YEAR TWO FINAL REPORT

I. PROJECT TITLE:

Formation of volatile sulfur compounds in Pinot noir post-fermentation.
Part 1: Role of grape amino acid content and wine lees composition
Part 2: Lees level and contact time on volatile sulfur compounds in wine

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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 contributing factors or conditions that impact VSCs is limited due in part to the complexity of their formation. This study focuses on the development of VSCs in Pinot noir during post-fermentation aging. During the first year of the study the impact of lees levels and composition on formation of VSCs was determined. Results showed that although lees levels and yeast strain impacted the amount of sulfur containing amino acids (pre-cursors for the formation of volatile sulfur compounds) in the wine, this did not result in an increase in the formation of volatile sulfur compounds.

Wine samples were also provided by collaborating wineries in 2013 and assessed for VSCs so as to determine the cause of early reduction issues in barrel. Wineries were instructed to take juice and wine samples from lots that traditionally had issues with VSCs. Wine samples were taken after pressing and after one, three, and nine months barrel aging. Analysis of these samples 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 such as mercaptens and disulfides. Where this H_2S is derived from and what factors impact its formation became the focus of future experiments. Firstly, experiments investigating the role of YAN concentration and content were undertaken. A synthetic grape juice was prepared where the amount and type of YAN (primary amino acids vs. ammonia from diammonium phosphate (DAP)) could be varied. H_2S production was measured throughout fermentation and finished wines were assessed for a range of other VSCs. Variation in YAN concentration as well as whether YAN was derived from amino acids or DAP impacted H_2S production during fermentation as well as formation of volatile sulfur compounds 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 in the wines post-fermentation. Concentrations of

methionine were higher in wines produced by P1Y2 while for both yeast higher methionine levels were present in wines produced from synthetic juice containing low amino acids. While wines produced by P1Y2 contained cysteine, only wines made from low amino acid juice contained cysteine when fermented by UCD522.

Experiments investigating the role of elemental sulfur in the formation of H_2S and other volatile sulfur compounds post-fermentation were also undertaken. Pinot noir grape fermentations were undertaken where an addition of 0, 5 or 15 μg/g elemental sulfur was made to the grapes. Fermentations were conducted by a high H_2S producing yeast strain (UCD522) or a no-H_2S producing yeast strain (P1Y2). Addition of elemental sulfur to the grapes 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 elemental sulfur resulting in increased production of H_2S. In addition, higher elemental sulfur additions also 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 elemental sulfur also resulted in wines containing higher concentrations of methyl thioacetate post-fermentation. Both of these findings suggest an important role for elemental sulfur in the formation of volatile sulfur compounds during and after fermentation. Wines fermented by P1Y2 with 0 ug/g S^0 contained the highest methionine concentrations followed by wines fermented by UCD522 that also contained no elemental sulfur. Cysteine concentrations were similar for wines produced with varying S^0 although concentrations were higher in wines fermented by P1Y2.

Overall, this study to date has demonstrated that lees levels impact the concentration of sulfur containing amino acids in the wine but may not directly impact formation of volatile sulfur compounds. Instead, the formation of H_2S late in fermentation or early post-fermentation may be the main cause of post-fermentation reduction soon after wine goes to barrel. Current experiments are investigating the impact of YAN, yeast strain, and elemental sulfur on the formation of H_2S and other volatile sulfur compounds post-fermentation. This work includes an ongoing effort to measure the amount of elemental sulfur present on grapes at harvest.

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.

In year one of the study experiments were conducted by the Osborne lab where Pinot noir wines were produced using two different commercial yeast strains. One set of wines was produced using the low/no H_2S producing yeast strain *Saccharomyces cerevisiae* P1Y2 while a second set were produced using *Saccharomyces cerevisiae* RC212. 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). These wines were stored at 13°C and sampled after 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 as described by Fang and Qian (2005).

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.

The impact of nitrogen concentration and composition on the formation of volatile sulfur compound was investigated. A synthetic grape juice was used so that the concentration and composition of the nitrogen in the juice could be tightly controlled. Synthetic grape juice was prepared by the Osborne lab where 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 Dr. Michael Qian's lab as part of a separately funded project. Volatile sulfur compounds were assessed by HS-SPME-GC-

PFPD as described by Fang and Qian (2005). Amino acids were assessed b HPLC-DAD according to Lee and Schriener (2010).

The role of elemental sulfur (S^0) in the formation of volatile sulfur compounds during and after fermentation was assessed in Pinot noir grape fermentations. Experiments were conducted by the Osborne lab where 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 Dr. Michael Qian's lab as part of a separately funded project. Volatile sulfur compounds were assessed by HS-SPME-GC-PFPD as described by Fang and Qian (2005). Amino acids were assessed b HPLC-DAD according to Lee and Schriener (2010). 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.

The potential role of S^0 in the formation of volatile sulfur compounds 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.

As in year one of the project, grape and and wine samples were collected from collaborating wineries. The sampling protocol was modified slightly from the previous year. Wineries were instructed to take wine samples after pressing and after 2 and 4 weeks in barrel. Grape samples were frozen for amino acid analysis by the Osborne lab while wine samples were assessed for volatile sulfur compounds by Dr. Michael Qian's laboratory. Five wineries provided samples for analysis.

V. SUMMARY OF MAJOR RESEARCH ACCOMPLISHMENTS AND RESULTS:

The quantification of the amino acid content of wines prepared with different lees levels using two different yeast has been completed. Samples were taken after 14, 30, 60, 180, and 270 days and assessed for amino acid content. 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. For example, in wines with heavy 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. 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. For example, after 270 days storage on heavy lees wine fermented by P1Y2 contained 5.9 mg/L methionine while in wine fermented by RC212 there was 4.5 mg/L methionine. Overall, 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.

While yeast strain and lees levels impact the concentration of sulfur containing amino acids, the increase in these compounds did not alter the formation of volatile sulfur compounds significantly during aging (**see Qian year one final report**). Based on this data it is likely that other factors beside the presence of sulfur containing amino acids drive the formation of volatile sulfur compounds in wine. At this point what these other factors may be is unknown. Furthermore, 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. Therefore, the role of H_2S in the early formation of volatile sulfur compounds post-fermentation as well as the source of H_2S is being investigated currently.

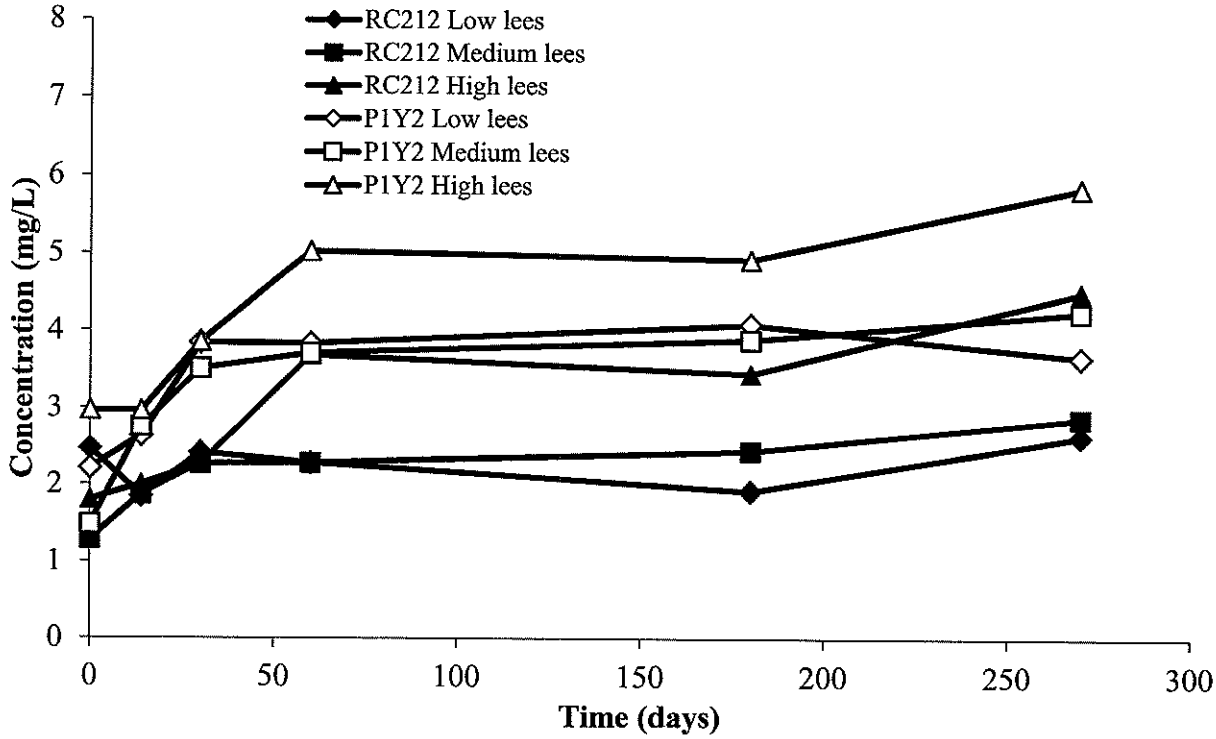

Figure 1. Concentration of methionine in Pinot noir wines fermented by *S. cerevisiae* strain RC212 (closed symbols) or strain P1Y2 (open symbols) and aged on light, medium, or heavy lees.

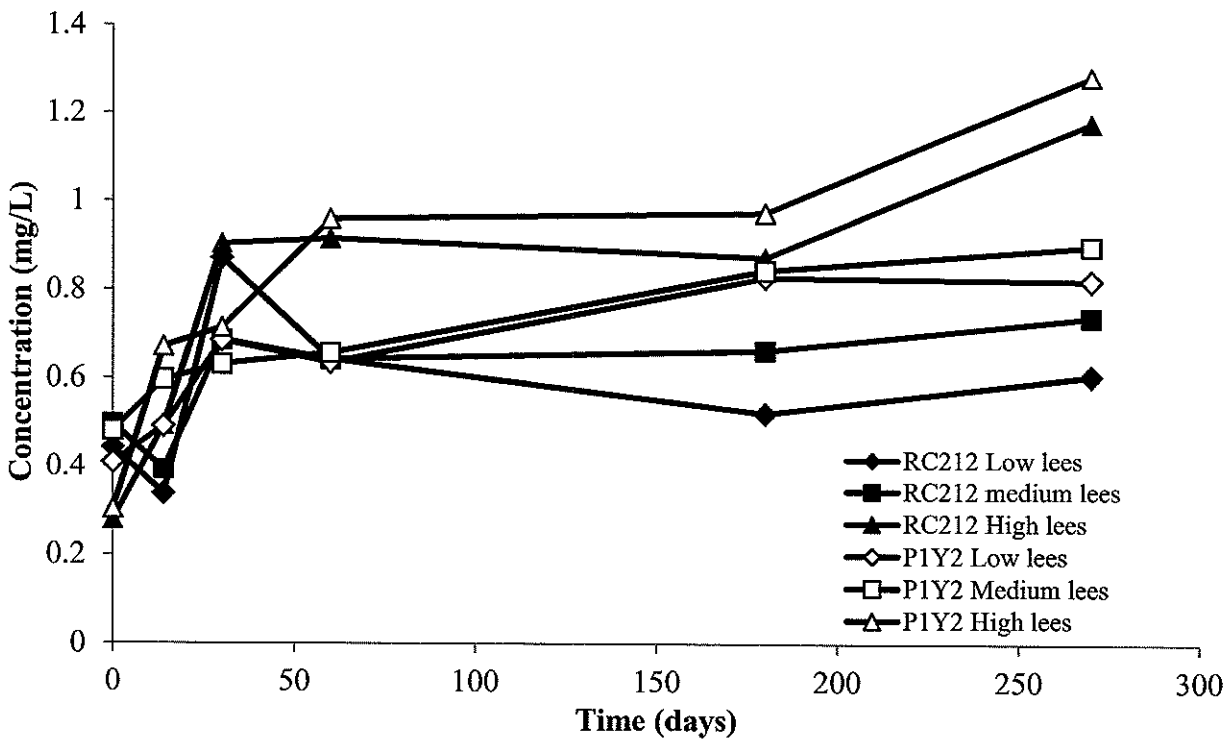

Figure 2. Concentration of cysteine in Pinot noir wines fermented by *S. cerevisiae* strain RC212 (closed symbols) or strain P1Y2 (open symbols) and aged on light, medium, or heavy lees.

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 as 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 1. 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).

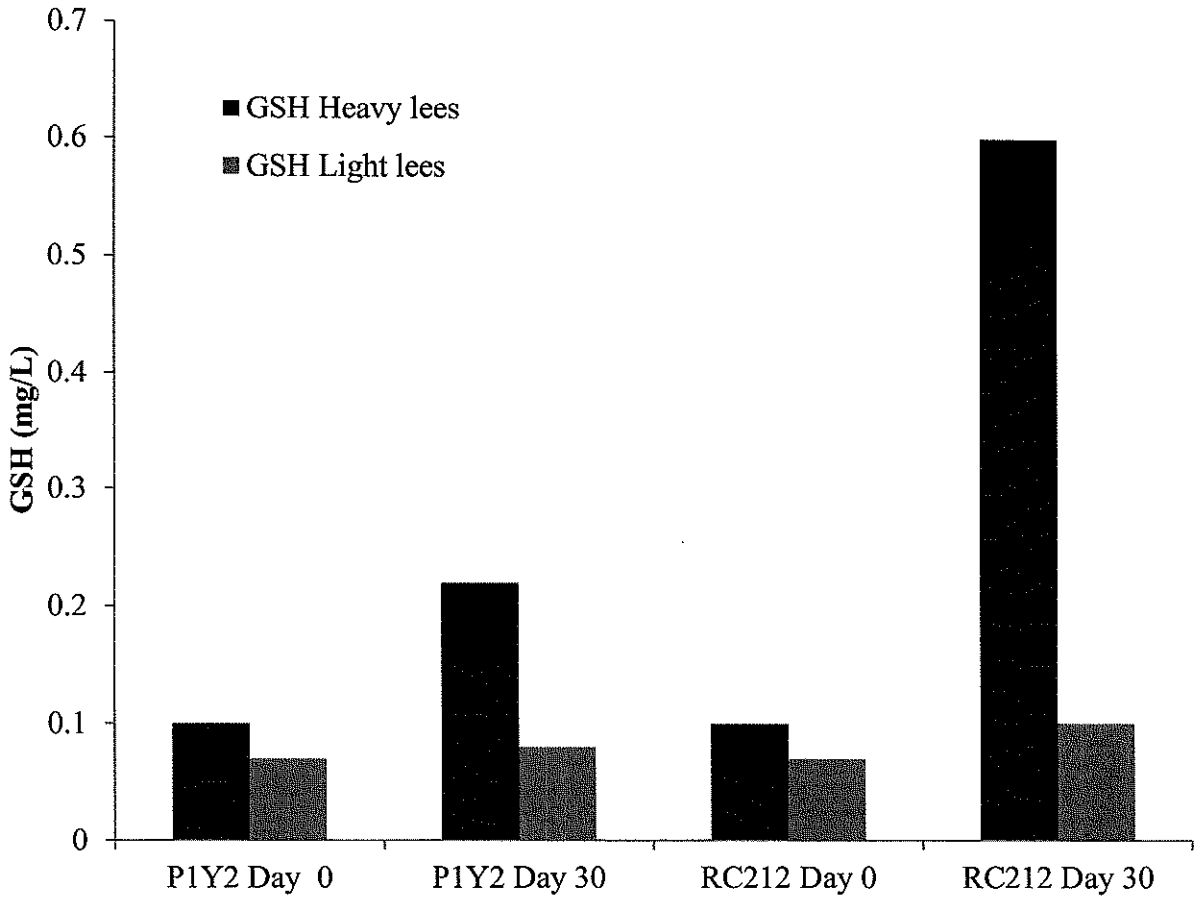

Figure 3. Concentration (mg/L) of glutatione (GSH) in Pinot noir wines fermented by *S. cerevisiae* P1Y2 or RC212 aged on light or heavy lees for 0 or 30 days.

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).

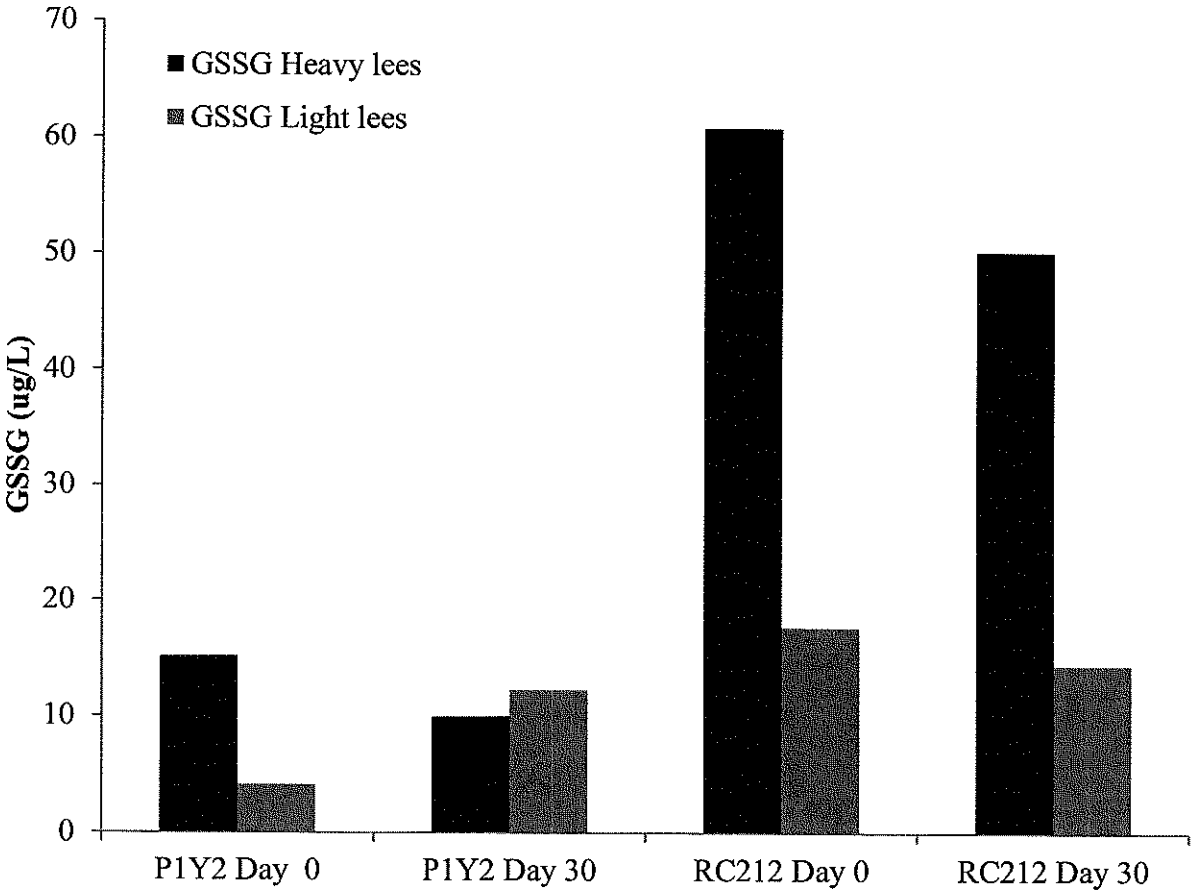

Figure 4. Concentration (ug/L) of oxidized glutatione (GSSG) in Pinot noir wines fermented by *S. cerevisiae* P1Y2 or RC212 aged on light or heavy lees for 0 or 30 days.

The effect of nitrogen concentration and composition on formation of volatile sulfur compounds was investigated 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 10^8 cfu/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 4. No H_2S was produced by yeast strain P1Y2 during fermentation in any of the treatments and so Figure 4 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 late formation of H_2S was observed contained the highest levels of methyl thioacetate and methanthiol (Table 1). Low concentrations of volatile sulfur compounds were present in wines fermented by P1Y2 while in wines.

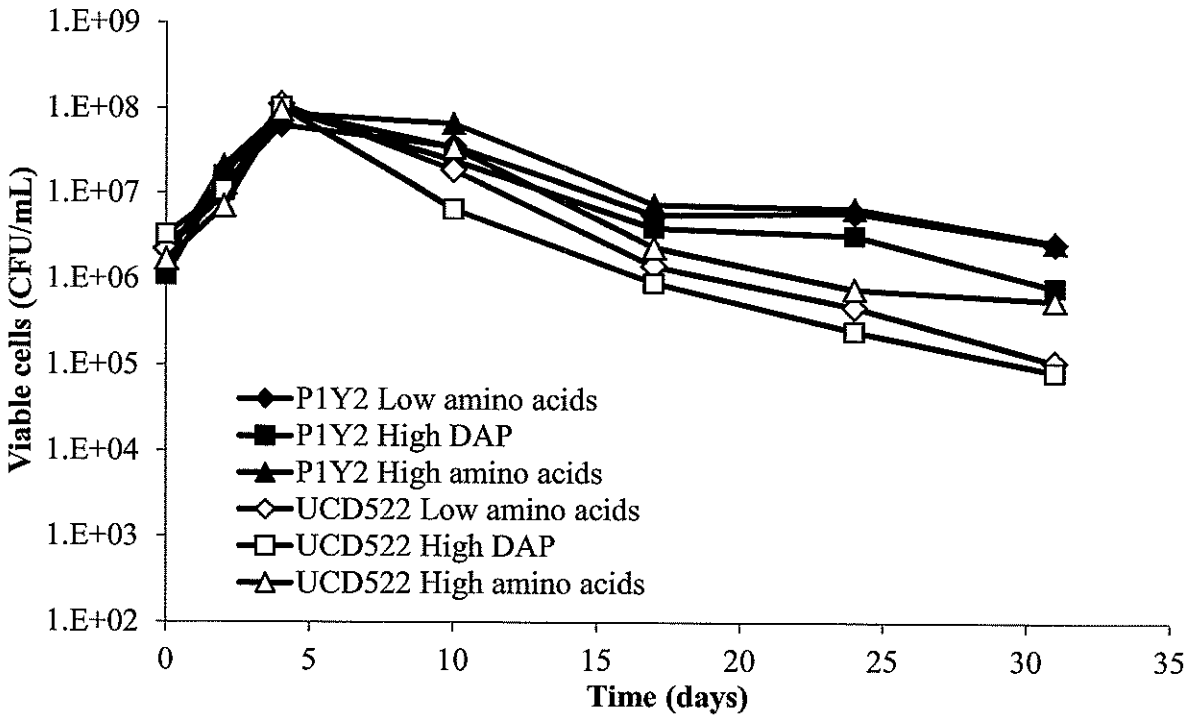

Figure 5. Growth of *S.cerevisiae* strain P1Y2 (open symbols) or UCD522 (closed symbols) in synthetic grape juice containing different concentrations of amino acids and/or diammonium phosphate (DAP).

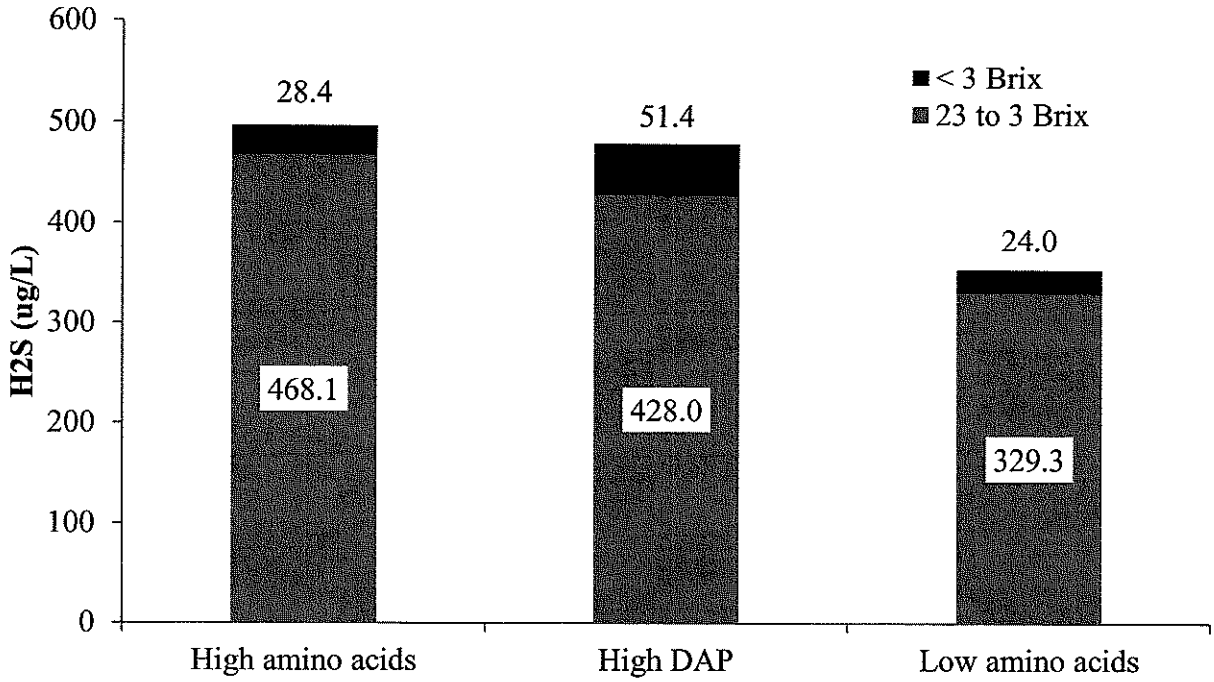

Figure 6. Total H_2S production by *S. cerevisiae* strain UCD522 during fermentation of synthetic grape juice containing different concentrations of amino acids and/or diammonium phosphate (DAP).

Table 1. 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

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 2. 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 2). 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 4) when cysteine utilization by yeast may lead to H_2S production (Rauhut, 1993).

Table 2. 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

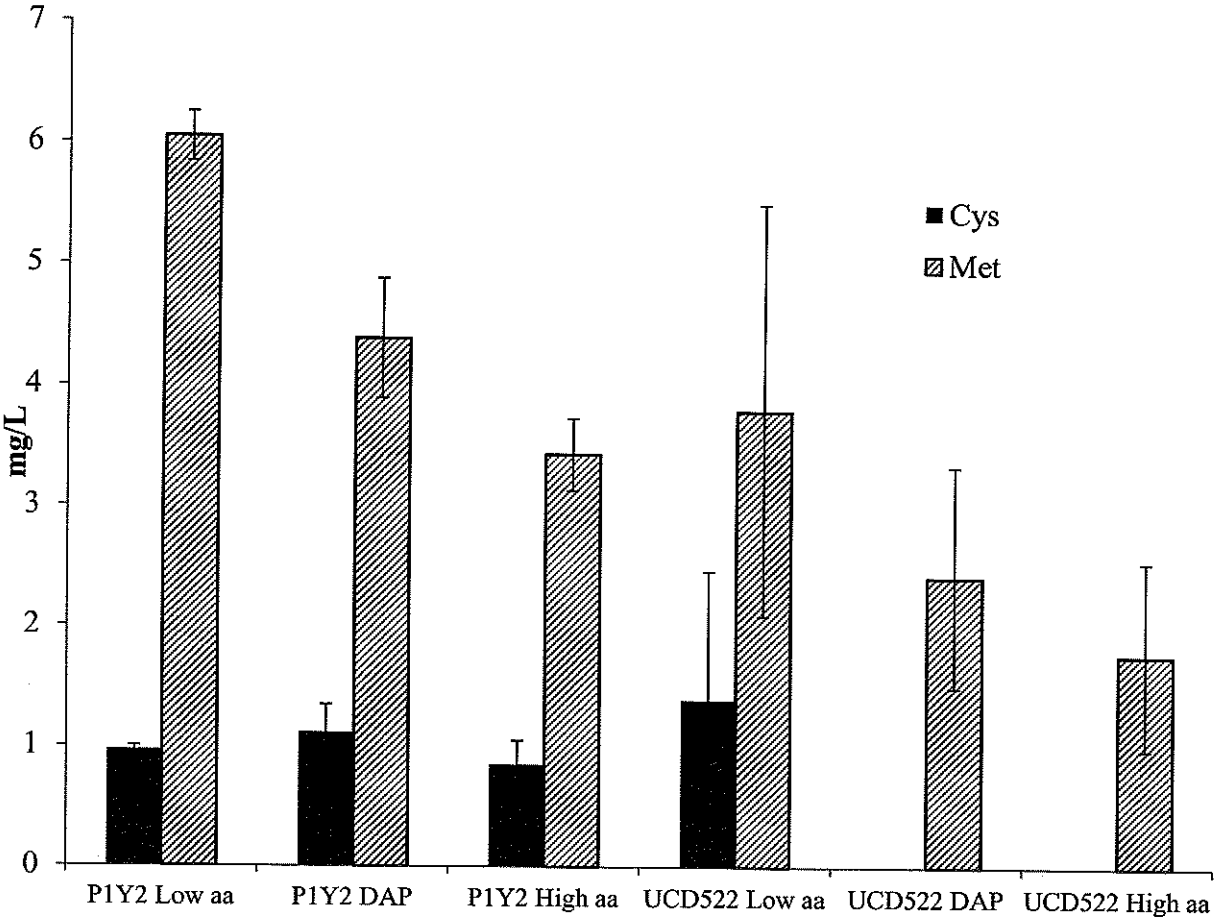

Figure 7. Concentration of cysteine and methionine in wine made from synthetic grape juice containing low amino acids, high amino acids, or low amino acids and diammonium phosphate (DAP). Ferments were conducted by *S. cerevisiae* strain P1Y2 or strain UCD522.

Glutatione and oxidized glutathione concentrations were also assessed in the syhthetic wines produced by different yeast from syhthetic juice contatining different nitrogen concentrations and composition. The results are shown in Figures 8 and 9. Interestingly there was higher GSH in the synthetic wines than in the Pinot noir wines. 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).

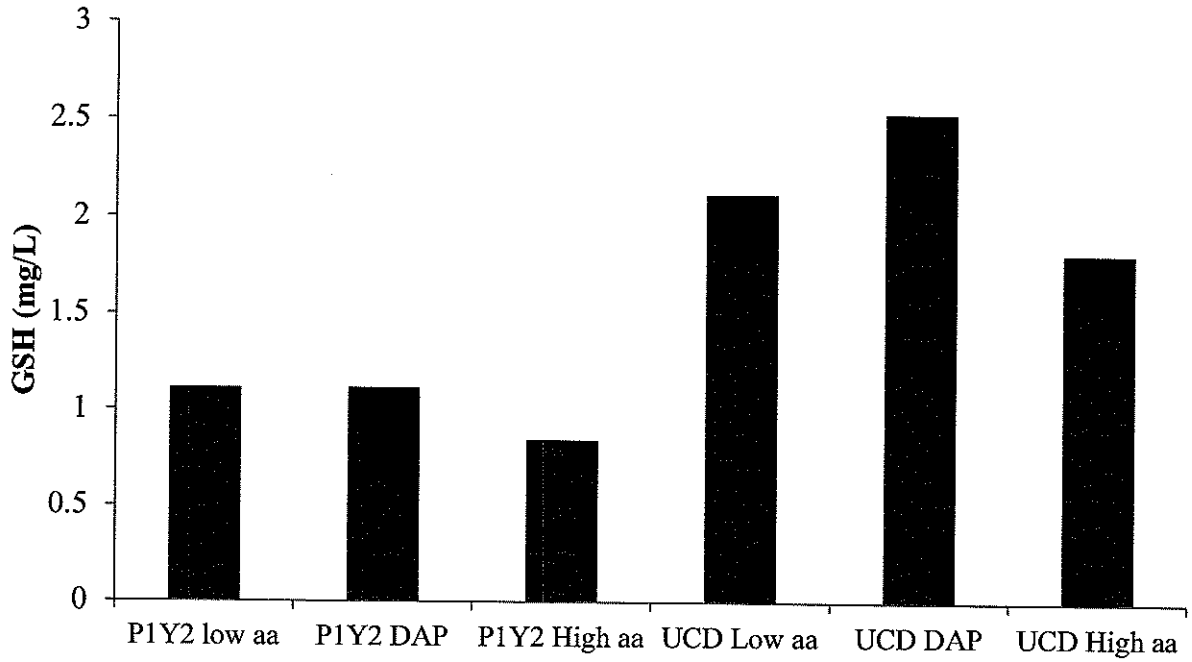

Figure 8. Concentration (mg/L) of glutathione (GSH) in wine made from synthetic grape juice containing low amino acids, high amino acids, or low amino acids and diammonium phosphate (DAP). Ferments were conducted by *S. cerevisiae* strain P1Y2 or strain UCD522.

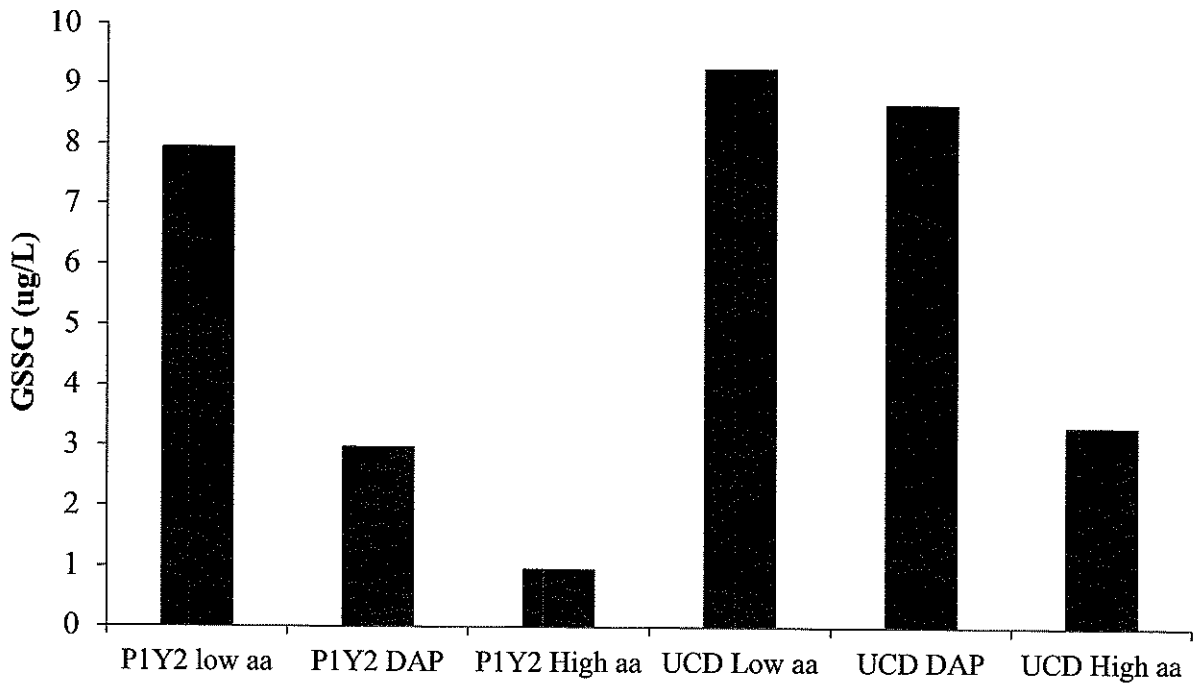

Figure 9. Concentration (ug/L) of oxidized glutathione (GSSG) in wine made from synthetic grape juice containing low amino acids, high amino acids, or low amino acids and diammonium phosphate (DAP). Ferments were conducted by *S. cerevisiae* strain P1Y2 or strain UCD522.

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.

All fermentations were complete in less than seven days. Fermentations conducted by UCD522 and containing the highest concentration of elemental S^0 took the longest time to complete (Figure 10). During fermentation the production of H_2S was measured using H_2S detection tubes. H_2S production peaked during the first 48-72 hrs 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 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 H_2S formation late in fermentation and additional experiments in year 3 will explore this finding in greater depth. In particular, the concentration of S^0 at the end of fermentation will be measured to determine whether S^0 present on the grapes can carry through to the wine or whether it is lost during fermentation.

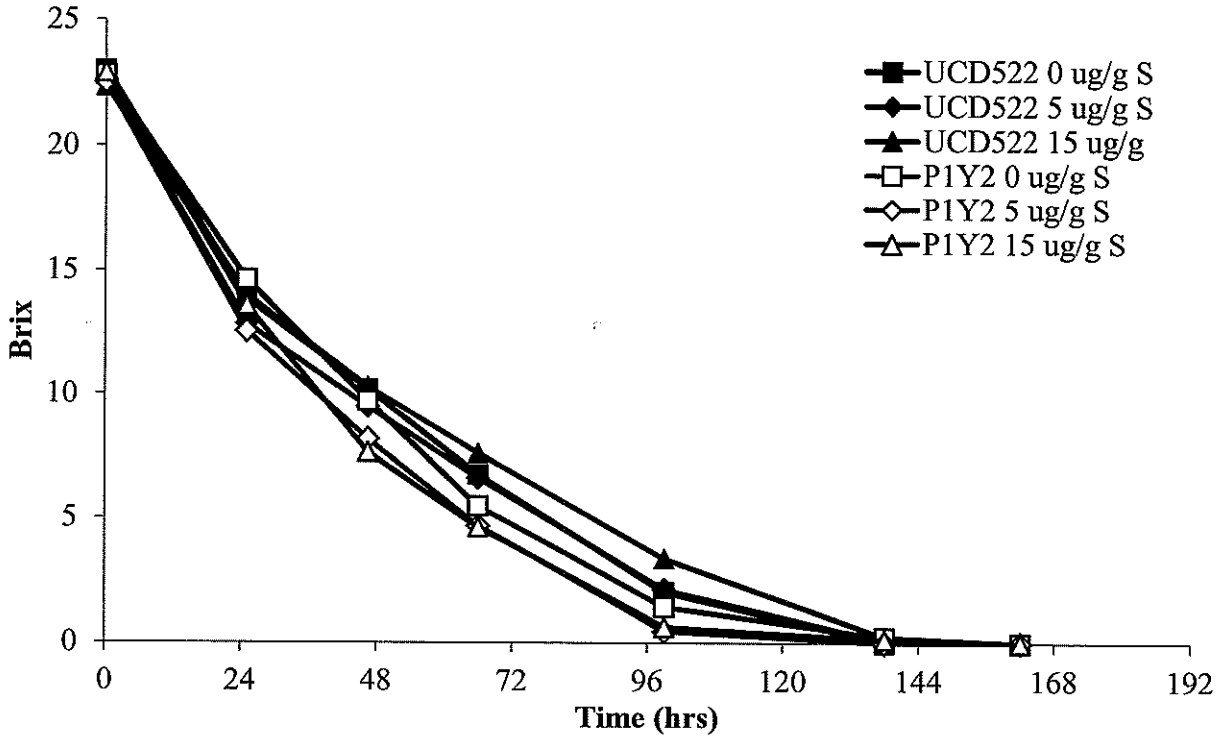

Figure 10. Change in Brix during fermentation by *S. cerevisiae* strain UCD522 or strain P1Y2 of Pinot noir grapes containing 0, 5, or 15 μg/g elemental sulfur.

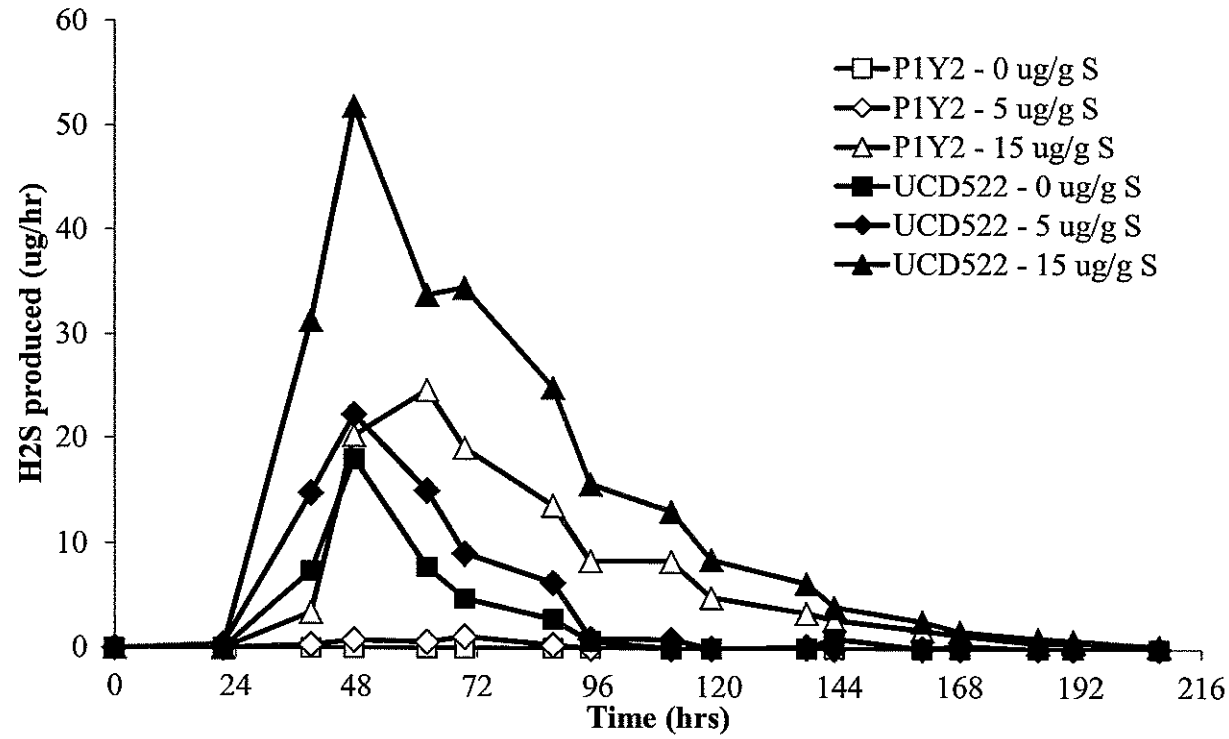

Figure 11. Production per hour of H_2S by *S. cerevisiae* strain UCD522 (closed symbols) or strain P1Y2 (open symbols) during fermentation of Pinot noir grapes with the addition of 0, 5, or 15 μg/g elemental sulfur.

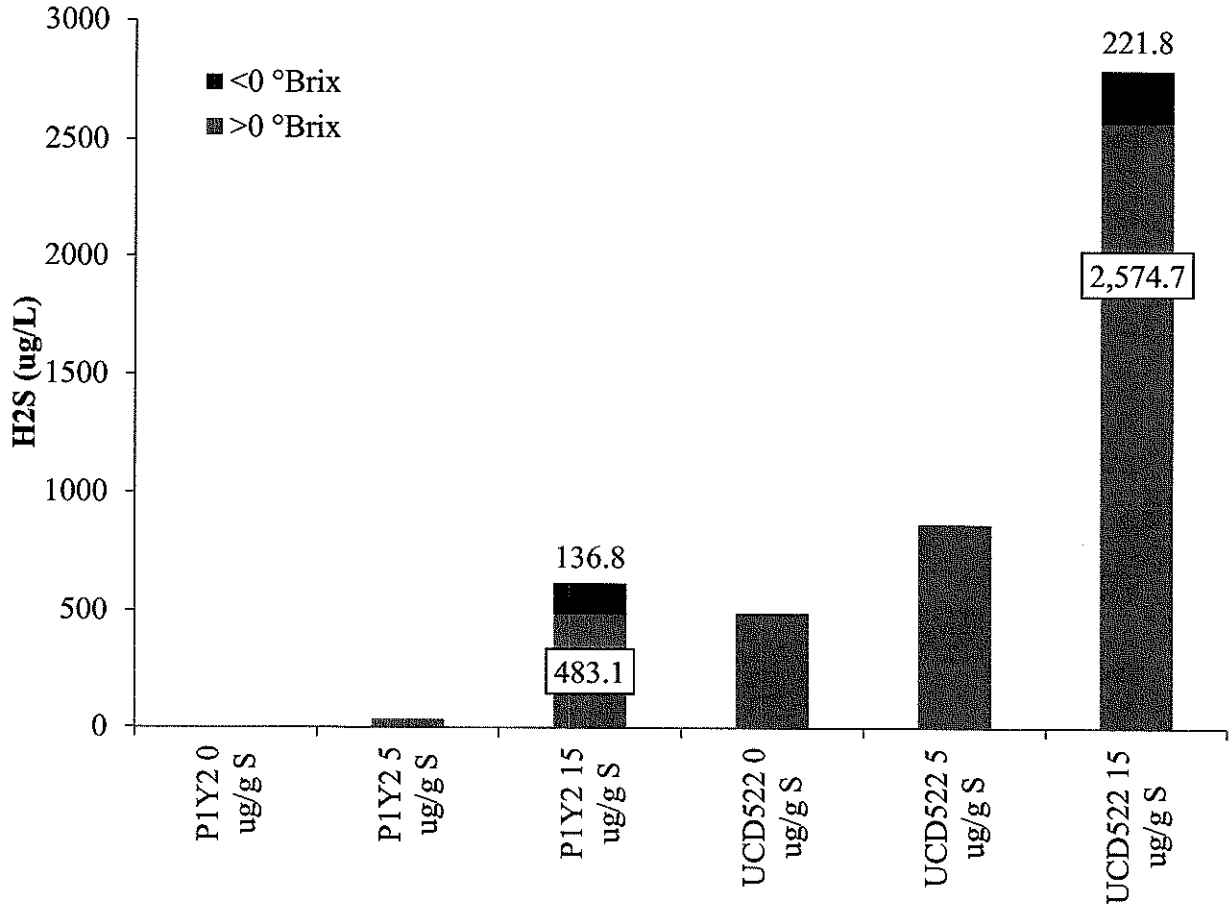

Figure 12. Total H_2S produced by *S. cerevisiae* strain UCD522 or strain P1Y2 during fermentation of Pinot noir grapes with the addition of 0, 5, or 15 ug/g elemental sulfur.

A number of volatile sulfur compounds were assesed in the Pinot noir wines by Dr. Micahel Qian's lab using HS-SPME-GC-PFPD. Of these compounds only two, carbon disulfide (CS_2) and methyl thioacetate (MeSOAc) were present in detectable amounts (Table 3). 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 (Figure 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 3. 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

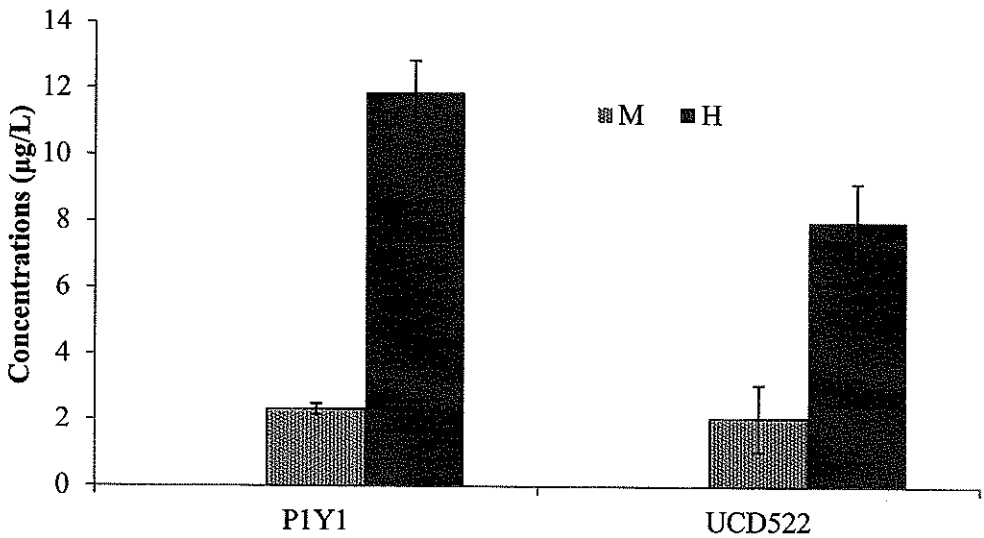

Figure 13. Concentrations of methyl thioacetate in Pinot noir wine fermented by *S. cerevisiae* strain UCD522 or strain P1Y2 with the addition of 0, (L) 5, (M) or 15 ug/g (H) elemental sulfur. Wines were assessed fourteen days post-pressing.

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). The significance of these different amounts of sulfur containing compounds in the wine during aging is at this point unknown.

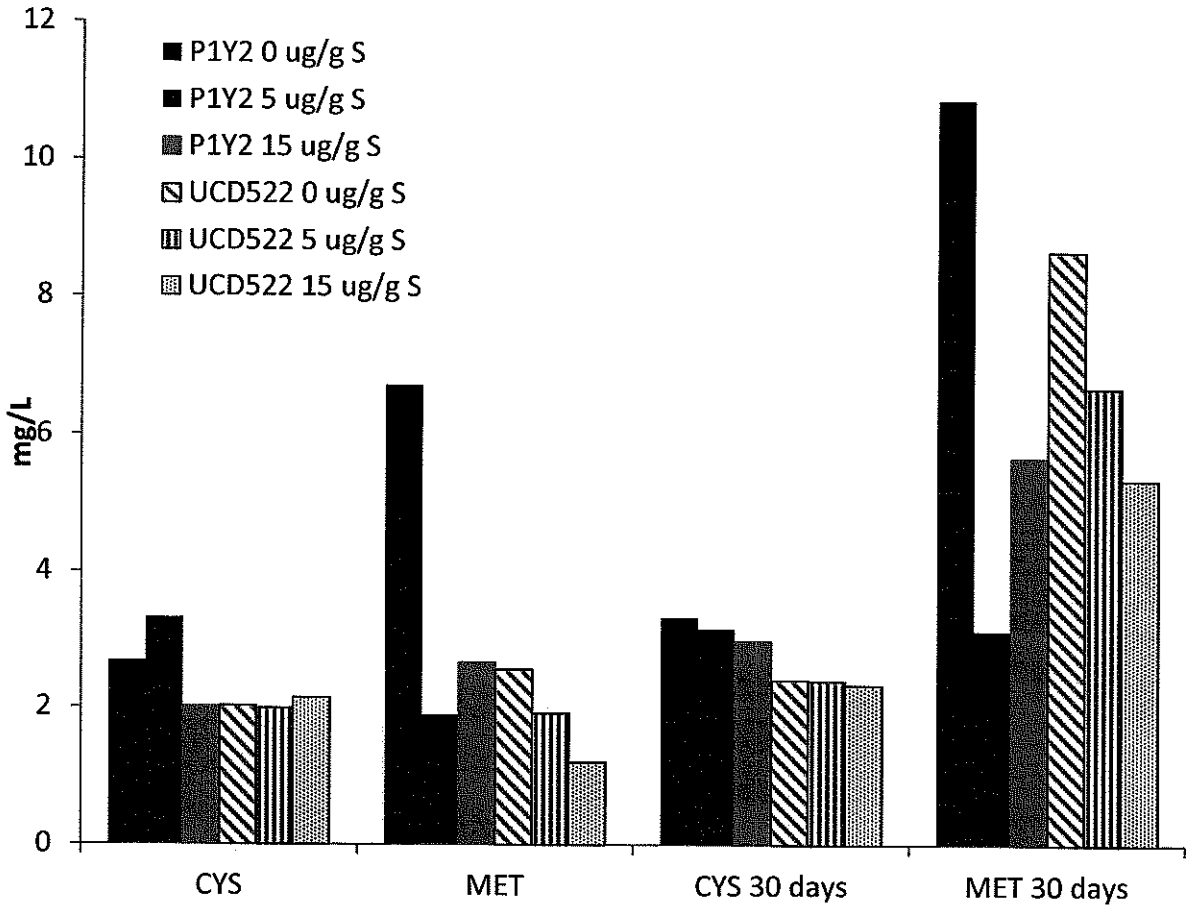

Figure 14. Concentration of cysteine and methionine in Pinot noir wine fermented by *S. cerevisiae* strain P1Y2 or strain UCD522 with the addition of 0, 5, or 15 ug/g elemental sulfur. Wines were assessed after pressing and after 30 days storage at 13°C.

Because of the role that elemental sulfur could play in the formation of volatile sulfur compounds an initial effort was made in 2014 to assess the amount of elemental sulfur present on grapes at harvest. 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. The amount present on the grapes will be impacted greatly by season and vineyard spray regimes (Kwasniewski et al. 2014) so 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. Assessment of grapes will continue independent of the current project. In 2014 twenty grape samples from six different wineries were assessed. As can be seen in Table 4 the majority of samples contained little to no elemental sulfur. There was only one sample that contained over 1 μg/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.

Table 4. Elemental sulfur measured on grape samples at harvest in 2014

Sample ID	Elemental S (μg/g)
BB2	0.02
BB3	0.00
THUP 9/25	0.09
3C 9/26/14	0.00
5B 9/26/14	0.00
6B 9/26/14	0.00
ST3 #1	0.00
ST3#2	0.00
ST7C	0.00
ST8C	0.00
JN1A 9/26/14	0.00
JN9 9/26/14	0.00
RN1A 9/26/14	0.00
RN3B 9/26/14	0.00
RN3C 9/26/14	0.00
BK PN 3Oct	0.00
777 Temp. Hill	0.06
SH Temp. Hill	0.09
PN "14"	0.00
PN "32"	0.70
Chard 11	1.30

VI. OUTSIDE PRESENTATIONS OF RESEARCH

A poster presentation was given at the 2014 "OWRI Grape Days" (March 31st). Two poster presentations will also be given at the annual American Society of Enology and Viticulture national meeting in Portland (June 17th – 18th). In addition results have been shared and discussed with the Willamette Valley Enology Technical group during two meetings in 2014.

VII. RESEARCH SUCCESS STATEMENTS:

The development of volatile sulfur compounds during winemaking is an ongoing issue in the wine industry and many of the factors impacting the formation of these compounds are unknown or poorly understood. The initial objective of the present study was to focus on the impact of wine lees levels and composition on sulfur containing amino acids and formation of volatile sulfur compounds post-fermentation. Results from the first year of the study showed that although lees levels and yeast strain impacted the amount of sulfur containing amino acids (pre-cursors for the formation of volatile sulfur compounds) in the wine, this did not result in an increase in the formation of volatile sulfur compounds.

Analysis of winery wine samples from 2013 indicated that the early formation of reductive smells soon after going to barrel are most likely due to H_2S rather than the formation of more

complex volatile sulfur compounds such as mercaptens and disulfides. This was an important finding as winemakers have more options at their disposal when trying to prevent or remove H_2S from their wine compared to dealing with mercaptens and disulfides. Where this H_2S is derived from and what factors impact its formation became the focus of future experiments. Firstly, experiments investigating the role of YAN concentration and content were undertaken. Variation in YAN concentration as well as whether YAN was derived from amino acids or DAP impacted H_2S production during fermentation as well as formation of volatile sulfur compounds 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 in the wines post-fermentation.

Experiments investigating the role of elemental sulfur in the formation of H_2S and other volatile sulfur compounds post-fermentation have produced some interesting results. An addition of elemental sulfur to the grapes resulted in H_2S formation during the alcoholic fermentation independent of which yeast strain was used (high H_2S producing yeast vs. no-H_2S producing yeast). These results demonstrated that H_2S can be produced no matter which yeast strain you use if elemental sulfur is present. If you are using a yeast strain that produces H_2S then the presence of elemental sulfur results in an even higher production of H_2S. Increasing amounts of elemental sulfur also resulted in increasing production of H_2S during fermentation. This finding is in contrast to Thomas et al (1993b) where the amount of elemental sulfur in the fermentation did not correlate with the amount of H_2S produced. In addition, higher elemental sulfur additions also resulted in increased concentrations of 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 elemental sulfur also resulted in wines containing higher concentrations of methyl thioacetate post-fermentation. Both of these findings suggest an important role for elemental sulfur in the formation of volatile sulfur compounds during and after fermentation.

Overall, this study to date has demonstrated that lees levels impact the concentration of sulfur containing amino acids in the wine but may not directly impact formation of volatile sulfur compounds. Instead, the formation of H_2S late in fermentation or early post-fermentation may be the main cause of post-fermentation reduction soon after wine goes to barrel. Current experiments are investigating the impact of YAN, yeast strain, and elemental sulfur on the formation of H_2S and other volatile sulfur compounds post-fermentation. This work includes an ongoing effort to understand how much elemental sulfur is present on grapes at harvest in the Willamette Valley.

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 collection of samples from collaborating wineries, winemaking, equipment and HPLC supplies for amino acid analysis, and supplies and service costs for glutathione analysis. In addition funds have been used to perform analysis of elemental sulfur on grapes. The majority of remaining funds are allocated for salary and supplies.

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Table 5. Concentrations of amino acids and ammonia in the synthetic grape juice

	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**	**346 mg/L**	**350 mg/L**