FINAL REPORT

I. PROJECT TITLE:

Formation of volatile sulfur compounds in Pinot noir post-fermentation. #2015-1545

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III. SUMMARY:

Development of volatile sulfur compounds (VSCs) post-fermentation can be a significant issue during both red and white winemaking. Unfortunately our understanding of 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. During the first year of the study the impact of wine lees levels and composition on formation of VSCs was determined. Although lees levels and yeast strain impacted the concentration of sulfur containing amino acids and glutathione (pre-cursors for the formation of VSCs) in the wines, this did not correlate to an increase in the formation of VSCs during the nine month duration of the experiment. Wine samples supplied by wineries who noted early formation of VSCs contained predominately H_2S and not mercaptans and disulfides. This indicated that the early formation of reductive smells soon after going to barrel were most likely due to H_2S rather than the formation of more complex volatile sulfur compounds.Based on these results the focus of the project shifted to identifying what factors may impact the late formation of H_2S during fermentation and/or the formation of H_2S post-fermentation.

Experiments in synthetic grape juice demonstrated that variations in yeast assimilable nitrogen (YAN) concentration as well as whether YAN was derived from amino acids or diammonium phosphate (DAP) impacted H_2S production during fermentation as well the concentration of otherVSCs post-fermentation. In particular, DAP supplementation increased the amount of H_2S formed late in fermentation and resulted in the highest amount of methyl thioacetate(MeSOAc) in the wines post-fermentation. Fermentations conducted using Pinot noir grapes where different concentrations of elemental sulfur (S^0) were added resulted in increased H_2S formation during alcoholic fermentation by both a high H_2S producing yeast (UCD522) and a no-H_2S producing yeast (P1Y2) demonstrating the impact of S^0 on yeast produced and non-yeast produced H_2S. H_2S production was higher in fermentations conducted by UCD522 with increasing amounts of S^0 resulting in increasing production of H_2S. In addition, higher S^0 concentrations resulted in higher H_2S production late in fermentation. This is particularly important as H_2S formation late in fermentation is more likely to be retained in the wine due to the reduced production of CO_2 by

yeast. Higher S^0 also resulted in wines containing higher concentrations of MeSOAcpost-fermentation. Both of these findings suggest an important role for elemental sulfur in the formation of VSCs during and after fermentation.Additional experiments using Pinot noir grapes showed that H_2S production during fermentation was also impacted by the type of nitrogen present. DAP additions caused an increase in H_2S formation while addition of amino acid decreased H_2S formation regardless of whether S^0 was added or not. Fermentations where DAP and S^0 were both added produced the highest amount of total H_2S with 35-45% more H_2S being produced in these ferments than produced in ferments where S^0 or DAP additions were made individually. Post-fermentation, YAN concentration and composition as well as S^0 impacted the concentration of VSCs other than H_2S. In particular, S^0 and high YAN increased the concentration of MeSOAc in the wines post-fermentation. The type of nitrogen added (ammonium vs. amino acid) had less of an impact on MeSOAc concentration then the increase in YAN. This may be related to the mechanism by which MeSOAC is formed during fermentation as well as the formation of methanethiol. These results indicate that the type and amount of nitrogen present during fermentation can impact formation of VSCs as can the presence of S^0. In particular, DAP additions led to increased H_2S formation during fermentation while high YAN resulted in elevated MeSOAC in the wines post-fermentation.

Because of the known role of elemental sulfur in the formation of VSCs, the concentration of this compound on Willamette Valley grapes at harvest was assessed as were techniques to reduce the amount of S^0 on red grapes pre-fermentation. For the two years that S^0 content on grapes was measured only low levels were noted. Only three out of the forty one samples analyzed contained S^0 concentrations greater than 1 ug/g. This may have been related to the warm, dry vintages in which these samples were collected from (2014 & 2015). Experiments conducted to explore ways to reduce the amount of S^0on red grapes prior to fermentation showed that cold soaking of Pinot noir grapes followed by draining off the juice reduced the amount of S^0on the remaining grapes. Cold settling the juice followed by racking and addition of the racked juice back to the original grapes allowed minimal loss of volume.

IV. OBJECTIVES AND EXPERIMENTS CONDUCTED TO MEET STATED OBJECTIVES:

Objective 1.Determine the effect of wine lees level and contact time on formation of volatile sulfur compounds and volatile sulfur compound pre-cursors during aging of Pinot noir wine.

Initial experiments have been undertaken where Pinot noir wines were be produced using grapes from the Oregon State University vineyard. Grapes were harvested and stored overnight at 4 °C before being destemmed and dispensed into 100 L stainless steel tanks. Samples were taken and assessed for pH, TA, °Brix and yeast assimilable nitrogen. Additional grape samples were frozen and will be assessed for amino acid and glutathione content by HPLC according to Lee and Schriener (2010) and Park et al. (2000). The grape must underwent a 5 day cold soak (8-9 °C) before being warmed and inoculated with one of two commercial yeast cultures. To one set of tanks (3 tanks) the low/no H_2S producing yeast strain *Saccharomyces cerevisiae* P1Y2 was added while to another set of tanks the yeast *Saccharomyces cerevisiae*RC212 was added.

Fermentations were undertaken at 27 °C and °Brix and temperature were monitored. At the completion of alcoholic fermentation wines were pressed and split into three different lees treatments based on settling time (0, 24, and 72 hrs settling at 4 °C). Wine turbidity was measured by a turbidity meter to help quantify the effectiveness of settling to remove lees. Initial samples were taken and assessed for volatile sulfur compounds by HS-SPME-GC-PFPD as described by Fang and Qian (2005). Separate samples were collected and frozen for later analysis of glutathione and amino acids by HPLC. After appropriate settling the wines were dispensed into three gallon carboys fully topped and closed with an airlock and 50 mg/L SO_2 was added before being stored at 13°C. Large stir bars were placed in the carboys to allow stirring to occur prior to sampling while minimizing exposure of the wine to air. These wines were stored at 13°C and sampled after 1, 2, 3, 6, and 9 months. Samples were assessed by the Osborne lab for amino acid content by HPLC according to Lee and Schriener (2010). Wine samples were also analyzed for volatile sulfur compounds by the Dr. Michael Qian's lab as part of a separately funded project. Volatile sulfur compounds were assessed by HS-SPME-GC-PFPD.

Objective 2.Investigate the impact of grape amino acid composition and concentration on formation of volatile sulfur compounds and volatile sulfur compound pre-cursors during aging.

Volatile sulfur compounds in winery samples
Wine samples were collected from collaborating wineries who noted volatile sulfur formation in wines soon after wine was placed in barrel. Wineries were instructed to take wine samples after pressing and after 2 and 4 weeks in barrel. Samples were assessed for volatile sulfur compounds by HS-SPME-GC-PFPD.

Role of nitrogen concentration and composition
A synthetic grape juice was utilized where concentration and composition of the nitrogen in the juice could be tightly controlled. Synthetic grape juice composition was based on Osborne and Edwards (2006). However, the amino acid content of the juice was modified to better reflect the amino acid content of Oregon Pinot noir grapes as reported by Lee and Schriener (2010). The yeast available 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 (Table 5). 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. The synthetic juice was sterile filtered and 2.5 L dispensed into sterilized 4 L Erlenmeyer flasks. Two different yeast strains were used for the fermentations. In one set of juices (high amino acids, low amino acids, high DAP) the non-H_2S producing yeast strain *S. cerevisiae* P1Y2 was inoculated while in another set of juices *S. cerevisiae* UCD522 was inoculated. UCD522 is reported to produce high amounts of H_2S during fermentation (Spiropoulos et al. 2000; Edwards and Bohlscheid 2007). All treatments were prepared in triplicate. After inoculation the flasks were stoppered with a modified fermentation

lock where fermentation gases were forced to pass through an H_2S detection tube (Gastec 4H tubes). These tubes can be used to quantify H_2S gas by the reaction between lead acetate and H_2S which causes a color change in the tube. 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). Tubes were regularly changed out during the course of the alcoholic fermentation in order to determine the production of H_2S over time. Fermentations were conducted at 21°C in a temperature controlled room. °Brix was monitored daily using an Anton-Paar DMA 35N Density Meter while samples were also taken daily and plated on YPD media to determine yeast viable cells. At the completion of alcoholic fermentation samples were taken for amino acid and volatile sulfur compound analysis. Wines were then transferred to smaller sterilized Erlenmeyer flasks so as to remove headspace. Wines were stored at 13°C, sampled after 1 and 3 months, and analyzed for volatile sulfur compounds by by HS-SPME-GC-PFPD.Amino acids were assessed by HPLC-DAD.

Role of elemental sulfur

Pinot noir wines were produced using grapes from the Oregon State University vineyard (Woodhall Vineyard, Alpine, OR). After destemming, three kilograms of grapes were placed in four liter red wine microfermenters as described by Takush and Osborne (2011). 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. All fruit received an addition of 0.2 g/L Fermaid K as well as an addition of 0.25 g/L DAP to raise the YAN content to approximately 150 mg/L. Elemental sulfur 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^0content of the Pinot noir grapes was measured utilizing the method outlined by Kwasniewski et al (2011) and no S^0 was detected on the grapes. Two different yeast strains were used for the fermentations. 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. After inoculation the fermenters were placed in a temperature controlled room held at 27°C.°Brix was monitored daily using a density meter and H_2S detection tubes were regularly changed out during the course of the alcoholic fermentation in order to determine the production of H_2S over time. At the completion of alcoholic fermentation the wines were pressed and settled. Fourteen days post-pressing samples were taken for amino acid and volatile sulfur compound analysis. Wines were then transferred to 2 L carboys and topped fully to prevent any headspace. Wines were stored at 13°C, sampled after 1 and 3 months, and analyzed for volatile sulfur compounds by HS-SPME-GC-PFPD and amino acids were assessed by HPLC-DAD. Glutathione was assessed by LC-MS/MS according to Roland and Schneider (2015). Glutathione analysis was performed in collaboration with the Oxidative/Nitrative Stress Core Laboratory (ONSL) facility housed in the Linus Pauling Institute at Oregon State University.

Combined impact of nitrogen composition and concentration, and elemental sulfur

A set of experiments investigating the impact of S^0, yeast strain, and concentration and composition of YAN on H_2S and VSC formation during and after fermentation was conducted using Pinot noir grapes harvested in September 2015 (Woodhall Vineyard, Alpine, OR). After

destemming, one kg of grapes was placed in four liter red wine microfermenters as described by Takush and Osborne (2011). 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 24.8°Brix, pH 3.42, 0.64 g/100 mL titratable acid and 110 mg/L YAN (78 mg N/L α-amino, 32 mg N/L NH_4^+). The following treatments were conducted in triplicate and fermented using either *S. cerevisiae* UCD 522 (high H_2S producing strain) or P1Y2 (no H_2S producing strain):

1) UCD522 Control
2) UCD522 + 10 ug/g S^0
3) UCD522 + DAP to achieve 250 mg N/L YAN
4) UCD522+ DAP + 10 ug/g S^0
5) UCD522 + amino acid mixture to achieve 250 mg N/L YAN
6) UCD522 + amino acid mixture + 10 ug/g S^0
7) P1Y2 Control
8) P1Y2 + 10 ug/g S^0
9) P1Y2 + DAP + 10 ug/g S^0

The amino acid mixture added to treatments 5 & 6 was based on the concentration of the individual amino acids in Pinot noir grapes (Lee and Schriener 2010). After inoculation fermenters were placed in a temperature controlled room held at 27°C.°Brix was monitored daily using a density meter (DMA) and Gastec 4H H_2S detection tubes were regularly changed out during the course of the alcoholic fermentation in order to determine production of H_2S over time. At the completion of alcoholic fermentation wines were pressed and settled. Samples were taken for VSC analysis by HS-SPME-GC-PFPD as described by Fang and Qian (2005).

Elemental sulfur on Willamette Valley grapes at harvest

The role of S^0 in the formation of VSCs in Oregon wines was investigated by the Osborne lab through the assessment of residual S^0 content of grapes at harvest. Grape samples from wineries in the Willamette Valley were solicited with wineries instructed to take a 2-3 cluster sample from vineyard lots that traditionally had problems with reduction during winemaking as well as any lots where later season sulfur sprays were applied. Samples were frozen at the wineries before being collected and transported to the Osborne lab at OSU. Residual S^0 content of the grapes was measured according to Kwasniewski et al (2011). In brief, grape samples were blended, heated in PEG 400 (to aid in solubilizing S^0), diluted with water, and de-aerated and pH adjusted to pH 6 by the addition of an antacid tablet (Alka-Seltzer). Following de-aeration, a reducing agent (dithiothreitol) was added to reduce S^0 to H_2S. The H_2S was sparged through a Gastec 4M H_2S detection tube via addition of two additional antacid tablets. H_2S concentration was determined by relating the distance of color change on the detection tube to that observed for calibration standards.

Methods to reduce the amount of S^0 on red grapes prior to fermentation were explored. While Kwasniewski et al. (2014) demonstrated that cold settling of white grape juice could reduce elemental sulfur levels by up to 95%; no method to reduce elemental sulfur content on red grapes has been described. Wettable or microthiol sulfur was applied at 5 lbs/acre to Pinot noir grapes at Woodhall vineyard (Alpine, OR) six days prior to harvest. Sulfur was applied using a hand

sprayer and a sticker (R56) was used. After harvest (by hand) grapes were destemmed and placed in 100 L stainless steel tanks. Grapes were split into two tanks per sulfur treatment (wettable and microthiol). Samples were taken prior to destemming and assessed for elemental sulfur content as previously described. Once grapes were placed in the tanks they were mixed and an inert gas (N_2) was blanketed on top before lids were securely placed onto the tanks. Each tank contained approximately 35 kg of fruit. Tanks were placed in a cold room set to 8°C and allowed to cold soak for seven days. After seven days the juice in each tank was drained from the bottom valve into a carboy. The S^0 content of the juice as well as the grapes left in the tanks was measured as previously described. For each treatment, 2 L of the drained off juice was placed in a cold room at 4°C for 24hrs. After this time the juice was racked off of the lees. The S^0 content of the racked juice as well as the lees was assessed.

One kg fermentations of each treatment were performed to determine the impact on H_2S production. The following treatments were conducted in triplicate using grapes treated with either wettable or microthiol sulfur:

1) Control – no removal of juice
2) Saignee grapes (juice removed after seven days cold soak)
3) Saignee grapes + cold settled juice (reconstituted to initial grape/juice ratio)

All treatments were fermented using the no-H_2S producing *S. cerevisiae* strain P1Y2 so that any H_2S produced could be attributed to S^0. After inoculation the fermenters were placed in a temperature controlled room held at 27°C.°Brix was monitored daily using a density meter and Gastec 4H H_2S detection tubes were regularly changed out during the course of the alcoholic fermentation in order to determine the production of H_2S over time. At the completion of alcoholic fermentation the wines were pressed and settled. Samples were taken for VSC analysis by HS-SPME-GC-PFPD as described by Fang and Qian (2005). Wines were assessed for free and bound H_2S using the TCEP method as previously described.

Investigating "bound" or "releasable" H_2S

It has been proposed that H_2S at the end of fermentation can be present bound to as yet unknown compound(s). This form of H_2S is no longer volatile and so will not be detected by smell. However, under reductive conditions (such as those present in a barrel or bottle) H_2S may be reduced back to its free form and become detectable by smell again. In order to test this hypothesis, Pinot noir wine previously produced from experiments investigating the impact of S^0 on H_2S production were assessed for free and "bound" H_2S. These wines were used as they represented wines where a range of H_2S was produced during fermentation. Free and "bound" H_2S was measured using a method based on the analysis of S^0 on grapes (Kwasniewski et al 2011). In brief, 20 mLs of wine was placed in a 250 mL Erlenmeyer flask along with 60 mL DI water. An antacid tablet (Alka-Seltzer) was added and any free H_2S sparged from the wine was measured by passing the gas through a Gastec 4LT H_2S detection tube. H_2S concentrations were determined by relating the distance of color change on the detection tube to that observed for calibration standards (Ugliano and Henschke 2010). To measure "bound" H_2S a strong reducing agent, *tris*-2-carboxyethyl phosphine (TCEP), was added to the sample. After 5 minutes an antacid tablet was added and any released H_2S was measured by passing the gas through a 4LT H_2S detection tube. A second antacid tablet was added after 2 minutes. TCEP was used as a

reducing agent rather than dithiothreitol (as used for S^0 analysis) as using dithiothreitol in wine can result in false positives due to reactions with reduced and oxidized glutathione present in wine (Rothwarf and Scheraga 1992).

V.SUMMARY OF MAJOR RESEARCH ACCOMPLISHMENTS AND RESULTS:

After processing, Pinot grapes were distributed into tanks and samples were taken and assessed for basic chemical parameters (Table 1). Wines fermented by RC212 had lower ethanol and free amino acid concentrations than wines fermented by P1Y2 and all wines have low residual ammonia (Table 2). Wines were prepared with different levels of lees by settling for various times. This resulted in wines with different turbidities (Table 3) where unsettled wine had very high turbidity while settling for 24rs resulted in a large reduction in turbidity. Settling wine for an additional 48 hrs did not result in a large difference in turbidity.

Table 1.2013 Pinot noir grape chemistry. n=6

°Brix	Titratable acidity (g/100 ml)	pH	Ammonia (mg/L)	Free amino acids (mg/L)
23.3 ± 0.5	0.56 ± 0.02	3.51 ± 0.21	28.2 ± 2.3	122.7 ± 5.2

Table 2. Basic chemistry of Pinot noir wines produced by two different *S. cerevisiae* commercial yeast. n=3

	Titratable acidity (g/100 ml)	pH	Ammonia (mg/L)	Free amino acids (mg/L)	Ethanol (% v/v)
RC212	0.67 ± 0.04	3.66 ± 0.01	5.8 ± 0.4	50.4 ± 1.9	12.6 ± 0.2
P1Y2	0.64 ± 0.01	3.70 ± 0.02	5.7 ± 0.6	61.7 ± 3.3	13.2 ± 0.1

Table 3. 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	72 hrs settling
RC212	> 10,0000	800	600
P1Y2	> 10,0000	800	600

Table 4. Concentrations of ammonia and free amino nitrogen in Pinot noir wines aged three months on heavy, medium, or light lees after fermentation by *S. cerevisiae* RC212 or *S. cerevisiae* P1Y2. n=3

	Ammonia (mg/L)	Free amino nitrogen (mg/L)
RC212 Heavy lees	1.5 ± 0.7	61.6 ± 2.6
RC212 Medium lees	0.9 ± 0.2	49.5 ± 0.7
RC212 Light lees	2.6 ± 0.8	48.4 ± 0.6
P1Y2 Heavy lees	0.4 ± 0.4	67.9 ± 0.8
P1Y2 Medium lees	0.8 ± 0.8	56.4 ± 0.7
P1Y2 Light lees	2.5 ± 0.3	52.7 ± 0.7

Initial concentrations of free amino nitrogen after pressing were different between the yeast strains with wines fermented by P1Y2 having almost 62 mg/L while RC212 fermented wines contained 50.4 mg/L FAN (Table 2). Considering that the initial grape FAN content was 122.7 mg/L, the amount of FAN still remaining in the wine after fermentation is quite high. After 3 months of aging on the lees the concentrations of FAN had increased in all wines with the concentration being the highest in the heavy lees treatments not matter what yeast was used (Table 4.). This is likely due to yeast autolysis and release of amino acids into the wine. There was very little ammonia remaining in the wine at this point. Settling the wines after pressing reduced the amount of FAN present in the wines during aging although the concentrations present are still > 50 mg/L. Settling for longer than 24hrs did not make a significant difference to the amount of FAN or YAN in the yeast lees. Data from this study suggests that a 24 hr settling time was sufficient to significantly reduce turbidity (and overall lees content) as well as decrease the concentration of FAN present.

Amino acid content of the wines was assessed after 14, 30, 60, 180, and 270 days. While the concentrations of all amino acids were assessed, only the concentrations of the sulfur containing amino acids, cysteine and methionine, will be discussed in this report. Aside from an initial decrease in methionine and cysteine concentrations after fermentation (Figures 1 and 2), concentrations of methionine and cysteine generally increased as storage time increased. Differences between lees levels and yeast strains were also noted. For both yeast strains, increasing lees levels resulted in higher amounts of methionine and cysteine being present in the wine. In addition, methionine and cysteine were higher in wines fermented by yeast strain P1Y2 than in wines fermented by RC212 at each lees level. After nine months 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). Sulfur containing amino acids can be a source of volatile sulfur compounds such as methanethiol, ethanethiol, dimethyldisulfide, and dimethyltrisulfide.

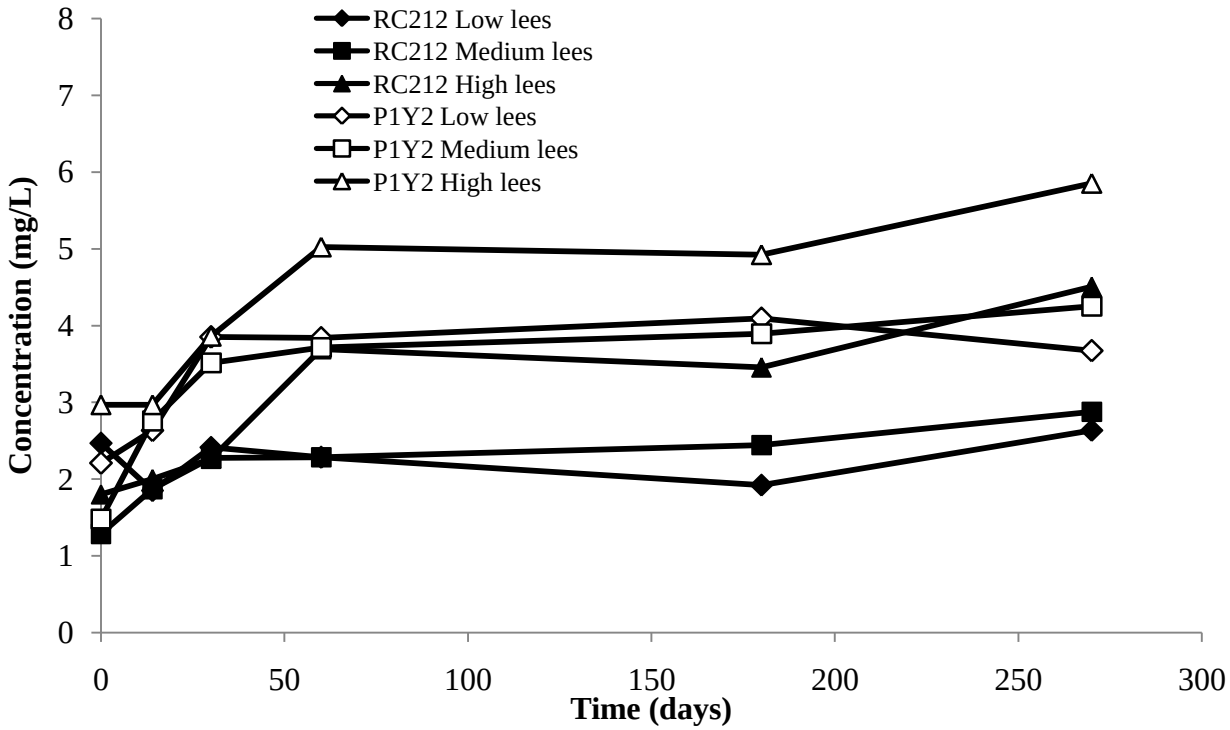

Figure 1. Concentration of methionine in Pinot noir wines fermented by *S. cerevisiae* strain RC212 (closed symbols) or strain P1Y2 (open symbols) and aged on light, medium, or heavy lees.

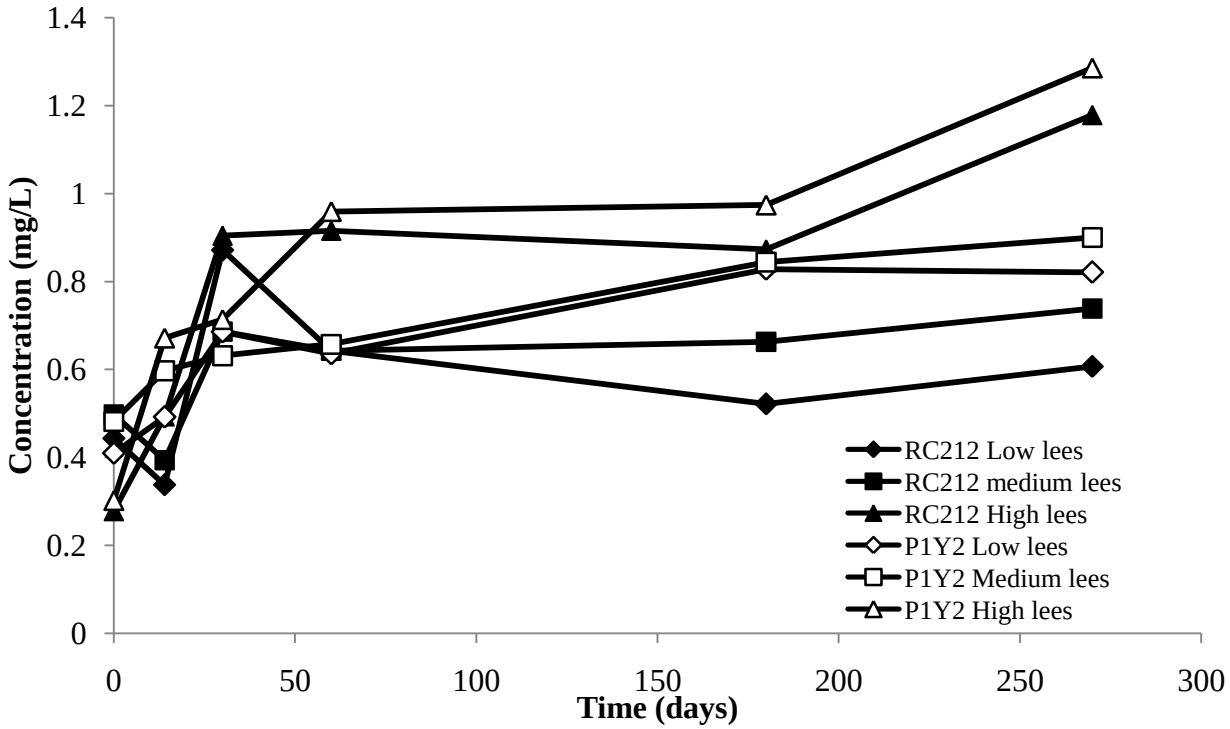

Figure 2. Concentration of cysteine in Pinot noir wines fermented by *S. cerevisiae* strain RC212 (closed symbols) or strain P1Y2 (open symbols) and aged on light, medium, or heavy lees.

In addition to the concentration of sulfur containing amino acids, the concentration of the sulfur containing tri-peptide, glutathione, was measured in wines aged with different levels of lees. While glutathione can represent a large pool of sulfur containing compounds in wine post-fermentation (Marchand and de Revel 2010), its' concentration in red wines and the impact of grape nitrogen content and lees composition is relatively unknown. The concentration of glutathione (GSH) in Pinot noir aged on heavy or light lees is shown in Figure 3. Overall, the GSH levels were low compared to previously published work (Roland and Schneider 2015; Marchand& de Revel 2010). After 0 days aging the GSH levels were very similar between wines fermented by the two different yeast and at the two lees levels. However, after 30 days aging there was a higher concentration of GSH in wines aged on the heavy lees with the highest levels being measured in wines fermented by RC212 (Figure 3). The concentration of the oxidized form of glutathione (GSSG) was also assessed. Glutathione is easily oxidized and so often the oxidized form is more prevalent in wine. The results of this analysis are shown in Figure 4. GSSG concentrations were significantly lower than corresponding GSH levels in the wines. At day 0 of aging, wines fermented by RC212 contained higher concentrations of GSSG than wines fermented by P1Y2. As seen with GSH levels, aging the wine on heavy lees resulted in elevated GSSG levels. However, unlike the GSH levels GSSG levels only increased in wines fermented by RC212 (Figure 4).

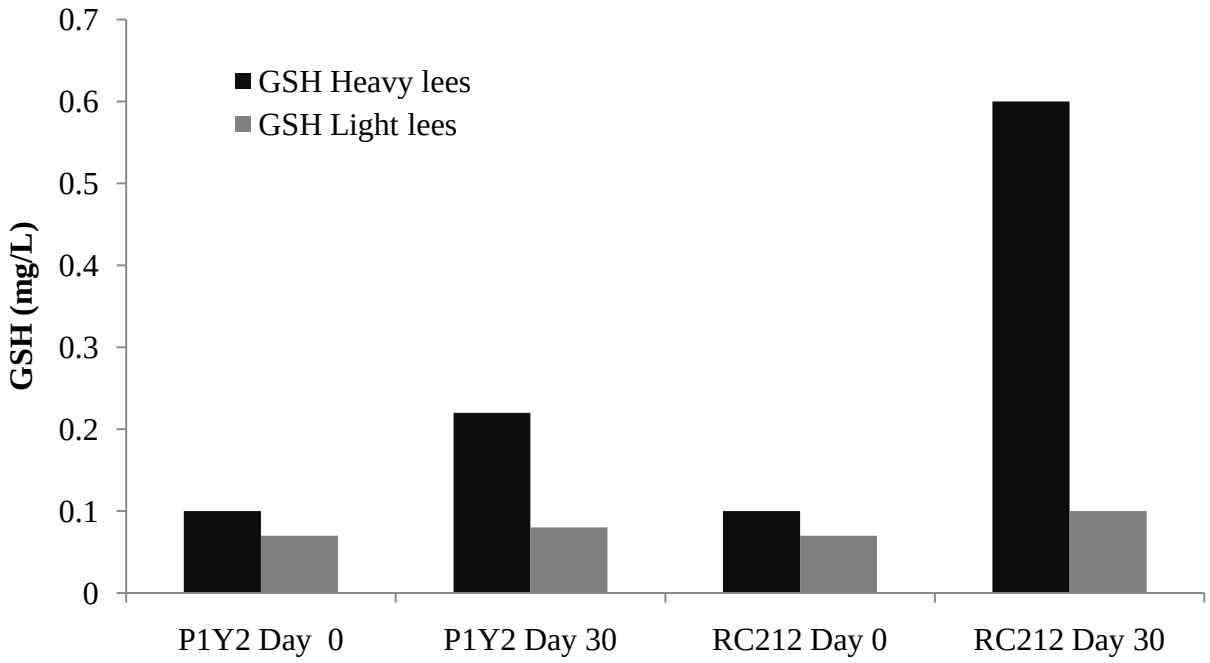

Figure 3. Concentration (mg/L) of glutatione (GSH) in Pinot noir wines fermented by *S. cerevisiae* P1Y2 or RC212 aged on light or heavy lees for 0 or 30 days.

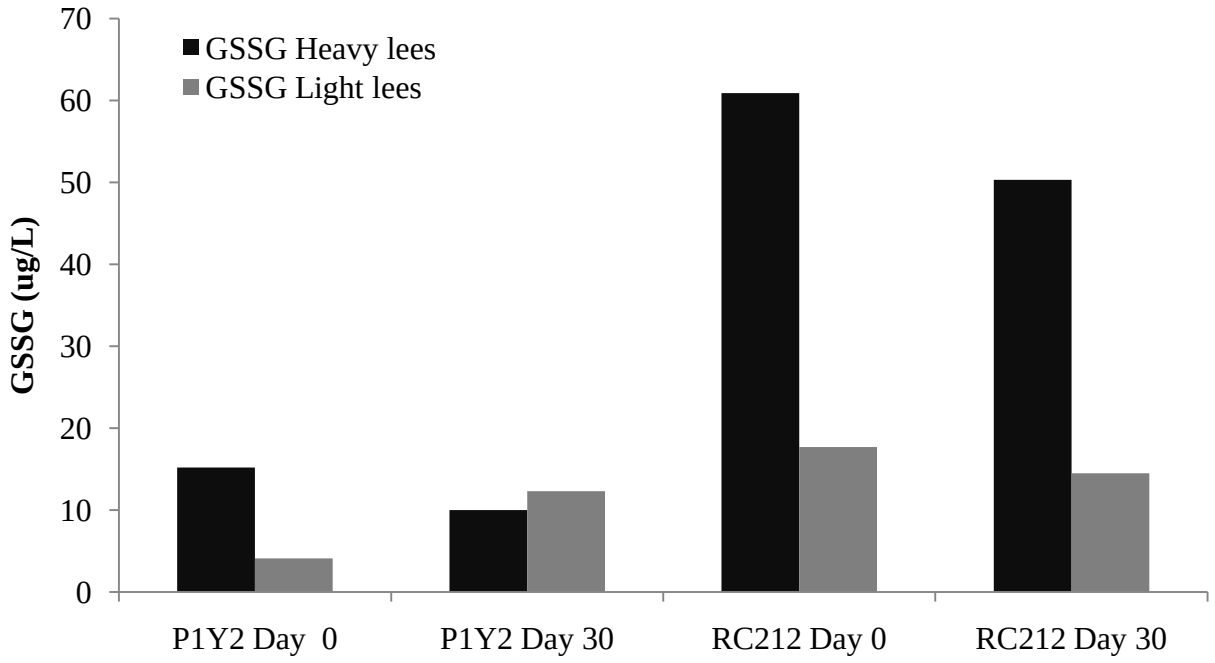

Figure 4. Concentration (ug/L) of oxidized glutatione (GSSG) in Pinot noir wines fermented by *S. cerevisiae* P1Y2 or RC212 aged on light or heavy lees for 0 or 30 days.

While yeast strain and lees levels impact the concentration of sulfur containing amino acids and glutathione, the increase in these compounds did not alter the formation of volatile sulfur compounds significantly during aging. H_2S was present in all wines after pressing with the highest amount in wines produced by RC212 (Table 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 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 5). While concentrations of methanethiol (MeSH) (Table 6) and dimethyl sulfide (DMS) (Table 7) were very similar between all the wine treatments, larger differences were noted for methyl thioacetate (MeSOAc) (Table 8). MeSOAc concentrations were significantly higher in wines produced by RC212 than in wines produced by P1Y2 (Table 8). These differences were present at every time point independent of the lees level. This trend was also apparent for methionol (Table 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 9). Based on this data it is likely that other factorsbesidesjust the presence of sulfur containing amino acids drive the formation of volatile sulfur compounds in wine during aging. In support, 2013 wine samples collected from wineries contained predominately H_2S when sampled early during barrel aging rather than more complex sulfur compounds typically derived from methionine and cysteine (Table 10).

Based on results from the first years studies the focus of the project shifted towards H_2S production during alcoholic fermentation with an emphasis on late formation of H_2S that may

lead to high H_2S in wines going to barrel. Initial experiments explored the role of nitrogen concentration and composition on formation of volatile sulfur compounds using a synthetic grape juice. Synthetic grape juices with varying YAN concentrations and compositions were inoculated with either a high (UCD522) or low (P1Y2) H_2S producing yeast strain. Fermentations proceeded well with yeast populations reaching at least 1 x 10^8cfu/mL (Figure 5). During the course of the fermentation H_2S production was measured using H_2S detection tubes. Total H_2S produced during fermentation is shown in Figure 6. No H_2S was produced by yeast strain P1Y2 during fermentation in any of the treatments and so Figure 6 illustrates production by yeast strain UCD522. The highest production of H_2S occurred in fermentations of synthetic juice containing high amino acids (346 mg/LYAN) 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. The timing of H_2S production was also followed. While the majority of the H_2S was produced during early to mid-fermentation, some late production of H_2S was observed. The highest amount of late H_2S formation occurred in fermentations of high DAP juice (Figure 6). This is important because while H_2S produced early in fermentation is typically 'blown off' by CO_2, late formation of H_2S when CO_2 production is minimal may result in higher levels of H_2S in the wine post-fermentation. However, when the wines were assessed for volatile sulfur compounds post-fermentation there was very little H_2S present in the wines. This lack of correlation between H_2S produced during fermentation and H_2S in the wines post-fermentation has been reported by others (Ugliano et al. 2009) and is usually attributed to the low solubility and high volatility of H_2S. In contrast, the concentrations of other volatile sulfur compounds did correlate to H_2S production during fermentation. For example, wines produced from the high DAP juice where

Table 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 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 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 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 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

Table 10. Concentration of volatile sulfur compounds (μg/L) in Pinot noir wine barrel samples from commercial wineries in the Willamette Valley.

Winery Code	H2S	MeSH	CS2	DMS	DES	MeSOAc	DMDS	EtSOAc	DEDS	DMTS	Methionol
117	27.03	2.62	0.04	2.49	N.D.	13.82	0.05	0.82	N.D.	0.04	7.66
643	24.14	1.94	0.04	1.45	N.D.	12.30	0.04	0.55	N.D.	0.08	4.19
463	23.24	2.49	0.27	2.49	N.D.	10.43	0.09	0.49	N.D.	0.14	4.53
629	18.61	2.30	0.04	2.07	N.D.	8.85	0.04	0.58	N.D.	0.10	3.33
439	26.83	1.90	0.04	2.34	N.D.	17.39	0.00	0.93	N.D.	0.06	4.05
751	9.45	1.91	0.22	4.17	N.D.	8.84	0.03	0.43	N.D.	0.04	3.25
108	57.43	3.26	0.28	3.94	N.D.	15.80	0.05	1.29	N.D.	0.11	3.55
336	96.22	4.03	0.31	4.21	N.D.	20.29	0.18	1.17	N.D.	0.27	4.08
117	8.55	3.49	0.19	3.58	N.D.	16.92	0.37	1.18	N.D.	1.14	1.65

N.D = non-detectable

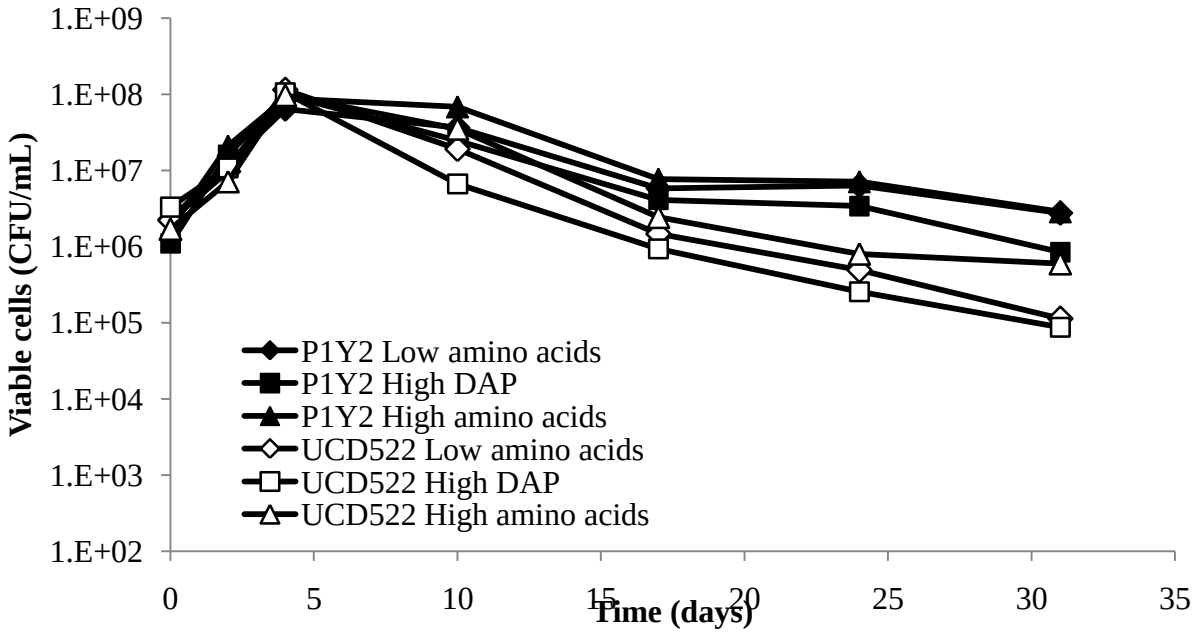

Figure 5. Growth of *S.cerevisiae* strain P1Y2 (open symbols) or UCD522 (closed symbols) in synthetic grape juice containing different concentrations of amino acids and/or diammonium phosphate (DAP).

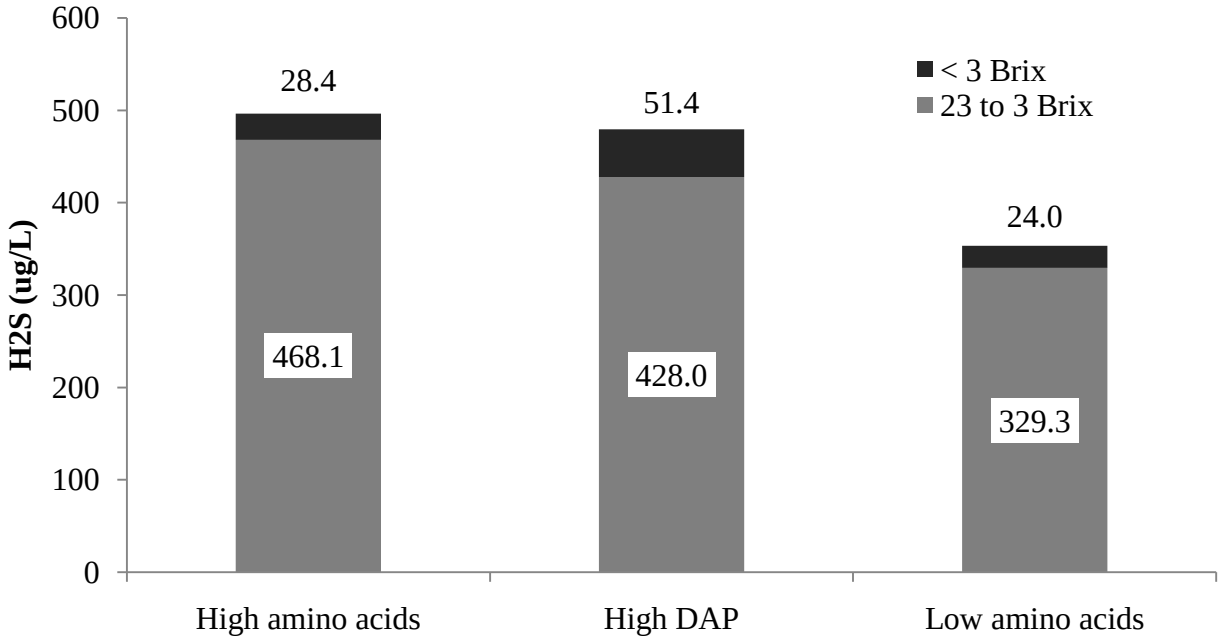

Figure 6. Total H_2S production by *S. cerevisiae* strain UCD522 during fermentation of synthetic grape juice containing different concentrations of amino acids and/or diammonium phosphate (DAP).

Table 11. Concentraion 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.

Volatile Sulfur compounds	H_2S	MeSH	CS_2	MeSOAc	DMDS	EtSOAc	DMTS
***Saccharomyces cerevisiae* P1Y2**							
P1Y2 Low	ND	5.2±0.0	2.7±1.3	ND	0.4±0.1	ND	0.06±0.02
P1Y 2 High	ND	ND	3.2±0.7	ND	1.3±0.2	ND	0.08±0.01
P1Y2 DAP	ND	2.5±0.0	3.4±0.8	ND	0.7±0.3	ND	0.09±0.00
***Saccharomyces cerevisiae* UCD522**							
UCD522 Low	ND	4.6±0.8	5.2±1.2	9.8±1.7	2.8±0.3	4.6±0.4	0.6±0.3
UCD522 High	3.1	4.4±0.7	5.6±1.3	8.2±0.7	1.5±0.5	3.2±0.1	0.3±0.1
UCD522 DAP	12.1	6.4 ±0.6	3.3±1.0	11.4±0.3	2.1±1.3	5.3±1.0	0.4±0.3

Low = Low amino acids (110 mg/L YAN)
High = High amino acids (350 mg/L YAN)
DAP = Low amino acids supplemented with DAP (350 mg/L YAN)
ND: Below the detection limit

late formation of H_2S was observed contained the highest levels of methyl thioacetate and methanthiol(Table 11). Low concentrations of volatile sulfur compounds were present in wines fermented by P1Y2 while in wines.

The concentration of amino acids in wines produced from the synthetic grape juice study were assessed by HPLC-DAD. Concentrations of all the amino acids are shown in Table 12. The concentration and type of nitrogen in the starting juice impacted the concentration of various amino acids in the resulting wines. In general wines produced from juice containing high amino acids contained the highest levels of residual amino acids. For example, wine produced by UCD522 from high amino acid juice contained 875 mg/L of arginine while wine produced from low amino acid juice only contained 116.8 mg/L. Interestingly, the low amino acid juice that had been supplemented with DAP so as to match the YAN value of the high amino acid juice also contained lower concentrations of most amino acids (Table 12). The concentrations of the sulfur containing amino acids are shown in Figure 7. Methionine concentrations were higher in wines made from the low amino acid juice for both P1Y2 and UCD522. Cysteine concentrations were similar in all wines fermented by P1Y2 no matter what the type and concentration of nitrogen in the juice had been. In contrast, in wines fermented by UCD522 only wines made from low amino acids juice contained any cysteine. Wines made from high amino acid juice or DAP supplemented juice contained no detectable cysteine. The significance of this at this point is unknown although it should be noted that wines produced from high amino acid or DAP supplemented juices produced higher concentrations of total H_2S during fermentation as well as H_2S late in fermentation (Figure 6) when cysteine utilization by yeast may lead to H_2S production (Rauhut, 1993).

Table 12. Concentrations (mg/L) of amino acids in wine made from synthetic grape juice containing low amino acids, high amino acids, or low amino acids and diammonium phosphate (DAP). Ferments were conducted by *S. cerevisiae* strain P1Y2 or strain UCD522.

	P1Y2 Low aa	P1Y2 DAP	P1Y2 High aa	UCD522 Low aa	UCD522 DAP	UCD522 High aa
ASP	6.62	9.44	54.29	8.36	8.77	54.12
GLU	12.19	55.26	342.47	34.08	53.90	339.54
ASN	2.64	18.67	15.78	5.05	45.23	19.01
SER	7.69	13.35	39.87	9.16	12.25	40.41
GLN	3.26	9.93	43.88	7.60	23.96	52.53
CIT	4.98	6.11	31.33	17.92	4.93	40.68
HIS	18.73	28.09	30.79	20.59	41.67	34.52
GLY	3.10	3.00	14.02	3.75	3.36	15.82
THR	5.62	5.48	18.01	8.83	7.88	22.48
ARG	119.27	315.94	588.97	116.79	488.71	875.70
ALA	37.46	69.68	130.61	30.37	62.26	124.19
TYR	5.55	5.14	10.53	5.67	3.93	11.34
CY	0.97	1.12	0.85	1.40	0.00	0.00
VAL	14.89	15.88	49.20	27.97	25.53	66.30
MET	4.46	4.39	3.44	3.81	2.43	1.78
TRP	12.31	18.42	19.12	24.79	55.54	21.88
PHE	7.01	6.14	19.94	6.67	15.21	27.64
ILE	12.01	3.61	6.20	22.96	4.28	13.10
LEU	9.20	10.85	17.32	9.45	3.20	25.51
LYS	9.46	6.52	8.56	6.41	10.91	3.39

Glutatione and oxidized glutathione concentrations were also assessed in the synthetic wines produced by different yeast from synthetic juice containing different nitrogen concentrations and composition. The results are shown in Figures 8 and 9. Interestingly there was higher GSH in the synthetic wines than previously measured in the Pinot noir wines (Figure3). Considering that a portion of the GSH in the Pinot noir wines would have been derived from GSH in the grapes while the GSH in the synthetic wines is exclusively derived from the yeast this was surprising. Unlike in the Pinot noir wines, yeast strain had an impact on GSH concentration as wines fermented with UCD522 had higher GSH concentrations than wines fermented by P1Y2. The concentration and type of nitrogen had less of an impact on GSG (Figure 8) then it did on GSSG levels (Figure 9).

In addition to nitrogen content, elemental sulfur was also investigated as a cause of the formation of volatile sulfur compounds post-fermentation. An addition of elemental sulfur was made to Pinot noir grapes which were then fermented by either a high (UCD522) or low (P1Y2) H_2S producing yeast strain. The amount of elemental sulfur added (either 5 or 15 μg/g) was based on a recent publication (Kwasniewski et al. 2014) that reported levels of residual sulfur on grapes in the Finger Lakes area (NY) ranged from 0 – 40 μg /g depending on harvest year and date of last spray application. These are significantly higher residual elemental sulfur levels than those previously reported by studies conducted in California (Thomas et al. 1993a). However, given the climate and vineyard disease pressures in the Finger Lakes area, the higher residual elemental sulfur levels may better reflect potential levels on Oregon grapes.

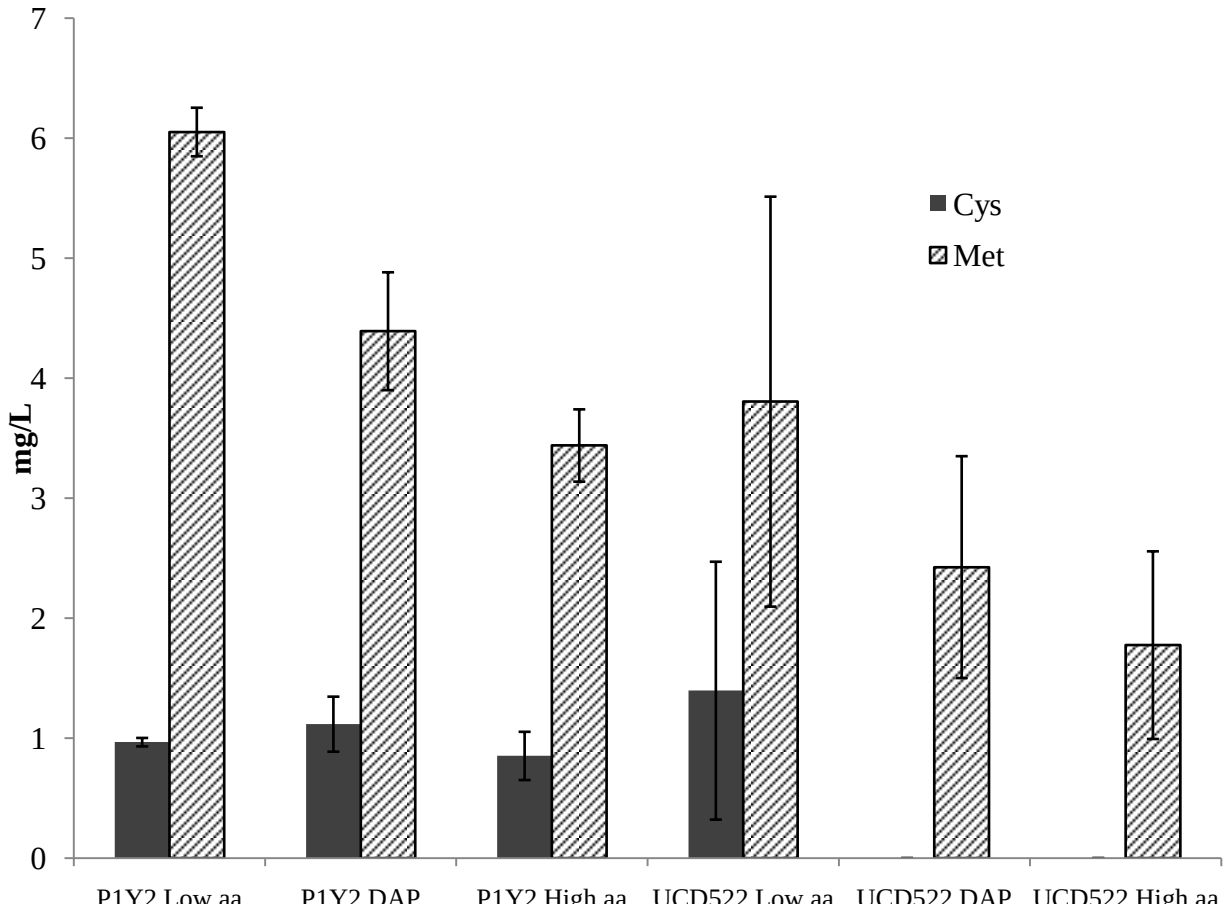

Figure 7. Concentration of cysteine and methionine in wine made from synthetic grape juice containing low amino acids, high amino acids, or low amino acids and diammonium phosphate (DAP). Ferments were conducted by *S. cerevisiae* strain P1Y2 or strain UCD522.

All fermentations were complete in less than seven days. Fermentations conducted by UCD522 that contained the highest concentration of elemental S^0 took the longest time to complete (Figure 10). H_2S production peaked during the first 48-72 hrs of fermentation with the highest rate of production occurring in fermentations containing 15μg/g S^0conducted by UCD522 (Figure 11). As expected, UCD522 produced H_2S at a higher rate during fermentation than the no-H_2S producing strain P1Y2. However, H_2S was still produced in fermentations conducted by P1Y2 when S^0 was added to the fermentations. Total H_2S produced during the fermentations is shown in Figure 12. No H_2S was measured in fermentations conducted by P1Y2 when no S^0 was added and only a low level was measured (<35 μg/L) when 5 μg/g S^0 was added. However, when 15μg/g S^0 was added a higher amount of H_2S was produced in ferments conducted by P1Y2 (620 μg/L). The highest total H_2S production occurred in UCD522 fermentations where 15 μg/g S^0 had been added (2700 μg/L)

While the majority of H_2S was produced during early and mid-fermentation (Figure 11), a significant amount of H_2S was produced near the end of the fermentations containing high S^0. As noted previously, late production of H_2S is more problematic for wine quality as this H_2S is less likely to be 'blown off' by CO_2 and so may be retained in the wine post-fermentation. Increased

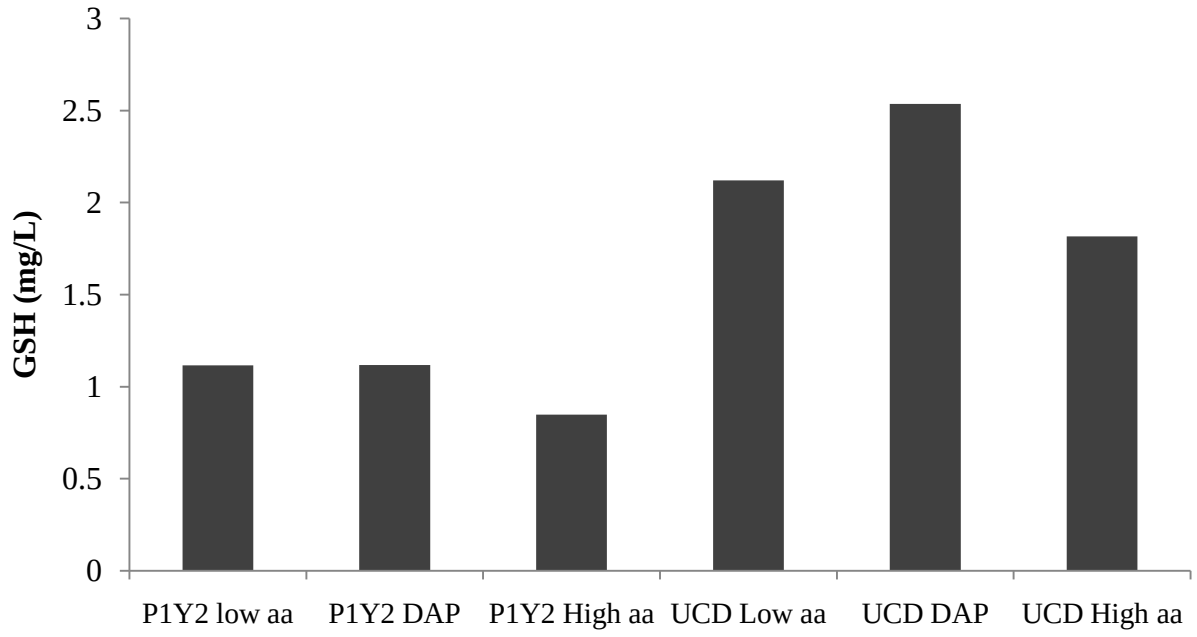

Figure 8. Concentration (mg/L) of glutathione (GSH) in wine made from synthetic grape juice containing low amino acids, high amino acids, or low amino acids and diammonium phosphate (DAP). Ferments were conducted by *S. cerevisiae* strain P1Y2 or strain UCD522.

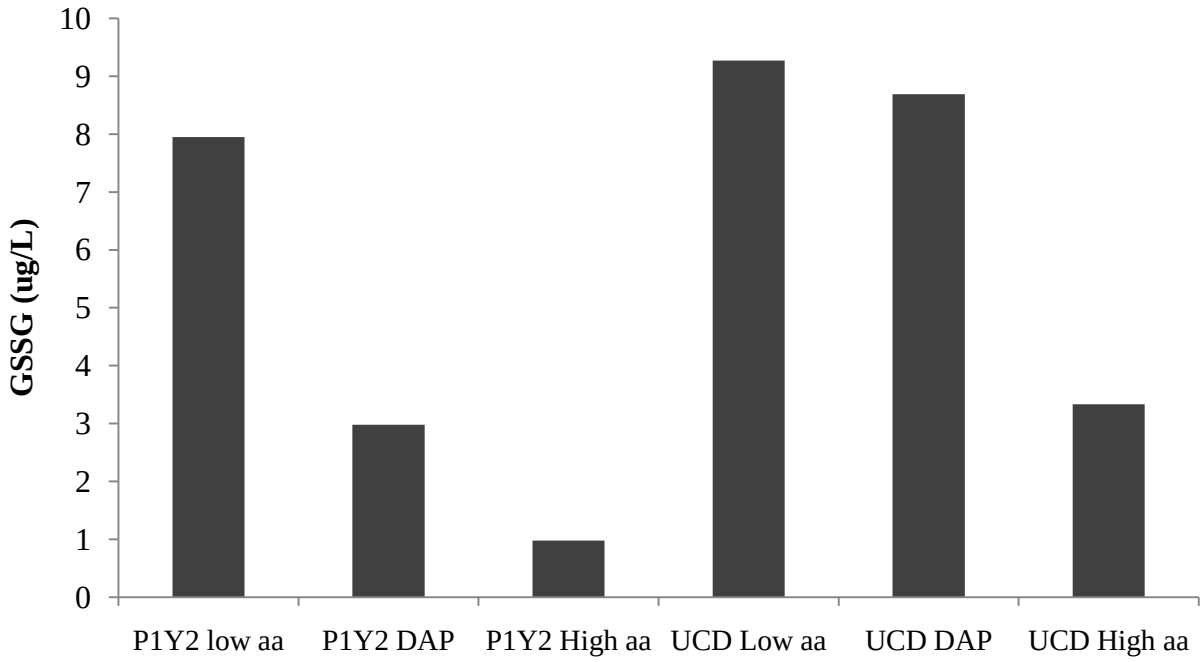

Figure 9. Concentration (ug/L) of oxidized glutathione (GSSG) in wine made from synthetic grape juice containing low amino acids, high amino acids, or low amino acids and diammonium phosphate (DAP). Ferments were conducted by *S. cerevisiae* strain P1Y2 or strain UCD522.

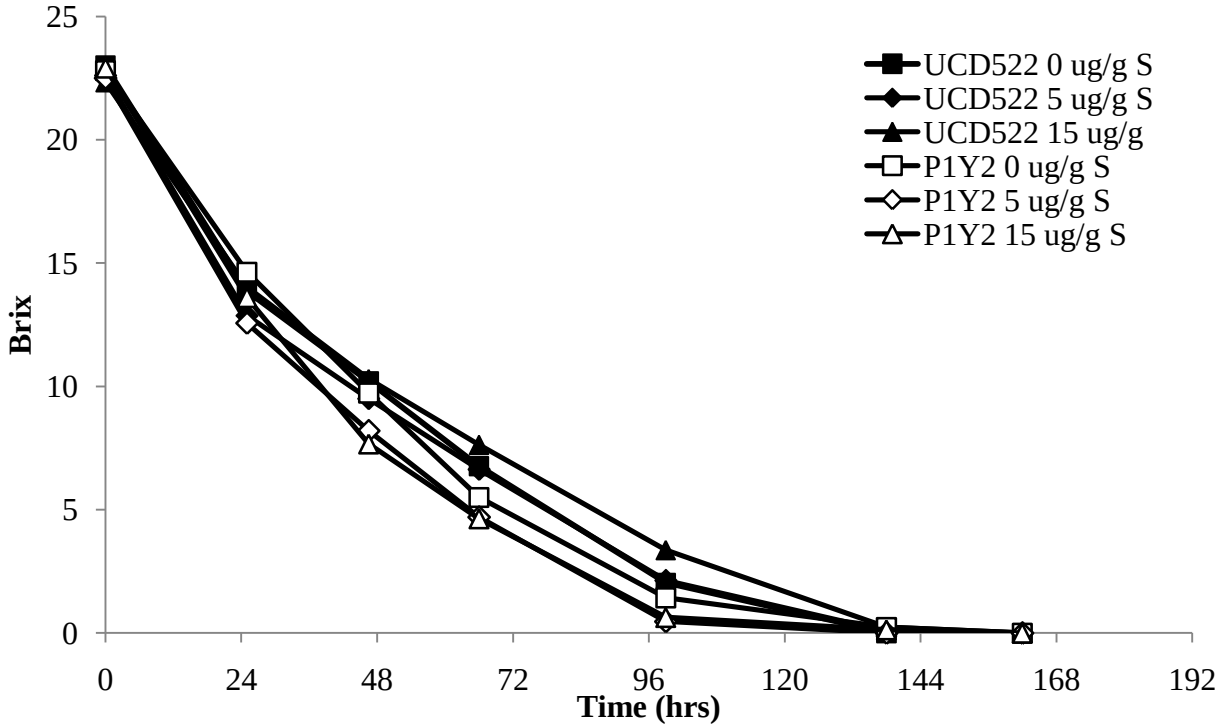

Figure 10. Change in Brix during fermentation by *S. cerevisiae* strain UCD522 or strain P1Y2 of Pinot noir grapes containing 0, 5, or 15 μg/g elemental sulfur.

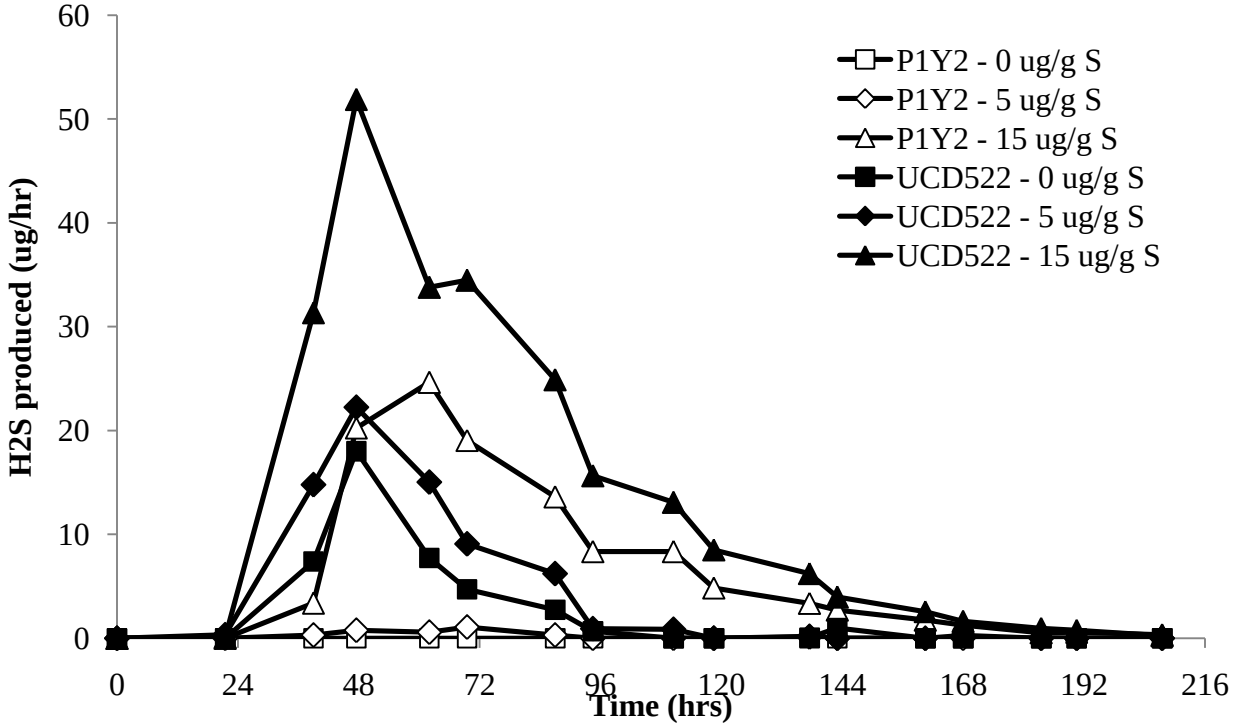

Figure 11. Production per hour of H_2S by *S. cerevisiae* strain UCD522 (closed symbols) or strain P1Y2 (open symbols) during fermentation of Pinot noir grapes with the addition of 0, 5, or 15 μg/g elemental sulfur.

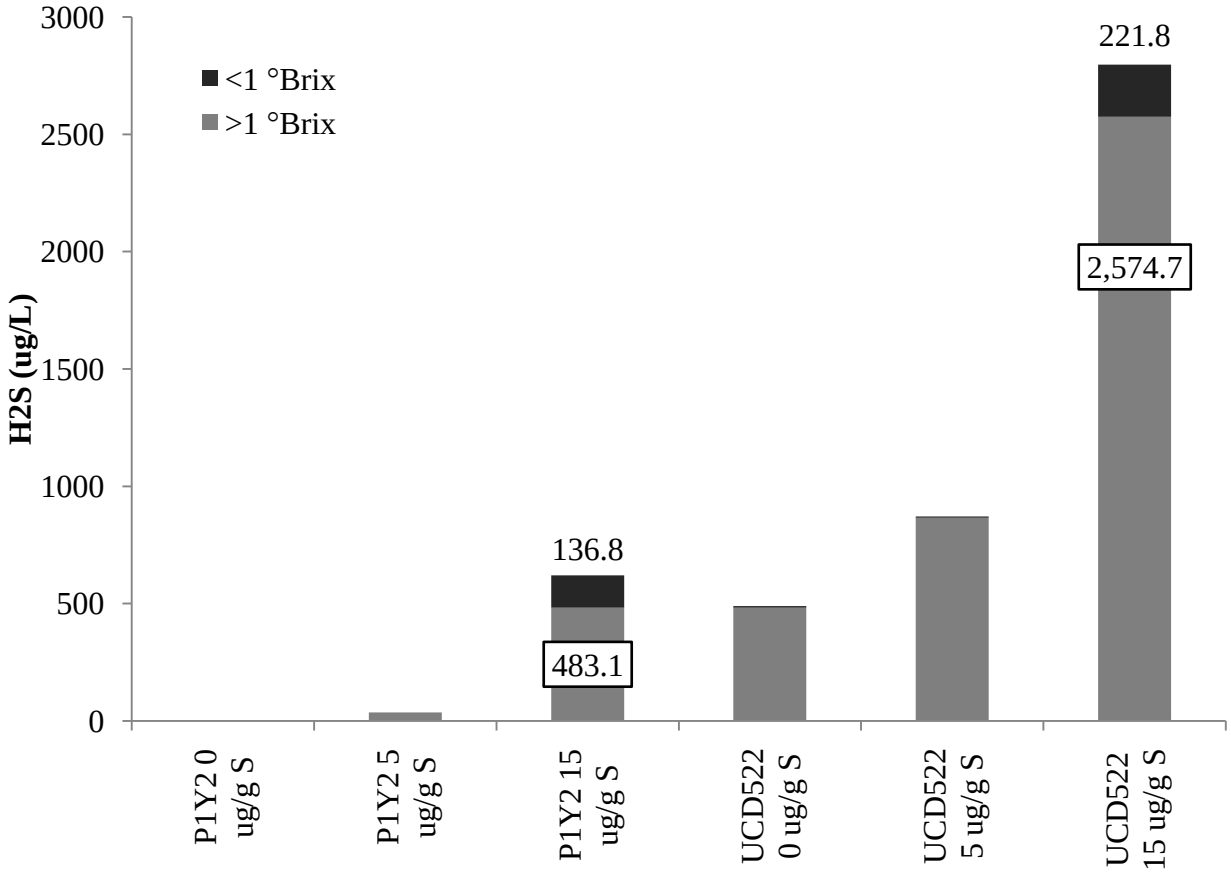

Figure 12. Total H_2S produced by *S. cerevisiae* strain UCD522 or strain P1Y2 during fermentation of Pinot noir grapes with the addition of 0, 5, or 15 ug/g elemental sulfur.

formation of H_2S late in fermentation has previously not thought to be related to S^0 content and was thought to be due most likely to breakdown of S-containing amino acids or release of H_2S from yeast as they die and breakdown (Rauhut 1993). The results from this study demonstrate that S^0 may contribute to formation of H_2Slate in fermentation which could contribute to reduction issues post-fermentation.

A number of volatile sulfur compounds were assesed in the Pinot noir wines. Of these compounds only two, carbon disulfide (CS_2) and methyl thioacetate (MeSOAc) were present in detectable amounts (Table 13). As was seen with the synthetic grape fermentations, H_2S formation during fermentation did not correlate with H_2S concentration in the wines post-fermentation. The exact reason for this is unknown but as previously noted this lack of correlation has been reported by others (Ugliano et al. 2009) and is usually attributed to the low solubility and high volatility of H_2S. In addition, because of the high reactivity of H_2S in wine the time between pressing the wines and sampling could have resulted in loss of H_2S. While H_2S produced during fermentation did not correlate with H_2S in the wines post-fermentation, it did correlate with the presence of MeSOAc. Wines where the highest H_2S was produced during fermentation contained higher amounts of MeSOAc. In addition, S^0 also impacted the concentration of MeSOAc as higher MeSOAc concentrations were present in wines produced

from grapes where high S^0 additions were made (Table 13). This may be related to the higher amount of H_2S formed late in fermentations where high S^0 had been added. The mechanism by which some of these volatile sulfur compounds are produced and the factors that drive their formation are still not well understood. However, our data suggest elemental sulfur may contribute to their formation either directly or indirectly by causing an increased formation of H_2S late in fermentation.

Table 13. Concentraion of volatile sulfur compounds (μg/L) in Pinot noir wines produced from grapes containing 0, 5, or 15 μg/g elemental sulfur (S^0) and fermented by either*S. cerevisiae* strain P1Y2 or UCD522. Wines were assessed fourteen days post-pressing.

Sulfur compounds	**CS_2**	**MeSOAc**
***Saccharomyces cerevisiae* P1Y2**		
P1Y2 0 μg/g S^0	0.04±0.01	ND
P1Y 2 5 μg/g S^0	0.08±0.01	2.3±0.2
P1Y2 15 μg/g S^0	0.11±0.01	11.9±0.9
***Saccharomyces cerevisiae* UCD522**		
UCD522 0 μg/g S^0	0.09±0.00	ND
UCD522 5 μg/g S^0	0.06±0.00	2.1±1.0
UCD522 15 μg/g S^0	0.08±0.02	8.0±1.2

ND: Below detection limit

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 (Figure 14) followed by wines fermented by UCD522 that also contained no elemental sulfur (Figure 14). Cysteine concentrations were similar for wines produced with varying S^0 although concentrations were higher in wines fermented by P1Y2 (Figure 14). While cysteine levels increased very little after 30 days aging, methionine levels increased (Figure 14).

In year three of the study interactions between nitrogen content, composition, and S^0concentration on H_2S production was investigated using Pinot noir fermentations. While all fermentations completed to sugar dryness (< 0.5 g/L) fermentations where no additions were made to the grapes took the longest to complete (Figure 15A). This was likely due to the relatively low YAN content of the Pinot noir grape must (110 mg/L). The treatment that completed fermentation the fastest was that where amino acids were added to boost the YAN to approx. 250 mg/L. This was followed by the treatment where DAP was added to also reach 250 mg/L YAN (Figure 15A). The addition of nitrogen and/or S^0 also impacted the production of H_2S during the fermentation. For all treatments H_2S production peaked during days 2-3 of the fermentation (Figure 15B). By the end of the fermentation H_2S production was only detectable in the treatments where DAP and S^0 had been added to the grapes. Total H_2S production during the fermentation was also impacted by the amount and type of nitrogen present as well as elemental

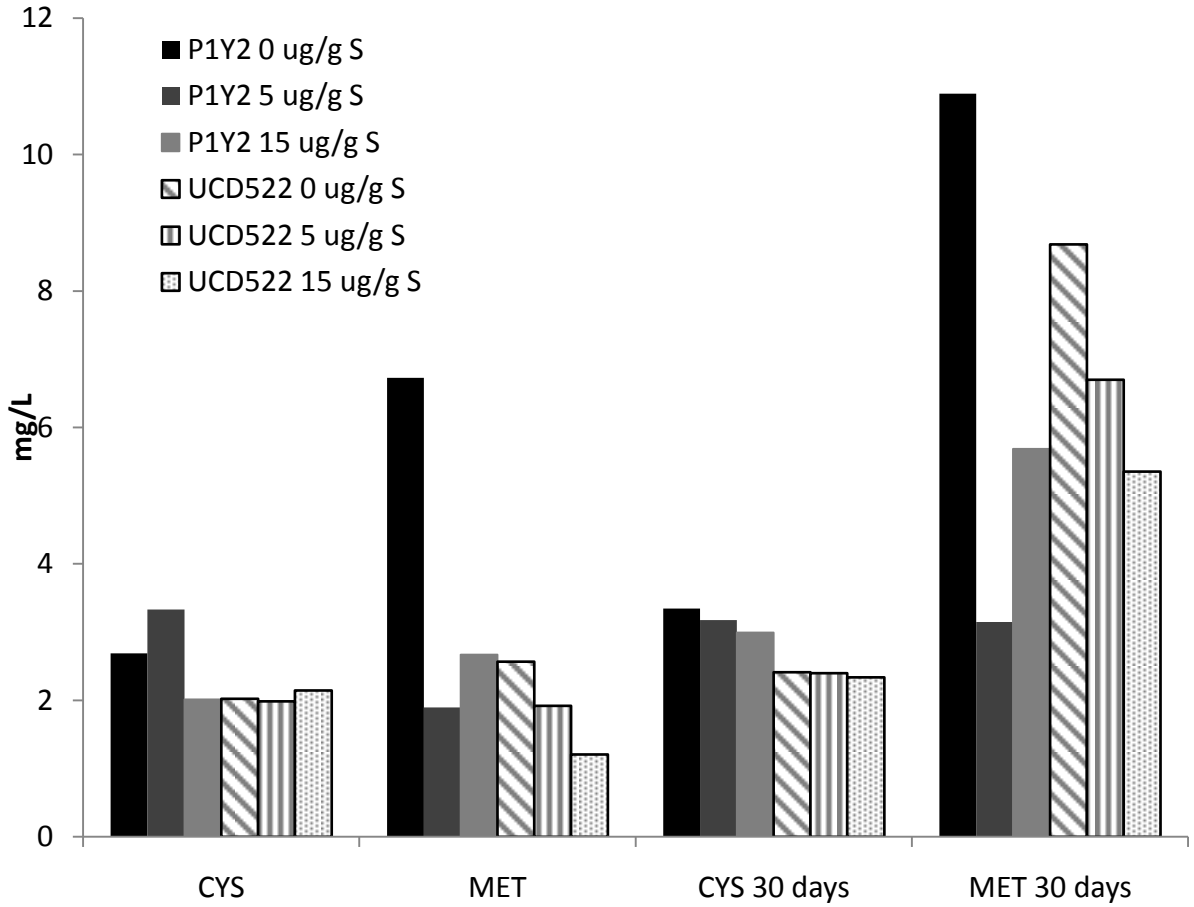

Figure 14. Concentration of cysteine and methionine in Pinot noir wine fermented by *S. cerevisiae* strain P1Y2 or strain UCD522 with the addition of 0, 5, or 15 ug/g elemental sulfur. Wines were assessed after pressing and after 30 days storage at 13°C.

sulfur (Figure 16). In wines fermented by UCD 522 the lowest amount of H_2S produced was in fermentations where amino acid had been added while the highest amount measured was in fermentations where DAP and S^0 had been added. Interestingly, the amount of H_2S produced in ferments containing S^0 increased if DAP was also added while it decreased if amino acids were added instead. Furthermore, adding only DAP to the grape must increased the amount of H_2S produced compared to the control ferment where no additions were made and where the starting YAN was relatively low. These results are supported by the recent findings of Uglanio et al (2009) where addition of DAP to a final YAN of 250 or 400 mg/L resulted in increased formation of H_2S compared to a non-supplemented fermentation with 100 mg/L YAN. While Uglanio et al (2009) only used DAP as a nitrogen supplement, the current study also compared the impact of adding amino acids on H_2S production. Our results suggest that it was not necessarily the increase in YAN that led to the higher amount of H_2S but rather the type of nitrogen added. For example, the addition of amino acids to the same YAN as the DAP supplementation resulted in lower H_2S production. This has implications for nitrogen supplementation of grape musts as both the type of nitrogen as well as the quantity must be considered if H_2S production is to be minimized.

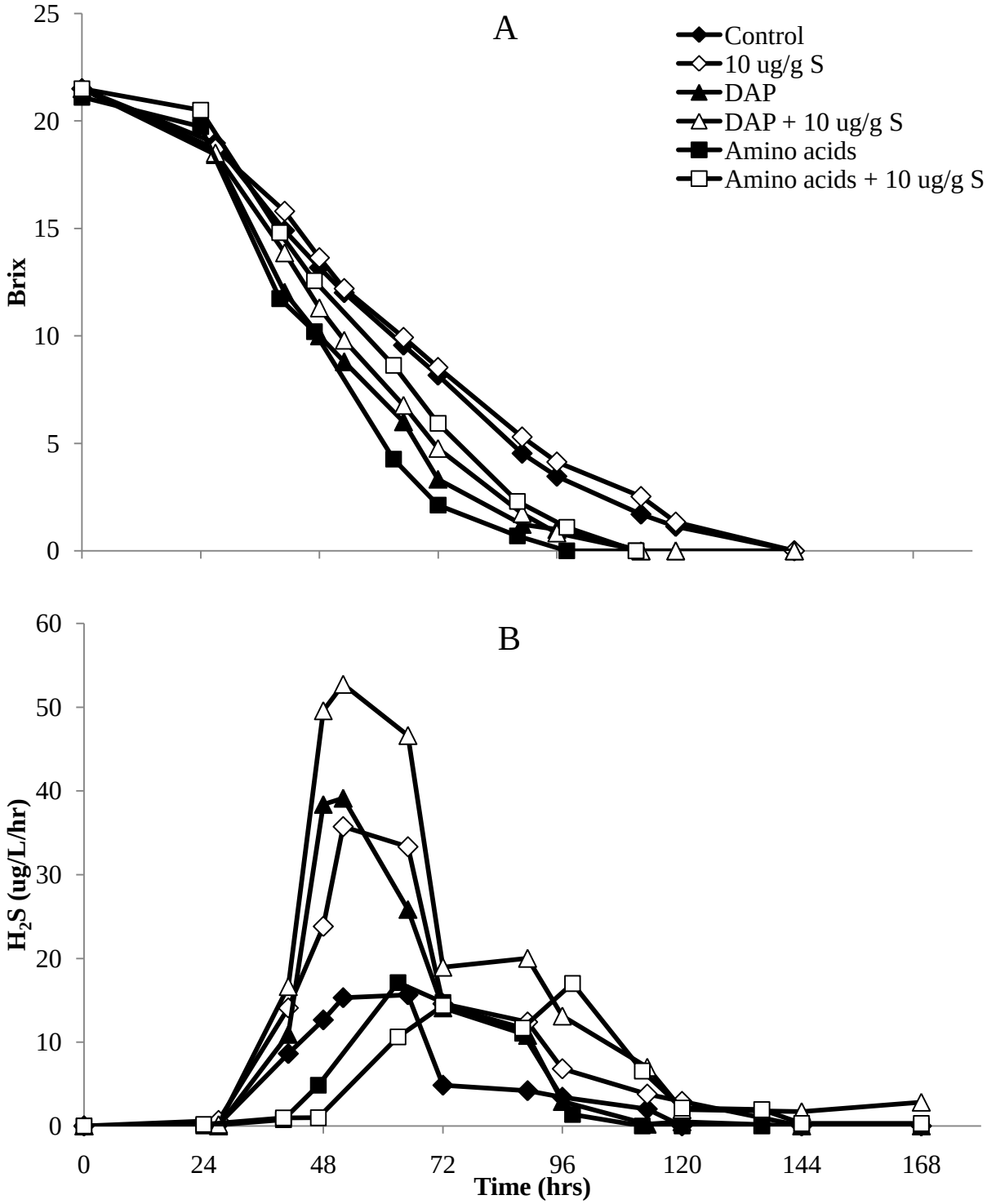

Figure 15. Change in Brix (A) and H_2S production per hour (B) during fermentation of Pinot noir grapes by *S. cerevisiae* UCD 522 with the addition of 10 ug/g elemental sulfur (S), diammonium phosphate (DAP) and/or amino acids (AA).

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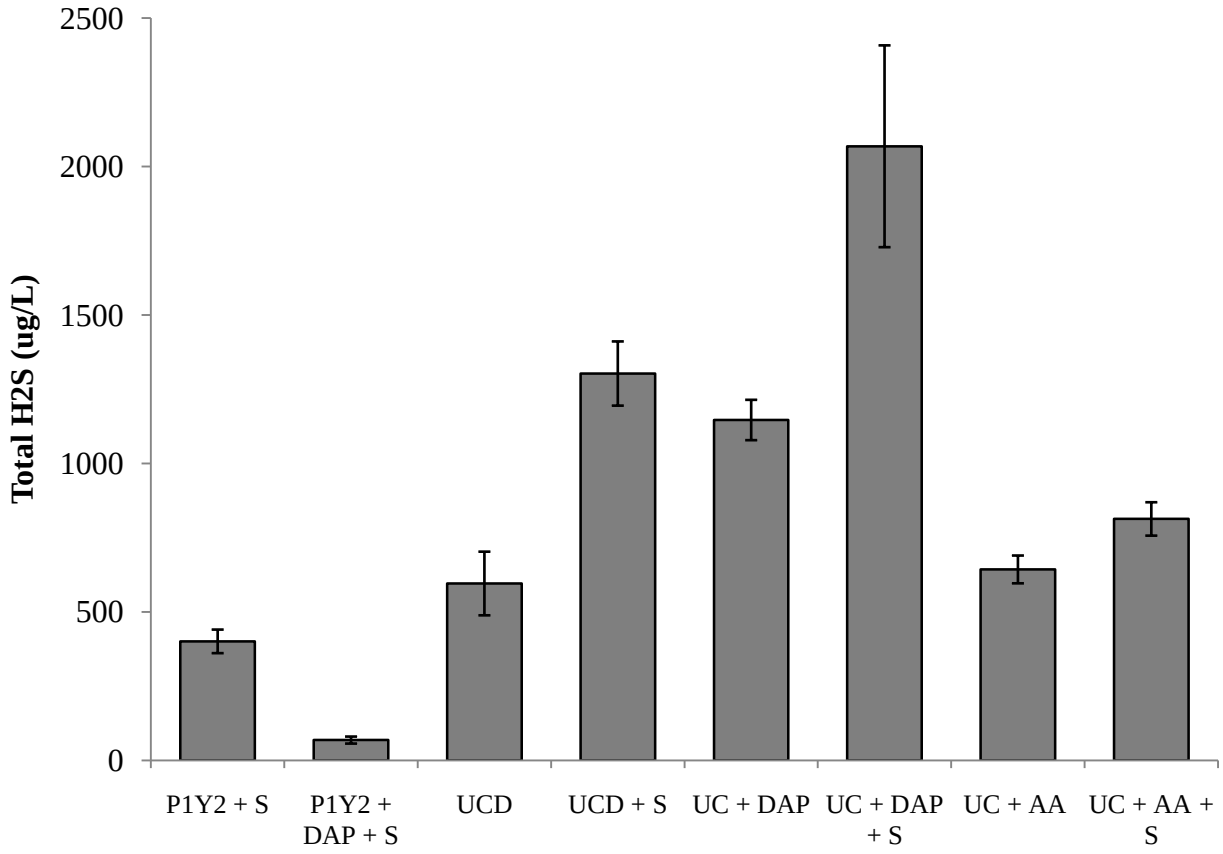

Figure 16. Total production of H_2S during fermentation of Pinot noir grapes by either *S. cerevisiae* P1Y2 or UCD 522 with the addition of 10 ug/g elemental sulfur (S), diammonium phosphate (DAP) and/or amino acids (AA).

While it was not surprising that the addition of S^0 at 10 ug/g resulted in elevated H_2S production, the large increase in H_2S production if DAP was also added was unexpected and this interaction has not been reported previously. The addition of DAP appears to only impact yeast produced H_2S as the same trend was not observed for the non-H2S producing yeast P1Y2 where any H_2S produced was due to the non-enzymatic reduction of elemental sulfur. In this case the addition of DAP to grape must containing elemental sulfur did not result in higher H_2S production compared to fermentations containing only elemental sulfur (Figure 16).

Pinot noir wines were also assessed for a number of other volatile sulfur compounds by GC-MS (Table 14). All wines contained VSCs aside from H_2S with methanethiol(MeSH), carbon disulfide (CS_2), and methyl thioacetate (MeSOAc) being present in all wines no matter the treatment. Wines fermented by UCD522 had higher MeSHcontent than P1Y2 fermented wines with concentrations above the reported sensory threshold for this cooked cabbage and rotten egg smelling compound (Rauhut 1993). While yeast strain was the dominant factor determining the concentration of VSCs, S^0 and YAN also played a role. For MeSH, the addition of S^0 did notimpact its concentration but increasing YAN, either through addition of DAP or amino acids,

Table 14. Concentration (μg/L) of volatile sulfur compounds in Pinot noir wines fermented by either *S. cerevisiae* P1Y2 or UCD 522 with the addition of 10 ug/g elemental sulfur (S), diammonium phosphate (DAP) and/or amino acids (AA).

	H_2S	MeSH	CS_2	DMS	DES	MeSOAc	DMDS	EtSOAc	DEDS	DMTS
P1Y2 + S	3.72	0.00	0.13	N.D	N.D	1.75	N.D	N.D	N.D	N.D
P1Y2 + DAP + S	7.41	0.40	0.22	N.D	N.D	4.73	N.D	N.D	N.D	N.D
UCD	N.D	0.83	0.16	N.D	N.D	2.88	N.D	N.D	N.D	N.D
UCD + S	1.25	0.53	0.24	N.D	N.D	6.83	N.D	N.D	N.D	N.D
UCD + DAP	0.00	1.54	0.16	N.D	N.D	11.76	N.D	N.D	N.D	N.D
UCD + DAP + S	2.60	1.75	0.15	N.D	N.D	16.08	N.D	0.71	N.D	N.D
UCD + AA	8.55	1.97	0.17	N.D	N.D	15.09	N.D	0.35	N.D	N.D
UCD + AA + S	5.70	1.66	0.26	N.D	N.D	16.50	N.D	0.93	N.D	N.D

resulted in higher MeSH concentrations in the wine (Figure 17A). MeSH is thought to be produced via sulfur-containing amino acids (Rauhut 1993) so an increase in YAN during fermentation may have led to increased formation of these amino acids and subsequent formation of MeSH.

The addition of S^0 and/or YAN (DAP or amino acids) impacted the concentration of MeSOAc in the wines (Figure 17B). In wines fermented by the no-H_2S producing yeast strain P1Y2 the addition of DAP resulted in higher MeSOAc concentrations (Figure 17B). This trend was seen more dramatically in wines fermented by UCD522 where the addition of just DAP increased MeSOAc concentrations from 2.88 ug/L to 11.76 ug/L while the addition of amino acids resulted in 15.09 ug/L MeSOAc. The addition of S^0 resulted in an increase in MeSOAc when DAP has also added but not when amino acids were added (Figure 17B). MeSOAc is thought to be produced via the reaction of methanethiol with acetyl-CoA and elevated formation of MeSOAc has been correlated with the production of high levels of H_2S by some yeast strains (Rauhut 1993). In the present study, high concentrations of MeSOAc in the wines did not necessarily correlate with high H_2S production during the fermentation. For example, while very similar amounts of MeSOAc were measured in wines fermented by UCD522 with the addition of either DAP or amino acids, very different amounts of H_2S were produced during fermentation (>2000 ug/L for DAP vs. <1000 ug/L for amino acids) (Figure 16). In general, the increase in YAN rather than the type of nitrogen was more predictive of whether wines would contain high concentrations of MeSOAc. This is in contrast to H_2S production where the type of nitrogen rather than YAN concentration had the largest impact (Figure 16). While the concentrations of

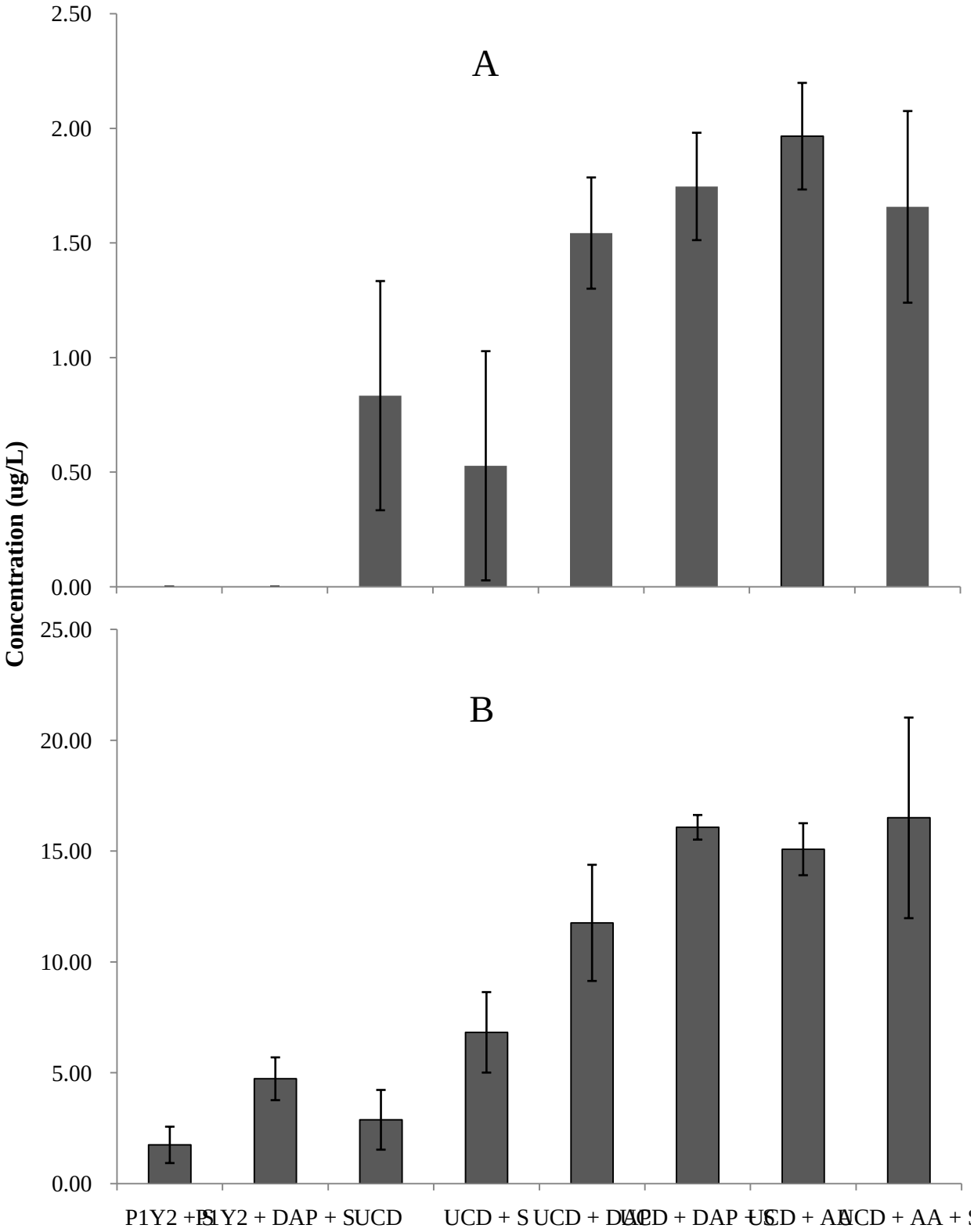

Figure 17. Concentration of methanethiol (A) and methyl thioacetate (B) in Pinot noir wines fermented by either *S. cerevisiae* P1Y2 or UCD 522 with the addition of 10 ug/g elemental sulfur (S), diammonium phosphate (DAP) and/or amino acids (AA).

MeSOAc in the wines was below the reported sensory threshold (> 20 ug/L) (Rauhut 1993), this compound can hydrolyse during aging back to MeSH which has a much lower sensory threshold (1.0–2.0 ug/L). Furthermore, MeSOAc cannot be removed by copper fining making it an important pre-cursor for sulfur off-odors in wine during aging.

The presence of a "bound" or "releasable" form of H_2S was assessed using Pinot noir wines produced from grapes containing different levels of S^0 and fermented by either a high or low H_2S producing yeast. All wines contained H_2S that was only detectable after treatment with a strong reducing agent (releasable H_2S). The amount of releasable H_2S differed between treatments (Figure 18). Wines produced from grapes where 15 ug/g S^0had been added containing the highest concentration (Figure 18) regardless of what yeast conducted the alcoholic fermentation. The amount of releasable H_2S did not necessarily correlate to the total amount of H_2S produced during fermentation. For example, wines fermented by UCD522 or P1Y2 where 15 ug/g S^0 had been added contained the same amount of releasable H_2S despite the fact that UCD522 produced over four times the amount of H_2S during the fermentation (Figure 12). Instead, the amount of releasable H_2S in the wines was more linked to late production of H_2S during fermentation and the concentration of methyl thioacetate (MeSOAc) in the wines. For example, wines produced from grapes containing 15 ug/gS^0 had very similar amounts of MeSOAc whether fermented by UCD 522 or P1Y2 (8.0 and 11.9 ug/L respectively) despite large difference in the total amount of H_2S produced during fermentation. These results indicate that late formation of H_2S during fermentation and the concentration of MeSOAc play a larger role in the concentration of releasable H_2S in the wines then the total amount of H_2S produced during fermentation.

Because of the role that elemental sulfur could play in the formation of volatile sulfur, grapes were collected at harvest in 2014 and 2015 and assessed for elemental sulfur content. The goal is to assess grape samples over a number of seasons so as to begin to determine exactly how much elemental sulfur may be on grapes when they enter the winery to be fermented. Because residual S^0 will be impacted greatly by season and vineyard spray regimes (Kwasniewski et al. 2014) a large number of samples over a number of seasons will need to be assessed before any trends or conclusions can be observed or made. Low levels of elemental sulfur were measured on most grape samples in 2014 and 2015 (Table 15). The majority of samples contained little to no elemental sulfur with only two samples containing over 1μg/g of elemental sulfur. The concentration of elemental sulfur on grapes that may cause H_2S issues has not been agreed upon by researchers. This is due mainly to the many variables aside from elemental S that can impact H_2S formation. Recently proposed values are < 1 μg/g for reds and < 3 μg/g for whites (Kwasniewski et al. 2014) but this will be highly dependent on other variables such as yeast strain, grape nutrient content, and fermentation conditions.

Methods to reduce the amount of residual S^0 on red grapes prior to fermentation were investigated. At harvest, Pinot noir grapes where either wettable or microthiol sulfur had been applied contained 2.6 or 2.8ug/g elemental sulfur (Figure 19). The amount of residual S^0 on the grapes was surprisingly low given that 5 lbs/acre of S^0 was applied only six days prior to harvest. This may be due to the period of hot weather that occurred during and after the S^0 had been applied as temperature is known to impact the persistence of sulfur on grapes (Bettiga et al 2013). Destemmed grapes were cold soaked for seven days at 8°C before the juice was drained from the bottom of the tank. The concentration of S^0 in the juice was higher than that on the

original grapes (Figure 19) indicating that a portion of the S^0 originally on the grape skins was now present in the juice. Decreased levels of S^0 were measured on the remaining grapes (Figure 19). Grapes treated with wettable or microthiol sulfur originally contained 2.6 or 2.8 ug/g S^0 respectively while after the cold soak/saignee treatment grapes contained only 0.8 or 1.0 ug/g. A large reduction in the amount of S^0 in the juice was also achieved by setting the juice for 24 hrs at 4°C and then racking off the lees (Figure 19). From a practical point of view this would allow a winemaker to drain juice from a tank, cold settle, rack, and then return the juice to the original grapes. This would minimize any loss of volume as in the current experiment the juice removed represented approximately 20% of the total weight of grapes. Alternatively, the settled juice could be utilized to produce a Rose while the original grapes could be fermented separately. In both cases the amount of S^0 present on the grapes could be reduced.

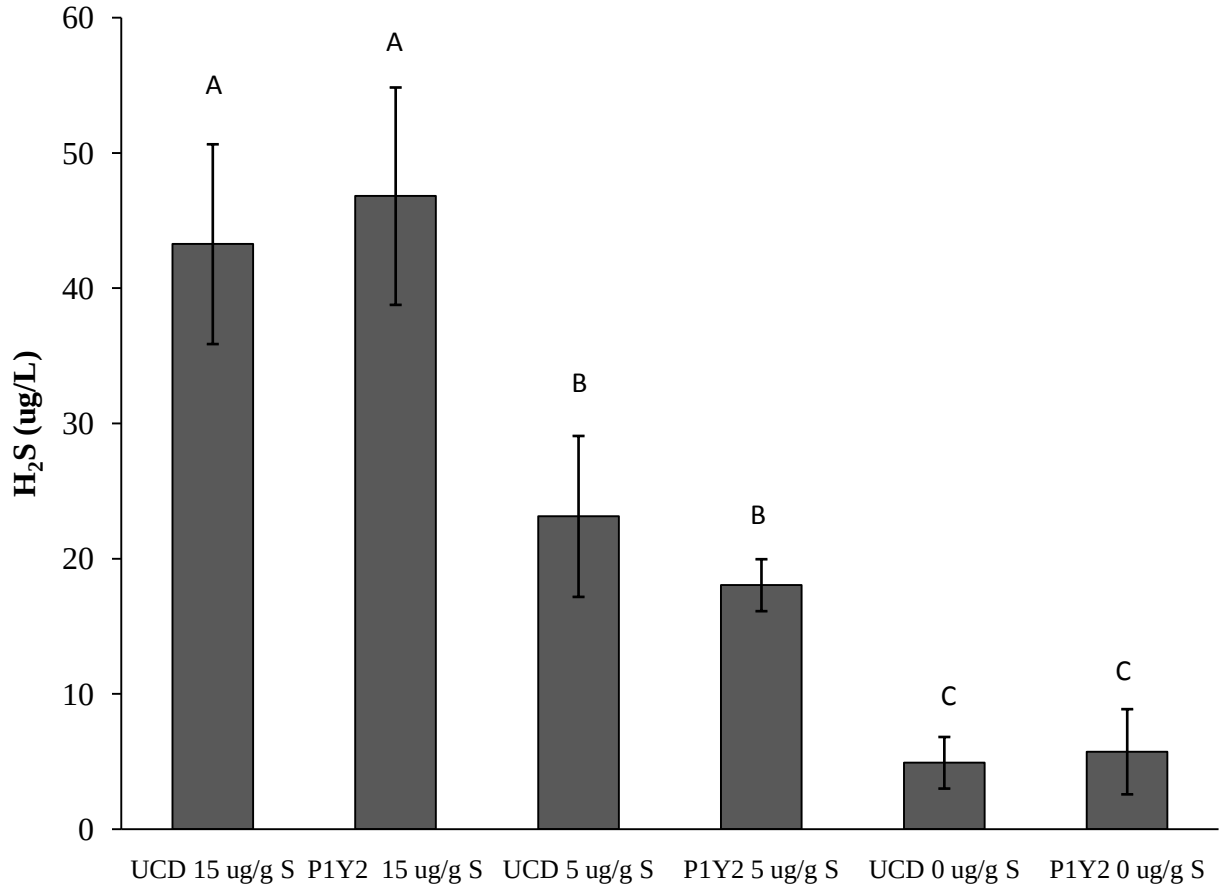

Figure 18. Releasable H_2S in Pinot noir wine produced from Pinot noir grapes where an addition of 0, 5, or 15 ug/g elemental sulfur (S)had been made. Fermentations were conducted by either *S. cerevisiae* UCD 522 or *S. cerevisiae* P1Y2. Columns with different letters are significantly different at $p \leq 0.05$.

Table 15. Concentration of elemental sulfur on Willamette Valley grapes at harvest in 2014 and 2015.

2014 samples			2015 samples	
Grape Sample ID	Elemental sulfur (ug/g)		Grape Sample ID	Elemental sulfur (ug/g)
111	0.2		503	0
445	0		533	0
696	0.1		763	0
378	0		450	1.6
553	0		959	0
211	0		858	0.2
539	0		397	0.8
460	0		694	2
464	0		613	0
933	0		298	0.4
631	0		427	0
959	0		961	0
831	0		987	0
267	0		539	0
928	0		728	0
840	0		237	0
278	0.1		418	0
398	0.1		103	0
552	0		691	0.3
781	0.7			
444	1.3			
111	0.2			

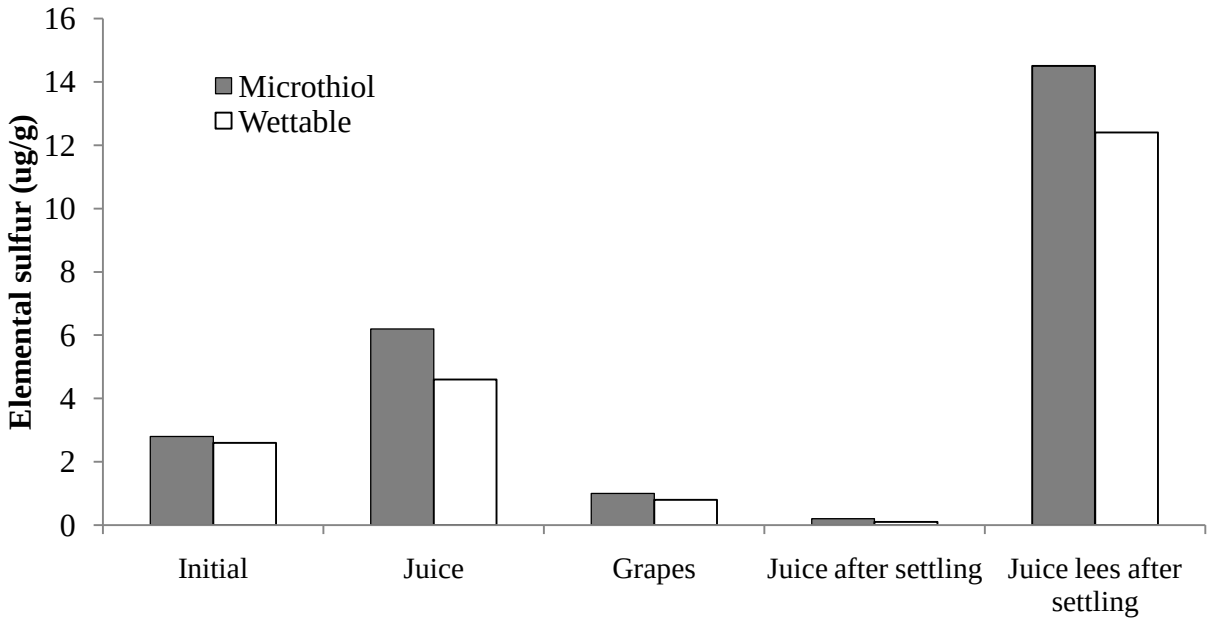

Figure 19.Concentration of elemental sulfur in Pinot noir grape and juice samples. "Initial" indicates grapes prior to cold soak and saignee treatments; "Juice" indicates juice removed by saignee after seven day cold soak; "Grapes" indicates grapes remaining after saignee; "Juice after settling" indicates juice removed by saignee and settled 24hrs at 4°C; "Juice lees after settling" indicates lees remaining after juice removed by settling for 24hrs at 4°C.

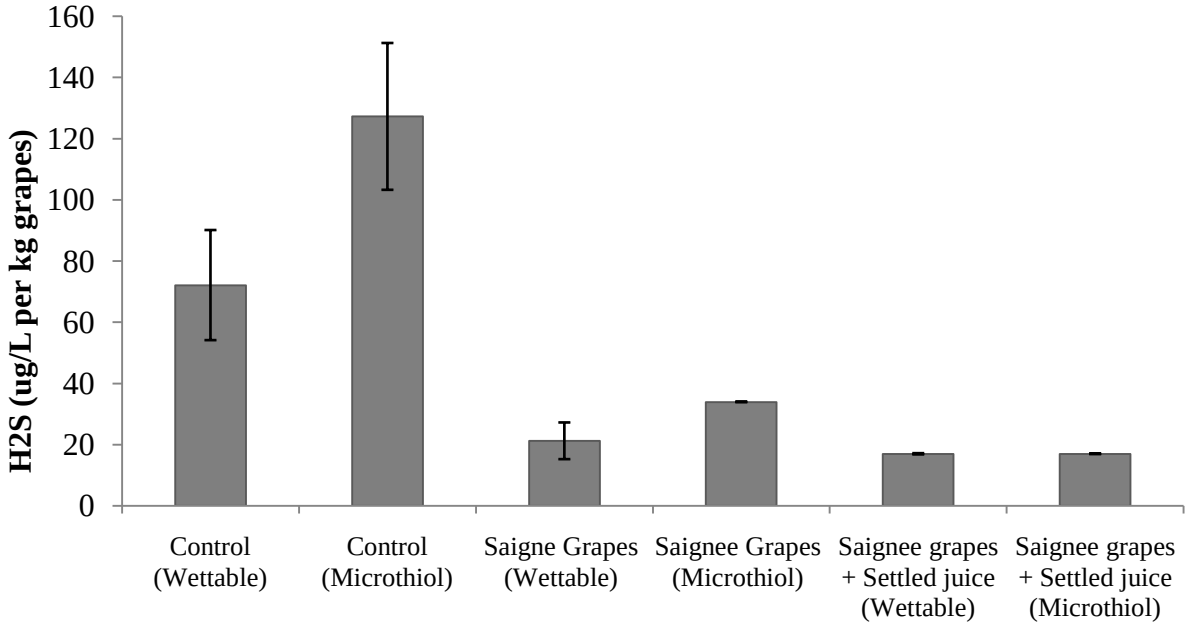

Figure 20. Total production of H_2S by *S. cerevisiae* P1Y2 during fermentation of Pinot noir grapes. "Control" indicates grapes that underwent cold soak but no juice was removed; "Saignee Grapes" indicates grapes where juice was removed at the end of a seven day cold soak; "Saignee grapes + settled juice" indicates grapes where juice was removed after a seven day cold soak and cold settled for 24hrs at 4°C before being returned.

The impact on H_2S formation by reducing the amount of S^0 present on the Pinot noir grapes can be seen in Figure 20. While fermentation of the original grapes containing wettable or microthiol sulfur resulted in high levels of H_2S production, lower amounts of H_2S were produced during fermentation of the grapes where the juice had been drained off (Figure 20). Low H_2S production was also noted where the drained off juice was returned to the fermenter after cold settling to remove S^0 (Figure 20).

VI. OUTSIDE PRESENTATIONS OF RESEARCH

Two poster presentations were given at the OWRI "Grape Days" research symposium (Corvallis, March 31st) and the annual American Society of Enology and Viticulture national meeting in Portland (June 17th – 18th). In addition, results have been shared and discussed with both the Willamette Valley and Southern Oregon Enology Technical groups. Results from this project were presented to Southern Oregon growers and winemakers during the OWRI Field Day on July 28th held at Del Rio Vineyards. Results were also presented to Willamette Valley growers and winemakers during a LIVE vineyard Field Day held on August 12th. Three manuscripts based on this study are currently in preparation.

Kraft, D.N., Zhou, Q., Qian, M.C., and Osborne, J.P. 2015. Impact of elemental sulfur and nitrogen on volatile sulfur compound formation during and after alcoholic fermentation *In*: Proceedings of Oregon Wine Research Institute "Grape Days", Corvallis, OR. March 31st.

Kraft, D.N., Zhou, Q., Qian, M.C., and Osborne, J.P. 2015.Impact of wine lees levels and composition on formation of volatile sulfur compounds during aging of Pinot noir wine. *In*: Proceedings of Oregon Wine Research Institute "Grape Days", Corvallis, OR. March 31st.

Kraft, D.N., Zhou, Q., Qian, M.C., and Osborne, J.P. 2015.Impact of elemental sulfur and nitrogen on volatile sulfur compound formation during and after alcoholic fermentation.Proceedings of the American Society of Enology and Viticulture Annual Meeting, June 17th-18th, Portland, OR.

Kraft, D.N., Zhou, Q., Qian, M.C., and Osborne, J.P. 2015.Impact of wine lees levels and composition on formation of volatile sulfur compounds during aging of Pinot noir wine.Proceedings of the American Society of Enology and Viticulture Annual Meeting, June 17th-18th, Portland, OR.

Daniel Kraft, an MS student working on this research project successfully defended his thesis on August 25th, 2015. His thesis is available online through the Oregon State University library: (https://ir.library.oregonstate.edu/xmlui/bitstream/handle/1957/57242/KraftDanielN2015.pdf?sequence=1).

VII. RESEARCH SUCCESS STATEMENTS:

Development of volatile sulfur compounds (VSCs) post-fermentation can be a significant issue during both red and white winemaking. This study focused on the development of VSCs in Pinot noir during post-fermentation aging. Results from this study have demonstrated that while higher amounts of lees in a wine post-fermentation increases the amount of VSC pre-cursors such as cysteine and glutathione, this alone does not result in increased formation of VSCs. Instead, the

early formation of VSCs soon after wines go to barrel is more likely due to H2S produced late in fermentation. Some of this H_2S may be present in a "bound" or "releasable" H_2S that is no longer volatile and so will not be detected by smell. However, under reductive conditions (such as those present in a barrel or bottle) H_2S may be reduced back to its free form and become detectable by smell again. The type and concentration of nitrogen present during fermentation as well as elemental sulfur impacts the formation of H_2S during fermentation as well as the concentration of MeSOAc in the wines post-fermentation. Of particular note is the finding that addition of DAP caused increased H_2S formation by *S. cerevisiae* UCD522 while the addition of the same amount of YAN as amino acids did not cause an increase in H_2S production. Post-fermentation, the dominant VSC compound in the wines was MeSOAc. High H2S production during fermentation resulted in high MeSOAc in the wines post-fermentation but there were conditions under which high MeSOAc was present in wines even though high H2S was not produced during fermentation. In these cases the wines a high YAN content was achieved by the addition of DAP or amino acids. These results suggest that while the presence of elemental sulfur is one factor in the formation of VSCs post-fermentation, high YAN concentrations can also be problematic.

At this point it is too early to conclude much regarding the amount of elemental sulfur present on Oregon grapes at harvest. The assessment of grapes at harvest will continue and be expanded over the next few years so that a more complete picture can be developed. However, if high levels of residual sulfur are present on red grapes at harvest, cold soaking of the grapes followed by draining off of the juice could reduce the amount of elemental sulfur on the grapes. Cold settling of the juice followed by racking and addition back to the original grapes allows minimal loss of volume. Overall, based on results from this study the best strategy to prevent formation of VSCs in wine is to firstly minimize the amount of residual elemental sulfur on grapes. Secondly, use a low H_2S producing yeast. Thirdly, measure YANs and do not add high amounts of DAP if supplementation is needed (use a mixture of DAP and organic nitrogen supplements). Lastly, settle wines post-fermentation to reduce the amount of lees present when going to barrel. reduce the amount of lees post-fermentation is important to prevent the formation of VSCs post-fermentation, managing YAN and using low H_2S producing strains is also recommended.

VIII. FUND STATUS:

The majority of the funds to date have been spent on salary and supplies for the graduate students performing the research. Funds have also been spent for, winemaking, equipment and HPLC supplies for amino acid analysis, supplies and service costs for glutathione analysis and supplies and equipment for GC-MS analysis of VSCs. The majority of remaining funds are allocated for salary and supplies to support an undergraduate student researcher and a PhD student who are currently completing work on experiments that began during the 2015 harvest as well as preparing manuscripts for publication.

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