YEAR ONE 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

II. PRINCIPAL INVESTIGATOR:

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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 on the wine lees and the impact of wine lees composition, quantity, and contact time. A particular emphasis of the study is to investigate the relationship between grape amino acid composition and concentration, wine lees composition, and production of VSCs and VSC pre-cursor compounds. A graduate student has begun work on the project and Pinot noir fermentations utilizing two yeast, a low/no H_2S producing yeast and an industry standard yeast, were undertaken in October 2013. Wine produced was split into three different lees treatments based on settling time (0, 24, and 72 hrs settling at 4 °C) and samples were assessed for volatile sulfur compounds. Later analysis will include grape and wine amino acid composition. Wines were stored at cellar temperatures and regularly sampled. Recent analysis of FAN content of wines during aging has shown that a significant amount of FAN remained after alcoholic fermentation was complete and that this concentration increased during aging on the lees. In addition, settling reduced the amount of FAN present in the wines. Preliminary amino acid analysis showed that both yeast strain and lees levels impacted both the total amount of amino acids present but also the relative concentrations of the sulfur containing amino acids methionine and cysteine. Methionine levels decreased after fermentation but then increased after 3 months storage for all lees treatments and for both yeast treatments. However, after 6 months aging on the lees the levels of methionine decreased. Wine fermented by RC212 contained relatively low levels of methionine during aging on the light and medium lees when compared to the same treatments in wine fermented by P1Y2. The heavy lees treatments for wines fermented by either yeast contained the highest amount of methionine with wine fermented by P1Y2 and aged on the heavy lees having the highest amount of methionine of all treatments after 3 months aging.

Similar trends were seen for cysteine where there were generally higher amounts of cysteine in wines fermented by P1Y2 and low levels in RC212 fermented wines except for the heavy lees treatments. These results demonstrate that both yeast strain and lees levels impacted the amount of total amino acids and specifically the sulfur containing amino acids methionine and cysteine. and the significance of this finding will become apparent once amino acid and analysis and glutathione concentration is completed.

In addition to the study at OSU, juice and wine samples were collected from collaborating wineries. Wineries identified vineyard lots that traditionally had issues with volatile sulfur compounds during and post fermentation and took grape samples at harvest. Wine samples were taken post pressing and at a 3 month intervals during barrel aging. These samples will be assessed for a number of parameters including amino acid content and volatile sulfur compounds. In conjunction with the experiments undertaken at OSU these results will help further our understanding of the links between grape amino acid content, wine lees composition, and volatile sulfur formation post-fermentation.

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 Dr. Michael Qian as part of a separate project "Formation of volatile sulfur compounds in Pinot noir post-fermentation -part 2: Lees level and contact time on volatile sulfur compounds in wine". 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. Samples were additionally taken after 2 and 4 weeks of storage and assessed for the same parameters. Further has occurred after 2, 3, and 6 months. A final sample at 9 months of storage will be taken in July.

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.

Grape and wine samples have been collected from collaborating wineries. Grape samples have been frozen for amino acid and glutathione analysis while wine samples were assessed for volatile sulfur compounds by Dr. Michael Qian as part of a separate project "Formation of volatile sulfur compounds in Pinot noir post-fermentation -part 2: Lees level and contact time on volatile sulfur compounds in wine". Wine samples will also be assessed for amino acid and glutathione content. To date wine samples have been taken after pressing and after one month storage and 6 months storage. In a number of cases wineries treated wine lots with copper sulfate or racked wines off their lees after noticing reductive characteristics. In these cases no further sampling was undertaken.

The graduate student working on this project is currently being trained to use the HPLC and implement the methodologies for amino acid and glutathione analysis. Analysis of samples for amino acids is currently underway.

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). The grape parameters were not unusually high or low for Pinot noir although the yeast assimilable nitrogen concentration was on the low end of the recommended range for a successful fermentation. However, the alcoholic fermentations for all treatments proceeded quickly and were completed in seven days. After pressing, wine samples were taken and assessed for chemical parameters (Table 2). Wines fermented by RC212 had lower ethanol and free amino acid concentrations than wines fermented by P1Y2 and all wines have low residual ammonia. 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. This difference in turbidity/lees between the treatments will allow the impact of lees content and composition on volatile sulfur compounds to be evaluated during ageing.

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

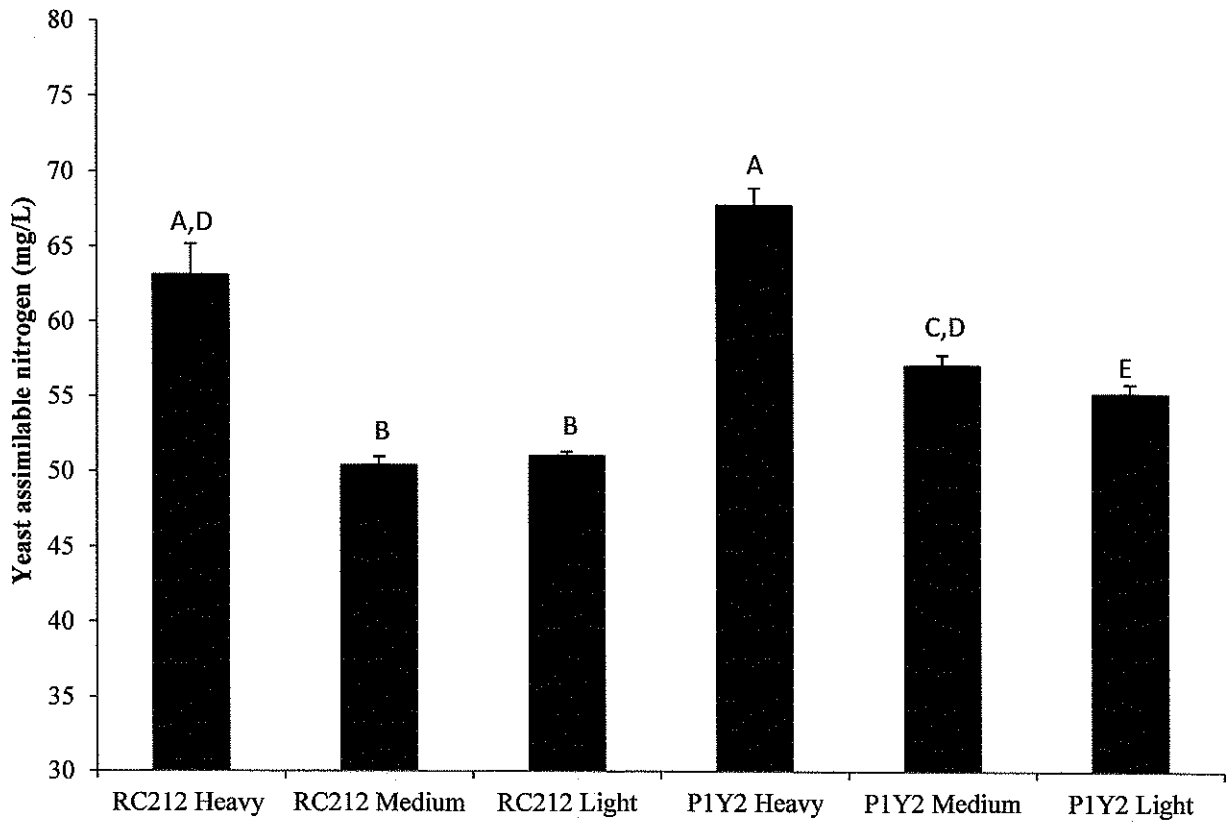

Figure 1. Concentration of yeast assimilable nitrogen in Pinot noir wine aged three months on either heavy, medium, or light lees after fermentation by *S. cerevisiae* RC212 or *S. cerevisiae* P1Y2. Different letters indicate significance at $p<0.05$ level. n=3

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 (Figure 1) except in the case of wine fermented by P1Y2 where there was a small decrease in FAN between the medium and light lees treatments (Figure 1). 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.

The free amino acid content of the Pinot noir juice and wines are shown in Table 1 and Table 2. At this point the data is presented as peak area values as quantification based on standard curves and internal standards is still underway. Never the less, peak area values allow us to compare the relative values of individual amino acids and to compare amino acid content during aging on different levels of lees. Specifically we can compare the amount of the sulfur containing amino acids methionine and cysteine in the different treatments. Methionine levels decreased after fermentation but then increased after 3 months storage for all lees treatments and for both yeast treatments (Figures 3 and 4). However, after 6 months aging on the lees the levels of methionine decreased. Wine fermented by RC212 contained relatively low levels of methionine during aging on the light and medium lees when compared to the same treatments in wine fermented by P1Y2. The heavy lees treatments for wines fermented by either yeast contained the highest amount of methionine. Wine fermented by P1Y2 and aged on the heavy lees had the highest amount of methionine of all treatments after 3 months aging. Similar trends were seen for cysteine (Figure 3) where there were generally higher amounts of cysteine in wines fermented by P1Y2 and low levels in RC212 fermented wines except for the heavy lees treatments. These results demonstrate that both yeast strain and lees levels impacted the amount of total amino acids and specifically the sulfur containing amino acids methionine and cysteine.

Initial analysis of volatile sulfur compounds (VSCs) present during the lees aging study show low concentrations of all sulfur compounds assessed including H_2S. To date, there has been no large increase in the amounts of volatile sulfur compounds present in the wines during the aging process. Results to date demonstrate that high lee levels are not necessarily corresponded with formation of VSCs and that other factors, such as the presence of VSC pre-cursors, may be necessary for the formation of VSCs during lees aging. Further detail regarding the concentrations of VSCs in the wines can be found in a separate report prepared by Dr. Michael Qian entitled "Formation of volatile sulfur compounds in Pinot noir post-fermentation. Part 2: Lees Level and Contact Time on Volatile Sulfur Compounds in Wine". In contrast, samples provided from wineries have in some cases contained high concentrations of certain VSCs and in particular H_2S. Winery samples were taken from wines early during the barrel ageing process and indicate that perhaps the formation of VSCs shortly after going to barrel is more driven by release of H_2S than the formation of other VSCs derived from sulfur pre-cursors in the lees. The

presence of high concentrations of H_2S in the wines after barreling is contrary to the observation from the wineries that no noticeable H_2S was produced during alcoholic fermentation. This may point to either the importance of small amounts of H_2S produced during fermentation or the binding or reacting of H_2S with compounds during fermentation that is subsequently released post-fermentation. Further details can be found in a separate report prepared by Dr. Michael Qian entitled "Formation of volatile sulfur compounds in Pinot noir post-fermentation. Part 2: Lees Level and Contact Time on Volatile Sulfur Compounds in Wine.

VI. OUTSIDE PRESENTATIONS OF RESEARCH

Due to the early stage of this research results have not yet been presented at a professional conference.

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 is to focus on the role of grape amino acid content, its' relationship to wine lees composition during aging, and the impact this has on the development of volatile sulfur content. Results from this study to date have indicated that the amount of wine lees do not by themselves cause formation of volatile sulfur compounds but more likely create conditions conducive to formation of volatile sulfur compounds (VSCs). Other factors such as the presence of VSC pre-cursors may be necessary for the formation of VSCs during lees aging. While preliminary amino acid analysis showed that both yeast strain and lees levels impacted the relative concentrations of the sulfur containing amino acids methionine and cysteine we have not seen this cause an increase in the formation of VSCs that may be derived from these amino acids. However, the 9 month samples are yet to be analyzed for VSC's and so this may provide additional answers. Samples collected from wineries give some indication that the early formation of reductive smells soon after going to barrel are due to release/formation of H_2S rather than the formation of more complex VSC's such as mercaptens and disulfides. Where this H_2S is derived from is unknown at this point and will be the focus of upcoming experiments. Two possible sources are formation of H_2S by yeast during fermentation or reduction of elemental sulfur present on the grapes. Both of these sources will be explored as explanations for the results gained from the winery samples including looking into the presence of entrapped H_2S that is subsequently released during barrel aging.

VIII. FUND STATUS:

A graduate student, Daniel Kraft, has been employed to work on the project. Funds have been spent for collection of samples from collaborating wineries, winemaking, and equipment and HPLC supplies for amino acid analysis. The majority of remaining funds have been spent on salary and supplies and those remaining are allocated for salary.

Table 5. Peak areas of Amino acids in Pinot noir juice and Pinot noir wine fermented by *S. cerevisiae* P1Y2 and aged on light, medium, or heavy lees.

		Press Wine			3 Months Aging			6 Months aging		
	Juice	Light	Medium	Heavy	Light	Medium	Heavy	Light	Medium	Heavy
GLU	4.08	2.80	13.27	1.81	3.21	1.68	8.18	6.76	1.33	7.96
ASN	5.71	1.09	1.32	1.31	1.30	2.39	4.19	2.64	1.39	3.91
SER	7.72	20.10	15.65	15.10	20.23	23.44	45.59	18.40	35.17	27.10
GLN	1.75	6.37	3.96	5.42	3.93	7.84	11.02	5.80	5.87	10.08
HIS	1.18	9.84	12.86	8.24	10.42	12.35	11.51	13.52	14.12	10.27
GLY	14.07	14.94	14.85	11.82	14.42	11.21	14.49	16.10	16.53	14.23
THR	26.45	13.00	11.84	9.96	12.23	13.05	14.35	13.18	14.05	14.29
ARG	20.22	36.25	40.77	35.17	34.17	37.99	34.67	38.30	39.96	34.94
ALA	0.80	19.83	17.69	16.09	19.07	19.90	19.70	21.46	18.33	18.74
TYR	8.52	12.19	10.93	10.00	12.05	11.28	11.78	14.13	11.45	11.47
CYS	0.27	1.32	0.96	0.30	1.41	1.88	2.45	1.00	1.85	1.99
VAL	8.52	4.54	4.31	6.29	5.81	6.06	5.96	6.69	5.86	5.53
MET	1.42	1.00	0.51	0.40	1.78	2.75	4.97	0.98	2.16	3.61
TRP	1.47	1.83	0.89	2.47	1.65	1.51	2.72	2.29	1.92	2.84
PHE	0.30	0.10	0.10	0.95	0.10	1.59	0.10	2.08	0.10	0.10
ILE	5.00	1.57	1.31	0.98	1.38	3.84	1.05	3.83	3.51	1.35
LEU	1.24	3.45	2.82	2.58	3.87	1.36	2.37	2.52	1.29	3.67
LYS	2.40	0.69	0.83	1.10	0.60	0.90	0.59	1.11	1.14	0.82

Table 6. Peak areas of Amino acids in Pinot noir juice and Pinot noir wine fermented by *S. cerevisiae* RC212 and aged on light, medium, or heavy lees.

		Press Wine			3 Months Aging			6 Months aging		
	Juice	Light	Medium	Heavy	Light	Medium	Heavy	Light	Medium	Heavy
GLU	4.08	2.64	0.10	9.79	2.64	1.40	7.15	1.52	0.60	7.34
ASN	5.71	1.50	1.45	4.45	1.27	1.48	4.08	1.06	1.05	3.75
SER	7.72	10.05	12.58	13.37	12.84	13.29	10.75	13.34	12.51	34.99
GLN	1.75	8.67	4.59	4.70	2.94	3.90	10.74	3.78	3.01	10.22
HIS	1.18	7.89	6.83	11.88	10.32	7.66	10.44	7.31	7.95	14.40
GLY	14.07	2.29	12.02	17.09	13.79	11.05	12.43	10.70	11.95	18.38
THR	26.45	7.42	7.64	8.64	5.68	9.65	11.02	9.71	5.50	10.71
ARG	20.22	38.50	35.71	40.38	36.74	33.66	36.11	37.47	35.79	36.73
ALA	0.80	17.25	19.55	17.57	17.40	17.70	19.74	17.80	17.31	19.23
TYR	8.52	10.99	10.36	10.21	11.43	11.19	11.51	11.10	9.19	12.40
CYS	0.27	0.75	0.48	0.43	0.83	0.58	2.16	0.88	0.56	1.73
VAL	8.52	5.58	4.47	1.82	5.51	4.92	5.95	5.50	4.69	5.24
MET	1.42	0.33	0.20	0.48	0.60	0.78	4.36	0.70	0.23	2.83
TRP	1.47	1.09	1.14	0.55	0.79	0.87	12.67	1.29	0.84	1.53
PHE	0.30	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
ILE	5.00	1.82	1.24	1.31	1.04	1.24	1.36	1.81	1.36	1.58
LEU	1.24	2.62	2.11	3.42	2.31	2.76	3.47	2.75	2.11	4.21
LYS	2.40	1.30	0.58	0.90	0.58	0.72	1.06	1.09	0.61	1.06

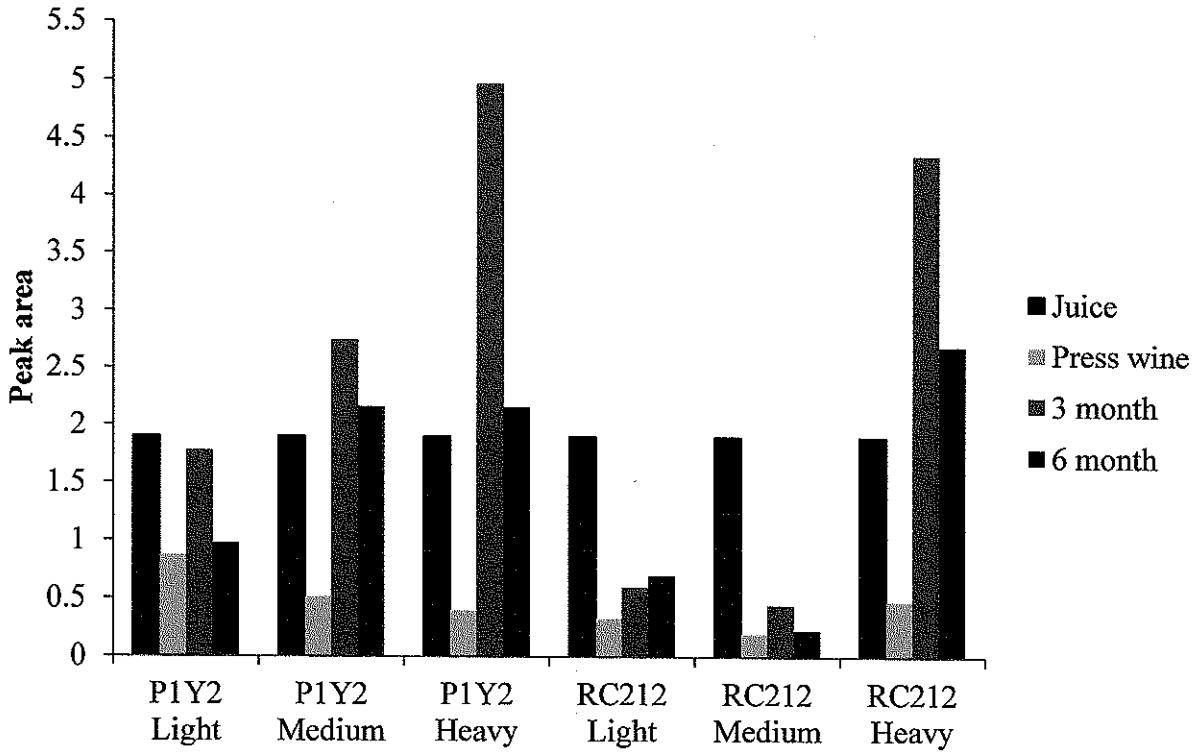

Figure 2. Concentration of methionine in Pinot noir juice and Pinot noir wine fermented by *S. cerevisiae* P1Y2 or RC212 and aged on light, medium, and heavy lees.

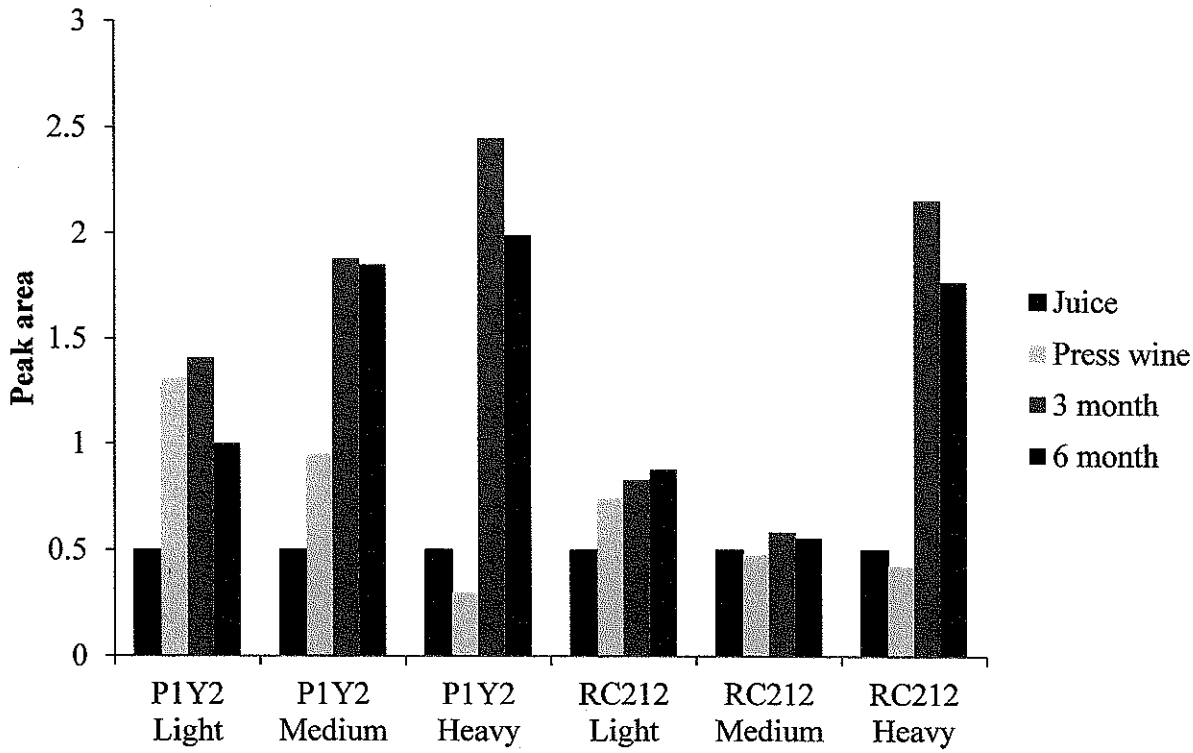

Figure 3. Concentration of cysteine in Pinot noir juice and Pinot noir wine fermented by *S. cerevisiae* P1Y2 or RC212 and aged on light, medium, and heavy lees.