation in the present study or the need for the development of a reductive environment in the fermentation to induce reduction of S^0 to H_2S. The use of a yeast that does not produce H_2S enabled the separation of yeast-produced H_2S and H_2S being formed from reduction of S^0. Because of this it was noted that yeast production of H_2S occurred earlier than formation of H_2S from S^0 reduction. Evidence for this is that 39 hrs after yeast inoculation over 30 ug/hr of H_2S was being released from fermentations conducted by UCD522 containing 15 ug/g S^0 while less than 4 ug/hr was measured in ferments conducted by P1Y2 (no yeast-produced yeast) with the same level of S^0.

Formation of H_2S during fermentation peaked mid-fermentation between 48 and 72 hrs after yeast inoculation. This finding is agreement with Thomas et al. (1993)

where it was reported that the main peak formation of H_2S occurred early to mid-fermentation. Thomas et al. (1993) also noted that a second peak of H_2S production occurred late in fermentation. In the present study it was also noted that in fermentations were 15 ug/g of S^0 had been added H_2S formation continued late in fermentation. This late formation of H_2S is more problematic as H_2S is more likely to be retained in the wine as the sparging effect of yeast produced CO_2 would be reduced near the end of fermentation (Zeeman et al., 1982).

Increasing amounts of S^0 in the fermentations resulted in larger amounts of H_2S being produced during the fermentation. When the amount of S^0 added tripled from 5 to 15 ug/g, the amount of additional H_2S produced increased by more than 10 times in P1Y2 fermentations and by more than 5 times in UCD522 fermentations. This finding is in contrast with Schutz and Kunkee (1977) where they reported a linear response of S^0 to H_2S produced. This difference may be due to the fact that Schutz and Kunkee (1977) used significantly higher amounts of S^0 during fermentation. Where in the present study concentrations of 5 and 15 ug/g S^0 were used, Schutz and Kunkee (1977) used 25, 50, 75, and 100 mg/L to measure the response of S^0 concentration to H_2S formation. It is possible that at lower levels a linear relationship does not exist.

The amount of S^0 necessary in a fermentation to cause production of H_2S at concentrations that may be problematic from a sensory point of view is not well defined. Thomas et al (1993) reported that 3.4 ug/g of S^0 was not sufficient to

reliably increase H_2S production during fermentation, while Kwasniewski et al

(2014) reported that concentrations as low as 1 ug/g S^0 correlated with increased

H_2S production. Neither Thomas et al (1993) or Kwasniewski et al (2014) used

yeast strains that could not produce H_2S via the SRS pathway so it is difficult to

separate yeast-produced H_2S from H_2S generated from S^0 reduction. In the present

study the presence of 5 ug/g of S^0 was sufficient to produce H_2S in fermentations

conducted with P1Y2 yeast. As this yeast cannot produce H_2S via the SRS pathway,

the formation of H_2S was due to reduction of S^0 to H_2S. Additional fermentations at

S^0 concentrations between 1-5 ug/g should be conducted using yeast strain P1Y2

to better understand the concentrations of S^0 that result in increased H_2S

formation. In addition, factors such as the oxidation-reduction state of the

medium/fermentation must be considered when determining what S^0

concentrations may be problematic as it is known that under more reductive

conditions the formation of H_2S is accelerated (Schutz and Kunkee, 1977).

Furthermore, it is currently unknown what concentrations of S^0 residue are

present on grapes in Oregon during wetter seasons with greater powdery mildew

pressure, when heavier spray regimes are required.

As was seen during the fermentations in SGJ, no H_2S was detected in the final

wines even when large amounts of H_2S were produced during fermentation. As

was previously noted, this may be due to the low solubility and high volatility of

H_2S as well as the potential reaction of H_2S to form more complex VSCs (Ugliano et

al., 2011; 2009; Rauhut, 1996; Schutz and Kunkee, 1977). This was still surprising as fermentations where 15 ug/g S^0 had been added produced H_2S very late in fermentation. The H_2S may have reacted to form other less volatile compounds that remain in the matrix (Rauhut, 1996). However, MeSH, which is perhaps the most likely culprit for this reaction, was also not found in these wines at detectable levels. MeSOAc, on the other hand, was measured in these wines and was significantly influenced by the amount of S^0 added to the fermentations. Although a number of studies have reported on the impact of S^0 on H_2S formation during fermentations, few if any have reported on the impact of S^0 on formation of VSCs post-fermentation. No MeSOAc was found in wines with no S^0 added, but higher levels were found in wines with 15 ug/g of S^0 added. In addition, significantly more MeSOAc was present in P1Y2 fermented wines than in UCD522 wines. This again is contrary to the supposition that MeSOAc content can be used as an indicator of high H_2S production during fermentation (Rauhut, 1999), as P1Y2 with 15 ug/g S^0 produced less than 20% as much total H_2S as UCD522 with 15 ug/g S^0. Despite the fact that no H_2S remained in these wines after pressing, the risk of MeSH formation via hydrolysis of MeSOAc is possible, depending on pH and storage conditions (Rapp, 1989). However, quinones may bind with MeSH produced from the hydrolysis of MeSOAc (He et al., 2013). Nevertheless, the wines produced in the present study with high amounts of S^0 contain sufficient MeSOAc that this could have a major sensory impact by MeSH.

While it has been demonstrated by many studies that S^0 can impact formation of H_2S during fermentation, little is known about whether it contributes to formation of VSCs post-fermentations. In previous studies that added S^0 to grape must or juice the wines produced were not analyzed to see if any residual S^0 remained. If S^0 remains in the wine during aging it is possible that it could result in formation of H_2S via reactions with glutathione (Rohwerder, 2003) or cysteine (Schutz and Kunkee, 1977). It is not known whether these reactions occur under winemaking conditions. In the present study S^0 content of the wines was assessed by HPLC. Aside from a small amount in one treatment, no S^0 was present in any of the wines. If this is the case then formation of VSCs post-fermentation is unlikely to be impacted by S^0.

Sulfur-containing amino acids were also quantified in this experiment. Cysteine concentrations were higher in these wines than those observed in the SGJ study, but did not appear to be dependent on addition of S^0. Methionine levels were similar or slightly higher than those measured in SGJ with the highest concentration being measured in wine produced by P1Y2 with no addition of S^0. The reason for this is unknown and requires further investigation before any conclusions can be made.

CONCLUSIONS

Both nitrogen concentration and elemental sulfur are known to contribute to the formation of H_2S during fermentation. In the present study both of these factors impacted how much H_2S was produced during the fermentation. However, no measurable H_2S remained in the wines at the conclusion of fermentation. Elemental sulfur was shown to influence the formation of H_2S during fermentation by a non-H_2S producing yeast allowing the two causes of H_2S formation to be separated. Methyl thioacetate concentrations were increased by the addition of elemental sulfur as well as the addition of DAP. The hydrolysis of this compound to methanethiol is a likely contributor to problematic wines that develop VSCs during aging. Future work should investigate the interactions between low concentrations of elemental sulfur and nitrogen type and concentration on formation of VSCs during post-fermentation aging.

GENERAL CONCLUSIONS AND SUMMARY

A lengthy list of factors have previously been found to influence the formation of H_2S during fermentation, yet winemakers across the world continue to frequently struggle with this issue. Many producers also incur problems with H_2S and VSC formation during post-fermentation aging, whether in barrel, tank, or bottle; the causes of this have not been adequately explored. This study investigated relationships between sulfur-containing amino acids and formation of VSCs as a product of lees content, nitrogen type and concentration, and elemental sulfur residue. In agreement with other work, these experiments found a lack of correlation between H_2S formed during fermentation and VSC concentration post-fermentation. However, the concentration of sulfur-containing amino acids did correlate with a post-fermentation production of H_2S between 14 and 30 days of aging. Further studies in the future should explore whether this formation of H_2S is a result of enzymatic activity, or if it is merely a chemical equilibrium dependent on concentrations of sulfur-containing precursor molecules.

The influence of DAP and amino acids is complex and is influenced by pantothenic acid concentration. The influence of fairly low pantothenic acid concentrations in these fermentations may have been responsible for greater H_2S production in high nitrogen treatments. The standard deviations of replicates within the same treatment were large and made it difficult to quantify the influence of these factors on the yeast.

It remains unknown what concentration of S^0 is required to risk VSC formation post-fermentation, but this study indicates that methyl thioacetate is influenced strongly by S^0, and the formation of methanethiol from this compound during aging has been previously been known to devastate the sensory profile of wines. While it is unlikely that reduction of S^0 is solely causing problems at levels found in most commercial wine grapes, additional factors including complexing with glutathione and other compounds may cause formation of H_2S and methanethiol during aging.

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Appendix A: Free Amino Acid concentrations (mg/L) in lees-aged wines

Treatment	Days	ASP			GLU			ASN		
RC212 Light	0	2.67			9.76			3.31		
RC212 Light	14	1.78	±	0.15	8.66	±	0.38	2.87	±	0.06
RC212 Light	30	0.76	±	1.03	8.83	±	0.18	2.77	±	0.12
RC212 Light	60	1.11	±	0.03	7.59	±	0.47	2.21	±	0.15
RC212 Light	180	1.84	±	0.28	7.17	±	1.11	1.91	±	0.27
RC212 Light	270	2.44	±	0.13	9.15	±	0.46	1.12	±	0.23
RC212 Medium	0	3.45			9.16			3.51		
RC212 Medium	14	1.71	±	0.17	8.80	±	0.35	3.37	±	0.19
RC212 Medium	30	0.31	±	0.27	8.72	±	0.56	3.01	±	0.35
RC212 Medium	60	1.11	±	0.03	7.59	±	0.47	2.21	±	0.15
RC212 Medium	180	2.36	±	0.34	8.97	±	0.74	2.60	±	0.01
RC212 Medium	270	2.98	±	0.52	9.89	±	0.31	1.51	±	0.28
RC212 High	0	1.86	±	0.22	17.80	±	1.47	4.66	±	0.48
RC212 High	14	1.87	±	0.16	17.97	±	2.13	4.89	±	0.20
RC212 High	30	1.18	±	0.20	15.21	±	1.11	4.92	±	0.20
RC212 High	60	4.15	±	1.20	13.04	±	1.54	4.38	±	0.37
RC212 High	180	4.34	±	0.36	13.42	±	1.49	4.21	±	0.28
RC212 High	270	6.71	±	0.57	16.55	±	0.94	2.52	±	0.31
P1Y2 Light	0	2.43			14.59			4.42		
P1Y2 Light	14	2.00	±	0.18	13.94	±	1.63	4.84	±	0.18
P1Y2 Light	30	3.24	±	0.34	13.55	±	0.28	3.97	±	0.16
P1Y2 Light	60	2.41	±	0.29	13.49	±	1.10	3.52	±	0.25
P1Y2 Light	180	3.50	±	1.03	14.12	±	1.06	3.25	±	0.18
P1Y2 Light	270	3.77	±	0.26	14.19	±	0.62	1.80	±	0.13
P1Y2 Medium	0	3.37			15.64			4.47		
P1Y2 Medium	14	2.24	±	0.13	16.31	±	1.34	4.97	±	0.39
P1Y2 Medium	30	2.35	±	0.28	14.47	±	0.66	4.05	±	0.27
P1Y2 Medium	60	2.64	±	0.19	14.66	±	0.73	4.18	±	0.08
P1Y2 Medium	180	2.85	±	0.53	14.32	±	1.11	3.80	±	0.09
P1Y2 Medium	270	4.06	±	0.57	15.27	±	1.40	2.05	±	0.08
P1Y2 High	0	2.83	±	0.08	26.89	±	1.17	7.06	±	0.36
P1Y2 High	14	2.50	±	0.21	24.65	±	1.00	6.82	±	0.33
P1Y2 High	30	3.17	±	0.81	20.11	±	0.95	5.89	±	0.28
P1Y2 High	60	5.20	±	0.78	22.07	±	0.94	6.24	±	0.24
P1Y2 High	180	4.83	±	0.25	19.47	±	0.92	4.71	±	0.32
P1Y2 High	270	7.38	±	0.13	22.55	±	0.45	2.90	±	0.18

Treatment	Days	SER			GLN			CIT		
RC212 Light	0	4.29			4.56			3.53		
RC212 Light	14	2.01	±	0.48	6.15	±	0.06	4.58	±	0.24
RC212 Light	30	3.09	±	0.10	6.74	±	0.12	5.15	±	0.54
RC212 Light	60	2.61	±	0.11	5.66	±	1.01	5.45	±	0.38
RC212 Light	180	0.05	±	0.09	5.80	±	0.69	3.86	±	0.53
RC212 Light	270	1.69	±	0.20	5.49	±	0.64	6.66	±	0.37
RC212 Medium	0	4.10			5.26			4.06		
RC212 Medium	14	2.39	±	0.22	6.83	±	0.80	4.60	±	0.17
RC212 Medium	30	3.09	±	0.13	7.16	±	0.49	5.57	±	0.25
RC212 Medium	60	2.92	±	0.11	5.66	±	1.01	5.45	±	0.38
RC212 Medium	180	0.62	±	0.14	6.33	±	1.86	4.56	±	0.48
RC212 Medium	270	1.95	±	0.14	6.19	±	0.23	7.53	±	0.69
RC212 High	0	1.61	±	0.26	15.72	±	2.44	4.20	±	0.34
RC212 High	14	3.07	±	0.08	10.28	±	0.49	5.42	±	0.33
RC212 High	30	3.59	±	0.22	9.44	±	0.37	7.01	±	0.22
RC212 High	60	3.74	±	0.46	8.55	±	0.79	7.79	±	0.74
RC212 High	180	1.51	±	0.32	7.67	±	0.12	5.68	±	0.66
RC212 High	270	2.90	±	0.14	7.86	±	0.33	10.19	±	0.15
P1Y2 Light	0	4.75			6.57			4.79		
P1Y2 Light	14	2.14	±	0.20	8.09	±	0.10	5.25	±	0.30
P1Y2 Light	30	3.26	±	0.13	6.66	±	4.48	7.50	±	0.45
P1Y2 Light	60	2.40	±	1.02	8.57	±	0.94	7.27	±	0.21
P1Y2 Light	180	0.86	±	0.02	8.97	±	0.31	6.45	±	0.51
P1Y2 Light	270	2.07	±	0.09	7.36	±	0.12	10.47	±	0.90
P1Y2 Medium	0	4.77			7.15			4.85		
P1Y2 Medium	14	2.33	±	0.15	9.12	±	0.45	5.70	±	0.67
P1Y2 Medium	30	3.04	±	0.40	9.45	±	1.42	7.40	±	1.15
P1Y2 Medium	60	1.34	±	0.11	10.10	±	0.59	7.46	±	0.40
P1Y2 Medium	180	1.15	±	0.18	9.06	±	0.45	7.05	±	0.77
P1Y2 Medium	270	2.22	±	0.28	7.66	±	0.56	11.04	±	0.70
P1Y2 High	0	2.41	±	0.16	18.06	±	0.56	6.44	±	0.28
P1Y2 High	14	3.35	±	0.13	12.10	±	0.76	7.33	±	0.36
P1Y2 High	30	3.58	±	0.41	10.78	±	0.68	8.38	±	0.15
P1Y2 High	60	2.59	±	0.09	11.73	±	0.53	9.06	±	0.31
P1Y2 High	180	2.73	±	0.72	8.54	±	1.06	8.81	±	0.19
P1Y2 High	270	3.17	±	0.09	9.25	±	0.36	12.87	±	0.46

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Treatment	Days	HIS			GLY			ALA		
RC212 Light	0	14.90			5.48			2.43		
RC212 Light	14	25.93	±	1.74	N.D.			1.19	±	0.22
RC212 Light	30	10.51	±	1.46	6.03	±	0.19	1.61	±	0.16
RC212 Light	60	9.16	±	0.24	5.65	±	0.67	1.35	±	0.27
RC212 Light	180	10.34	±	0.72	4.90	±	0.41	1.02	±	0.15
RC212 Light	270	10.09	±	0.14	4.55	±	0.50	0.96	±	0.14
RC212 Medium	0	14.97			5.14			3.57		
RC212 Medium	14	25.98	±	0.82	N.D.	±		1.90	±	0.41
RC212 Medium	30	9.07	±	0.74	6.18	±	0.47	2.38	±	0.67
RC212 Medium	60	9.16	±	0.24	5.81	±	0.32	2.80	±	0.10
RC212 Medium	180	12.24	±	0.34	4.96	±	0.48	2.65	±	0.17
RC212 Medium	270	11.24	±	0.18	4.14	±	0.11	2.10	±	0.10
RC212 High	0	11.90	±	1.38	3.83	±	0.25	1.09	±	0.13
RC212 High	14	27.70	±	0.31	N.D.			3.00	±	0.10
RC212 High	30	12.29	±	0.59	N.D.			N.D.		
RC212 High	60	13.43	±	0.18	5.73	±	0.65	6.66	±	0.50
RC212 High	180	15.50	±	0.90	4.76	±	0.59	6.14	±	0.59
RC212 High	270	14.94	±	0.13	4.67	±	0.20	5.32	±	0.05
P1Y2 Light	0	17.78			6.08			3.79		
P1Y2 Light	14	27.39	±	0.46	N.D.			2.60	±	0.28
P1Y2 Light	30	12.22	±	0.10	6.15	±	0.42	3.12	±	0.20
P1Y2 Light	60	12.51	±	0.65	5.62	±	0.34	2.97	±	0.02
P1Y2 Light	180	14.51	±	0.97	5.48	±	0.65	2.97	±	0.34
P1Y2 Light	270	12.51	±	0.25	4.64	±	0.16	2.15	±	0.04
P1Y2 Medium	0	17.61			5.70			4.08		
P1Y2 Medium	14	27.19	±	1.47	N.D.			2.95	±	0.62
P1Y2 Medium	30	11.89	±	0.78	5.42	±	0.49	3.73	±	0.80
P1Y2 Medium	60	13.11	±	0.53	5.24	±	0.43	3.90	±	0.39
P1Y2 Medium	180	14.53	±	0.83	5.00	±	0.22	3.86	±	0.21
P1Y2 Medium	270	13.23	±	0.81	4.44	±	0.24	3.16	±	0.26
P1Y2 High	0	16.50	±	1.74	5.05	±	0.42	3.21	±	0.12
P1Y2 High	14	28.87	±	0.33	####	±	0.07	4.07	±	0.08
P1Y2 High	30	14.20	±	0.91	5.00	±	0.33	5.42	±	0.40
P1Y2 High	60	16.94	±	0.49	5.49	±	0.22	7.15	±	0.36
P1Y2 High	180	16.23	±	1.50	2.97	±	1.83	5.96	±	0.43
P1Y2 High	270	17.32	±	0.23	4.74	±	0.09	5.92	±	0.03

Treatment	Days	THR			ARG			TYR		
RC212 Light	0	2.11			45.52			1.53		
RC212 Light	14	3.24	±	0.19	37.60	±	2.49	1.21	±	0.03
RC212 Light	30	2.71	±	1.53	42.94	±	0.88	1.29	±	0.24
RC212 Light	60	3.22	±	0.18	39.81	±	2.30	1.18	±	0.11
RC212 Light	180	3.38	±	0.24	35.17	±	2.03	1.81	±	0.22
RC212 Light	270	5.07	±	0.39	39.30	±	2.72	1.41	±	0.14
RC212 Medium	0	2.93			47.74			1.34		
RC212 Medium	14	3.64	±	0.31	41.96	±	1.32	1.02	±	0.05
RC212 Medium	30	3.89	±	0.30	45.48	±	2.31	1.17	±	0.18
RC212 Medium	60	3.22	±	0.18	39.81	±	2.30	1.18	±	0.11
RC212 Medium	180	4.57	±	0.21	45.96	±	1.12	2.09	±	0.09
RC212 Medium	270	5.98	±	0.12	46.15	±	0.74	1.51	±	0.11
RC212 High	0	4.87	±	0.46	60.68	±	8.04	1.50	±	0.10
RC212 High	14	6.07	±	0.31	63.41	±	2.50	1.27	±	0.05
RC212 High	30	6.37	±	0.33	69.22	±	3.04	1.90	±	0.06
RC212 High	60	7.52	±	0.47	72.64	±	3.55	2.36	±	0.11
RC212 High	180	8.36	±	0.59	70.53	±	5.65	3.31	±	0.25
RC212 High	270	10.42	±	0.03	71.45	±	0.52	3.15	±	0.26
P1Y2 Light	0	5.29			65.40			2.03		
P1Y2 Light	14	5.33	±	0.63	57.76	±	2.22	1.58	±	0.27
P1Y2 Light	30	6.16	±	0.09	65.08	±	1.64	1.84	±	0.11
P1Y2 Light	60	6.05	±	0.07	62.84	±	0.59	1.98	±	0.41
P1Y2 Light	180	12.26	±	5.38	66.11	±	2.52	3.06	±	0.44
P1Y2 Light	270	7.34	±	0.12	60.21	±	0.46	1.97	±	0.09
P1Y2 Medium	0	5.19			66.93			1.83		
P1Y2 Medium	14	6.44	±	0.45	64.00	±	1.26	1.79	±	0.16
P1Y2 Medium	30	6.71	±	0.67	69.64	±	4.34	1.91	±	0.12
P1Y2 Medium	60	7.06	±	0.06	68.28	±	1.10	2.39	±	0.07
P1Y2 Medium	180	7.05	±	3.40	72.93	±	2.84	2.94	±	0.22
P1Y2 Medium	270	6.73	±	3.74	68.33	±	3.03	2.36	±	0.22
P1Y2 High	0	8.77	±	0.51	96.21	±	5.27	2.28	±	0.03
P1Y2 High	14	9.16	±	0.06	89.14	±	1.59	1.99	±	0.16
P1Y2 High	30	9.00	±	0.59	91.57	±	4.39	2.06	±	0.21
P1Y2 High	60	11.47	±	0.39	99.80	±	2.83	3.67	±	0.12
P1Y2 High	180	11.85	±	0.65	91.43	±	4.18	3.33	±	0.45
P1Y2 High	270	13.62	±	0.29	97.51	±	0.25	4.03	±	0.23

Treatment	Days	CYS			VAL			MET		
RC212 Light	0	0.44			25.48			2.46		
RC212 Light	14	0.34	±	0.06	15.64	±	1.17	1.84	±	0.21
RC212 Light	30	0.87	±	0.11	20.86	±	0.73	2.41	±	0.19
RC212 Light	60	0.64	±	0.04	18.35	±	0.90	2.28	±	0.14
RC212 Light	180	0.52	±	0.07	16.68	±	0.67	1.92	±	0.34
RC212 Light	270	0.61	±	0.04	15.89	±	1.14	2.63	±	0.19
RC212 Medium	0	0.50			24.89			1.28		
RC212 Medium	14	0.39	±	0.04	15.41	±	0.48	1.87	±	0.15
RC212 Medium	30	0.69	±	0.06	19.46	±	0.73	2.27	±	0.17
RC212 Medium	60	0.64	±	0.04	18.35	±	0.90	2.28	±	0.14
RC212 Medium	180	0.66	±	0.04	17.91	±	0.63	2.44	±	0.18
RC212 Medium	270	0.74	±	0.21	15.26	±	0.64	2.87	±	0.18
RC212 High	0	0.28	±	0.04	20.40	±	1.51	1.80	±	0.42
RC212 High	14	0.49	±	0.11	16.66	±	0.20	2.00	±	0.09
RC212 High	30	0.90	±	0.21	19.17	±	1.23	2.26	±	0.45
RC212 High	60	0.91	±	0.23	21.02	±	1.34	3.69	±	0.47
RC212 High	180	0.87	±	0.11	20.23	±	0.99	3.45	±	0.22
RC212 High	270	1.18	±	0.13	17.41	±	0.17	4.50	±	0.24
P1Y2 Light	0	0.41			34.55			2.21		
P1Y2 Light	14	0.49	±	0.04	22.73	±	0.54	2.63	±	0.14
P1Y2 Light	30	0.68	±	0.03	26.99	±	0.60	3.84	±	0.13
P1Y2 Light	60	0.63	±	0.01	26.05	±	0.19	3.83	±	0.09
P1Y2 Light	180	0.83	±	0.12	25.24	±	0.84	4.09	±	0.40
P1Y2 Light	270	0.82	±	0.02	19.95	±	0.58	3.67	±	0.13
P1Y2 Medium	0	0.48			35.59			1.48		
P1Y2 Medium	14	0.60	±	0.03	21.63	±	0.77	2.75	±	0.01
P1Y2 Medium	30	0.63	±	0.02	26.06	±	1.21	3.51	±	0.34
P1Y2 Medium	60	0.66	±	0.05	24.84	±	0.31	3.71	±	0.11
P1Y2 Medium	180	0.84	±	0.11	24.19	±	0.52	3.89	±	0.41
P1Y2 Medium	270	0.90	±	0.15	20.11	±	0.88	4.25	±	0.14
P1Y2 High	0	0.30	±	0.02	31.77	±	1.07	2.96	±	0.54
P1Y2 High	14	0.67	±	0.07	22.65	±	0.71	2.96	±	0.07
P1Y2 High	30	0.71	±	0.17	26.08	±	1.47	3.85	±	0.66
P1Y2 High	60	0.96	±	0.09	28.34	±	0.57	5.01	±	0.34
P1Y2 High	180	0.97	±	0.08	24.91	±	1.22	4.92	±	0.50
P1Y2 High	270	1.28	±	0.06	23.42	±	0.76	5.84	±	0.66

Treatment	Days	PHE			ILE			LEU		
RC212 Light	0	3.10			3.47			1.80		
RC212 Light	14	2.42	±	0.23	2.27	±	0.28	1.43	±	0.12
RC212 Light	30	2.92	±	0.17	2.47	±	0.23	1.39	±	0.09
RC212 Light	60	2.89	±	0.26	2.38	±	0.42	1.32	±	0.09
RC212 Light	180	2.35	±	0.27	1.95	±	0.27	1.11	±	0.17
RC212 Light	270	3.28	±	0.41	2.32	±	0.49	1.18	±	0.12
RC212 Medium	0	2.76			3.07			1.66		
RC212 Medium	14	2.38	±	0.19	2.33	±	0.08	1.21	±	0.07
RC212 Medium	30	2.62	±	0.05	2.52	±	0.35	1.21	±	0.20
RC212 Medium	60	2.89	±	0.26	2.38	±	0.42	1.32	±	0.09
RC212 Medium	180	3.16	±	0.18	2.92	±	0.52	1.19	±	0.06
RC212 Medium	270	3.60	±	0.11	2.87	±	0.16	1.00	±	0.09
RC212 High	0	2.11	±	0.22	3.67	±	0.28	1.30	±	0.09
RC212 High	14	2.65	±	0.08	3.57	±	0.30	1.45	±	0.04
RC212 High	30	3.66	±	0.26	4.17	±	0.62	1.25	±	0.17
RC212 High	60	6.43	±	0.43	7.84	±	0.53	1.32	±	0.13
RC212 High	180	6.48	±	0.57	7.17	±	0.79	1.30	±	0.08
RC212 High	270	8.97	±	0.47	7.90	±	0.15	1.33	±	0.10
P1Y2 Light	0	4.88			5.81			2.07		
P1Y2 Light	14	4.29	±	0.03	4.22	±	0.20	1.67	±	0.17
P1Y2 Light	30	4.61	±	0.12	4.28	±	0.58	1.75	±	0.07
P1Y2 Light	60	4.32	±	0.24	3.71	±	0.33	1.59	±	0.15
P1Y2 Light	180	5.54	±	1.22	4.78	±	1.75	1.74	±	0.44
P1Y2 Light	270	5.18	±	0.24	3.60	±	0.40	1.50	±	0.07
P1Y2 Medium	0	4.68			5.43			2.03		
P1Y2 Medium	14	4.04	±	0.24	4.65	±	0.25	1.50	±	0.09
P1Y2 Medium	30	4.29	±	0.10	3.81	±	0.39	1.65	±	0.05
P1Y2 Medium	60	5.05	±	0.30	4.78	±	0.64	1.69	±	0.38
P1Y2 Medium	180	5.93	±	0.63	5.57	±	1.01	1.49	±	0.13
P1Y2 Medium	270	6.18	±	0.63	4.86	±	0.41	1.58	±	0.13
P1Y2 High	0	4.03	±	0.14	7.41	±	0.30	1.83	±	0.27
P1Y2 High	14	4.49	±	0.26	6.20	±	0.20	1.52	±	0.16
P1Y2 High	30	4.97	±	0.50	5.88	±	1.07	1.66	±	0.20
P1Y2 High	60	8.57	±	0.41	9.20	±	0.36	1.78	±	0.13
P1Y2 High	180	8.77	±	0.56	8.92	±	0.57	1.47	±	0.17
P1Y2 High	270	11.65	±	0.16	11.33	±	0.58	1.64	±	0.11

Treatment	Days	LYS		
RC212 Light	0	6.04		
RC212 Light	14	2.57	±	0.35
RC212 Light	30	3.19	±	0.48
RC212 Light	60	2.87	±	0.19
RC212 Light	180	2.59	±	0.18
RC212 Light	270	2.42	±	0.29
RC212 Medium	0	6.04		
RC212 Medium	14	2.39	±	0.05
RC212 Medium	30	2.94	±	0.10
RC212 Medium	60	2.87	±	0.19
RC212 Medium	180	2.78	±	0.18
RC212 Medium	270	2.53	±	0.18
RC212 High	0	3.81	±	0.10
RC212 High	14	2.48	±	0.10
RC212 High	30	2.89	±	0.13
RC212 High	60	2.91	±	0.24
RC212 High	180	2.87	±	0.13
RC212 High	270	2.39	±	0.14
P1Y2 Light	0	6.37		
P1Y2 Light	14	3.01	±	0.12
P1Y2 Light	30	3.65	±	0.02
P1Y2 Light	60	3.37	±	0.12
P1Y2 Light	180	3.17	±	0.22
P1Y2 Light	270	2.79	±	0.12
P1Y2 Medium	0	6.54		
P1Y2 Medium	14	2.88	±	0.10
P1Y2 Medium	30	3.41	±	0.26
P1Y2 Medium	60	3.32	±	0.06
P1Y2 Medium	180	3.24	±	0.11
P1Y2 Medium	270	2.80	±	0.14
P1Y2 High	0	5.14	±	0.45
P1Y2 High	14	2.90	±	0.10
P1Y2 High	30	3.31	±	0.44
P1Y2 High	60	3.41	±	0.18
P1Y2 High	180	2.93	±	0.08
P1Y2 High	270	3.03	±	0.10

Appendix B: Synthetic Grape Juice Recipe

Vitamins	mg/L
Myo-inositol	100
Pyridoxine HCl	2.0
Nicotinic acid	2.0
Pantothenic acid	0.05
Thiamin HCl	0.50
p-amino Benzoic acid	0.20
Riboflavin	0.20
Folic acid	0.20

Minerals	mg/L
Magnesium chloride	0.1982
Zinc chloride	0.1355
Iron chloride	0.0502
Copper chloride	0.0136
Boric acid	0.0057
Cobalt nitrate	0.0291
Sodium molybdemate	0.0242
Potassium iodate	0.0108

Sugars, acids, and salts	g/L
Glucose	115
Fructose	115
Tartaric acid	3.32
L-malic acid	3.0
Potassium tartrate	5.0
Potassium chloride	3.2
Calcium chloride	0.44
Potassium phosphate	1.14
Magnesium sulfate	1.23

Amino acid and ammonia content

	Low YAN (mg/L)	High YAN (mg/L)	High DAP (mg/L)
Alanine	46	163	46
Arginine	419	1468	419
Aspartic acid	28	96	28
Cysteine	0	0	0
Glutamic acid	98	342	98
Glycine	2	6	2

Histidine	21	74	21
Isolueucine	15	52	15
Leucine	16	56	16
Lysine	4	15	4
Methionine	3	11	3
Phenlyalanine	9	33	9
Proline	331	937	331
Serine	31	108	31
Threonine	27	95	27
Tryptophan	12	41	12
Tyrosine	3	9	3
Valine	19	67	19
Asparagine	7	23	7
Glutamine	90	314	90
Citrulline	9	31	9
DAP	148	148	1045.9
Total YAN from amino acid	**81**	**315**	**81**
Total YAN from ammonia	**31**	**31**	**269**
Total YAN	**112.6 mg/L**	**347 mg/L**	**350 mg/L**

Appendix C: Free amino acid content (mg/L) in synthetic grape juice 60 days after inoculation

	P1Y2 Low amino acids	P1Y2 DAP	P1Y2 High amino acids	UCD522 Low amino acids	UCD522 DAP	UCD522 High amino acids
ASP	6.56	9.36	53.83	8.29	8.70	53.67
GLU	9.95	45.09	279.44	27.81	43.98	277.04
ASN	2.66	18.79	15.89	5.08	45.54	19.14
SER	7.69	13.34	39.83	9.15	12.24	40.38
GLN	3.26	9.92	43.83	7.59	23.94	52.48
CIT	4.98	6.10	31.30	17.90	4.93	40.63
HIS	18.72	28.06	30.76	20.57	41.63	34.49
GLY	3.10	2.99	14.01	3.75	3.36	15.81
THR	5.61	5.47	17.99	8.82	7.87	22.46
ARG	119.13	315.58	1284.35	116.66	488.15	1570.74
ALA	37.42	69.61	130.47	30.34	62.19	124.06
TYR	5.54	5.14	10.51	5.66	3.92	11.32
CY	0.97	1.12	0.85	1.39	0.00	0.00
VAL	14.87	15.86	49.14	27.93	25.49	66.22
MET	4.46	4.39	3.43	3.80	2.42	1.77
PHE	9.95	14.88	15.44	20.03	44.87	17.68
ILE	5.56	4.87	15.81	5.29	12.06	21.92
LEU	11.99	3.60	6.19	22.93	4.27	13.08
LYS	10.24	12.08	19.27	10.52	3.56	28.39