

AN ABSTRACT FOR THE THESIS OF

Tresider R. Burns for the degree of Master of Science in Food Science and Technology presented on September 21, 2011.

Title: Impact of Malolactic Fermentation on Red Wine Color and Color Stability.

Abstract approved: _____
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Malolactic fermentation (MLF) is an important step in the production of wines and is commonly performed in red or cool climate wines to reduce acidity. In this study the impact of MLF on red wine color and the ability of *Oenococcus oeni* to degrade compounds important to the development of stable color were studied. Pinot noir and Merlot wines were produced, where simultaneous alcoholic and malolactic fermentations were induced in half of the wines. At dryness, all wines were pressed prior to sterile-filtration through 0.45 μm membranes. Wines that had not undergone malolactic fermentation (MLF (-)) were then either (a) inoculated with one of three strains of *O. oeni* (MLF (+)) or (b) pH adjusted to the same pH as MLF (+) wines. All wines were sterile-filtered, bottled, and stored at 13°C for analyses. MLF (+) wines had lower concentrations of acetaldehyde, pyruvic acid, and tartaric acid than MLF (-) wines. MLF (+) wines had significantly

lower color and polymeric pigments than MLF (-) wines while containing significantly higher monomeric anthocyanins. These differences were consistent throughout 12 months of storage demonstrating that MLF can affect red wine color independent of pH change and that *O. oeni* can impact phenolic and non-phenolic compounds involved in red wine color development.

Wine produced in a subsequent year was used to investigate possible reasons for the color loss caused by MLF as well as practical strategies to minimize these losses. One such strategy was to delay MLF, a practice that winemakers believe results in greater red wine color. Wines were held at 13°C for 0, 14, 28, 100, and 200 days before inoculation with *O. oeni* VFO to induce MLF at 25°C. Delaying MLF did not impact loss of color @ 520nm as all wines still experienced a color loss. However, as MLF was delayed for increasing time periods the polymeric pigment content and monomeric anthocyanin concentration of MLF (+) wines became more similar to those of the control. After 200 days delayed MLF, there was no statistically significant difference between the MLF (+) and control wine for polymeric pigment and only a minor difference in concentration of monomeric anthocyanins.

The reduced loss of polymeric pigment in delayed MLF wines may have been due to acetaldehyde being in the wine for a longer period of time as demonstrated in experiments investigating the impact of *O. oeni* metabolism of acetaldehyde and pyruvic acid metabolism. Wines that had undergone MLF were supplemented with

acetaldehyde and pyruvic acid to the levels measured in MLF (-) wines. Wines with acetaldehyde or acetaldehyde and pyruvic acid additions showed higher color and polymeric pigment than MLF (+) wine with no additions while addition of only pyruvic acid addition showed no improvement in color or polymeric pigment in comparison to standard MLF (+). However, acetaldehyde additions did not completely prevent a loss of color at 520nm after MLF. Whether this color loss was due to fining by *O. oeni* was investigated through exposure of wine to live or inactivated *O. oeni* for differing time periods. Wines that did not undergo MLF but were exposed to live or inactivated cells showed no difference in color, polymeric pigment, and monomeric anthocyanin compared to the control suggesting that loss of color during MLF was not due to fining by *O. oeni* cells

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IMPACT OF MALOLACTIC FERMENTATION ON RED WINE COLOR AND COLOR
STABILITY

by
Tresider R. Burns

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I understand that my thesis will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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CHAPTER 1

LITERATURE REVIEW

Wine Quality

The quality of a wine, while highly subjective, is established through vineyard practices and resulting fruit quality (Reynolds et al. 2007; Peterlunger et al. 2002), enological methods (Harbertson et al. 2009), and even marketing (Charters and Pettigrew 2007). The parameters used by consumers to determine quality include grape variety, color, flavor, and production region (Perrouy et al. 2006).

Wine Color

Visual assessment is the first step in the varied sensory experience that is the consumption of wine. For red wines, increased phenolic content and red color intensity correlate to a higher level of claimed quality (Mercurio et al. 2010) and consumers may perceive a lighter-colored red wine to be of lower quality. Lightly pigmented wines, such as Pinot noir and Gamay Noir, may suffer from this consumer misperception. Red wine color is primarily determined by the anthocyanin content of the wine. Anthocyanins are phenolic plant metabolites belonging to the flavonoid family which are responsible for most red and blue colors in fruits, berries, and flowers. In grapes, the majority of the anthocyanins are located in the grape skins. These compounds are water-soluble pigments that are extracted into the wine during alcoholic fermentation (Zoecklein et al. 1995).

In *Vitis vinifera* grapes, the anthocyanins extracted into the must are predominantly 3-monoglucosides of five aglycones: malvidin, peonidin, petunidin, cyanidin, and delphinidin (Brouillard et al. 2003). Interestingly, Pinot noir wines do not contain acylated forms of these monoglucosides. These simpler structures of the monomeric anthocyanin are more susceptible to chemical reactions including oxidation as reactive sites are not protected by the steric hindrance of cofactors or bulky side chains.

The color of an anthocyanin is highly influenced by the pH of the solution. At wine pH, a monomeric anthocyanin would be expected to be hydrated and thus, colorless. The presence of red color in anthocyanins at pH 3-4 suggests stabilization through reactions which impede nucleophilic addition of water to the pigment (Cheynier et al. 2006). Among the anthocyanins present in finished wine, malvidin-3-glucoside is found in the highest concentration (Ribereau-Gayon 1982). In Merlot grapes, malvidin-3-glucoside represents approximately 62% of anthocyanin content in crushed skins and 55% of anthocyanin content in the finished wine (Romero-Cascales et al. 2005). In Pinot noir, malvidin-3-glucoside has been reported as 65-67% of total anthocyanin content in crushed skins (Peterlunger et al. 2002). Monomeric anthocyanin concentration peaks as early as three days after onset of ferment (Zimman and Waterhouse 2004) and as late as five to seven days (Mayen et al. 1994) as the monomeric forms begin to react with cofactors in the must.

However, the color of a red wine is not solely determined by the concentration of monomeric anthocyanins in the wine. Following extraction, anthocyanins rapidly form polymeric pigments (Zimman and Waterhouse 2004), copigmentation complexes (Boulton 2001), and vitisins A and B (Schwarz et al. 2003; Morata et al. 2003). Development of these stable anthocyanin complexes is impacted by the concentration of compounds such as *p*-coumaric acid, caffeic acid, catechin, and quercetin for copigmentation reactions and pyruvic acid and acetaldehyde for vitisin formation. Alternatively, reaction of the monomeric anthocyanin with sulfur dioxide can result in a colorless adduct (Berké et al. 1998). Polymeric pigments are the result of a condensation reaction between the anthocyanin and a flavanol (tannin). Formation of stable polymeric pigments begins during early stages of fermentation and continues with time. During aging, levels of monomeric anthocyanins decrease as they are incorporated into polymeric pigments which represent 50 to 70 percent of a one year old wine's color (Nagel and Wulf 1979). Polymeric pigments are resistant to oxidation and sulfur dioxide bleaching although some oligomeric forms may be rendered colorless by reaction with sulfur dioxide (Versari et al. 2007). While stabilizing the pigment, the condensation of the tannin and anthocyanin also results in a hyperchromic shift increasing the absorbance of the anthocyanin at 520nm. After reaction with the anthocyanin, the flavanol structure continues to polymerize to various degrees making polymeric pigments a heterogeneous mixture of varying molecular weight compounds.

Copigmentation is the noncovalent association of an anthocyanin monoglucoside and a phenolic acid, flavonoid, or flavonol derivative (Boulton 2001). This association results in a hyperchromic shift at 520nm as well as a bathochromic shift in which the maximum absorbance wavelength of the anthocyanin is shifted upward towards the blue end of the visible spectrum. The resulting association is responsible for a “purplish” tint described in new wines. In copigments, the planar “stacking” of the anthocyanin and cofactor protect the hydration, and resultant color loss, of the anthocyanin (Fulcrand et al. 2006). Copigments disassociate during the aging of a wine and account for a reducing proportion of the wine’s color eventually disappearing almost entirely after one year of aging (Schwarz et al. 2005).

Another form of stabilized pigment occurs when an anthocyanin covalently reacts with pyruvic acid or acetaldehyde to form vitisins A and B, respectively. Vitisins are extremely stable forms of color which can still be detected in a wine after fifteen years of aging, long after the monomeric anthocyanins are no longer detectable (Schwarz et al. 2003). Also known as pyranoanthocyanins, vitisins comprise approximately 5% of the colored pigments in a wine although these levels can rise above 15% with different wine varieties (Alcalde-Eon et al. 2006).

Winemaking Impacts on Color

While the anthocyanin content of a grape is primarily determined by the grape’s variety and vineyard practices employed to produce it, enological methods can

manipulate the degree to which those anthocyanins are extracted during vinification. In a review of the effect of winemaking techniques on phenolic extraction (Sacchi et al. 2005), six winemaking techniques or variables were found to increase the phenolic composition of a wine. These techniques included fermentation temperature, thermovinification, must freezing, saignée, pectolytic enzyme treatments, and extended maceration. Other variables such as yeast selection and carbonic maceration, the partial fermentation of whole berries in a carbon dioxide environment, were found to have mixed results.

An increase of fermentation temperature from 20° to 30°C has been shown to increase color in Pinot noir (Girard et al. 1997), Shiraz (A. Reynolds et al. 2001), and Cabernet Sauvignon (Monticelli et al. 1999). While anthocyanin content increased slightly, these studies found that phenolic increases in the wine were largely driven by greater extraction of tannin from the grape skin and seeds. The greater concentration of tannin in the wine favored the condensation of polymeric pigments thus resulting in wines with a higher proportion of polymeric pigments and higher color. An extreme example of temperature impacting wine color is thermovinification. This is where the grape must is heated to 60° to 70°C for a short time before cooling and initiating ferment. The high temperature damages grape skin cells and releases anthocyanins while also denaturing the enzyme polyphenol oxidase which causes oxidative browning. In comparison to high fermentation temperatures, thermovinification favors the release of anthocyanins

as opposed to tannins (Auw et al. 1996; Gao et al. 1997). Gao et al. (1997) reported that color was increased in wines undergoing thermovinification, but over time, little difference was detected in polymeric pigment formation given the reduced extraction of tannin.

Extended maceration allows the grape pomace longer contact with the ethanol produced during fermentation. As tannin and anthocyanin are ethanol soluble, winemakers may extend the time period between the end of fermentation and pressing to allow for greater extraction of phenolic compounds. As with elevated fermentation temperature, extended maceration favors the extraction of tannins in relation to anthocyanins (Zimman et al. 2002). Extended maceration results in wines with higher levels of tannin and polymeric pigment with little to no increase in anthocyanins. Many of the tannins extracted during this process are derived from the grape seed which can impart extreme bitterness and negative sensory impacts on the wine (Harbertson et al. 2009).

Saignée is a prefermentation juice run off to increase the skin surface area to juice ratio. The amount of juice to solubilize the anthocyanins is reduced but anthocyanin extraction and color are increased (Harbertson et al. 2009). This suggests that anthocyanin extraction is not limited by solubility constraints during the winemaking process. In comparison to the previous winemaking techniques, the juice runoff increases both tannin and anthocyanin concentrations. Increased polymeric pigment and monomeric anthocyanins can still be detected years after

bottling (Gerbaux 1993). Interestingly, research on juice run off in Syrah wines by (Gawel et al. 2001) showed increased anthocyanin content in only one of six juice runoff treatments. After six months of aging, there was no detectable difference between treatments suggesting there was little value in the treatment for the Syrah variety.

A winemaking technique commonly employed to improve color, particularly in Pinot noir production in Oregon, is a cold soak or pre-fermentation maceration of the grape must before alcoholic fermentation. In a cold soak treatment, the grape must temperature is lowered to 10° to 15° and held for one to fourteen days (in extreme cases) before being warmed to initiate alcoholic fermentation. The purported goal of the cold soak is to allow for aqueous extraction of anthocyanins before fermentation. Research has provided conflicting results as to the color enhancement benefits of cold soak treated fermentations. (Poussier et al. 2003) found no increase in color or anthocyanins in Merlot wine with a sixty hour cold soak prior to fermentation. However, higher levels of resveratrol were observed and attributed to microbiological activity during the cold soak, a time when yeast has yet to produce ethanol which will inhibit other organisms. Cold soaks performed on Shiraz grapes (A. Reynolds et al. 2001) increased the anthocyanin content of the wine but only if the alcoholic fermentation was conducted at a lower temperature (15 and 20°C) and not at a higher temperature (30°C). (Marais 2003) actually found reduced levels of anthocyanins and tannins in Pinotage wine which

had undergone one, two, and four day cold soaks. The cold soak treatment wines were preferred by a sensory panel due to their increased aromatic characters, a result of glycosidase activity which liberated bound flavor molecules. The sensory impact of cold soak treatments may result in a more aromatic wine but not necessarily a better colored one.

Microorganisms in Wine

Aside from the techniques employed by the winemaker to increase phenolic extraction, microorganisms can also influence red wine color. A number of recent studies have focused on the influence of the wine yeast *Saccharomyces cerevisiae* on red wine color. For example, (Hayasaka et al. 2007) reported that Cabernet Sauvignon wines produced by two different yeast strains, a *Saccharomyces cerevisiae* strain and a *Saccharomyces bayanus* strain, resulted in significantly different color properties. The authors attributed the differences to the higher production of acetaldehyde by the *S. bayanus* strain leading to greater vitisin B formation. In another study, Morata et al. (2003) demonstrated a strong correlation between pyruvate and acetaldehyde production by yeast and vitisin A and B formation. The other mechanism by which yeast influences color in wine is adsorption of anthocyanins to the cell wall. (Medina et al. 2005) found that yeast adsorb anthocyanins of higher polarity and can greatly reduce anthocyanin concentrations. Interestingly, (Palomero et al. 2007) found that autolysis of yeast

may lead to the release of polysaccharides which stabilize anthocyanins. These and other studies have clearly shown that the action of yeast during alcoholic fermentation can influence the formation of red wine color. However, the development of red wine color is an ongoing process that continues long after the alcoholic fermentation is complete. In particular, the concentration of compounds like pyruvic acid and acetaldehyde do not remain stable and can be affected by other winemaking practices such as the malolactic fermentation (MLF).

The MLF is the enzymatic decarboxylation of D-malic acid to L-lactic acid by wine lactic acid bacteria which results in a decrease in acidity (Pilone and Kunkee 1972; Rossi and Clementi 1984). It is usually performed by *Oenococcus oeni* and is particularly important for wines produced in cool climates which often contain high acidity. Since early work by (Kunkee et al. 1965) and (Pilone et al. 1966) observed few differences in the sensory characteristics and chemical composition of wines fermented by various strains of bacteria, it appeared that any strain could be used to induce MLF. However, this does not appear to be the case since strains have varying tolerances to low pH, sulfur dioxide, and temperature (Henick-Kling et al. 1989; Wibowo et al. 1988; Izuagbe et al. 1985) and can variably alter sensory quality (Boido et al. 2002; Delaquis-Pascal et al. 2000; Rodriguez et al. 1990; McDaniel et al. 1987; Giannakopoulos et al. 1984). With regard to wine color, winemakers have reported that MLF causes a decrease in color, an observation noted in some studies investigating the MLF (Rankine et al. 1970; Husnik et al.

2007) and typically attributed to pH increase of 0.1 to 0.3 units that occurs post MLF (Rankine et al. 1970). Other studies have demonstrated that *O. oeni* can impact the concentration of compounds involved in red wine color such as acetaldehyde (Osborne et al. 2000) and even SO₂ bound acetaldehyde (Osborne et al. 2006), a compound important in the development of red wine color. In addition, *O. oeni* has been shown to degrade pyruvic acid in wine (Asentorfer et al. 2003) and subsequently impact the formation of vitisin A. So although yeast strains may produce varying amounts of acetaldehyde and pyruvic acid, the malolactic bacteria may have the last say as to the concentration of these compounds in wine available for reaction with anthocyanins. Additionally, there is some evidence that malolactic bacteria are capable of degrading the compounds involved in co-pigmentation such as *p*-coumaric acid, and caffeic acid (Hernandez et al. 2007; Hernandez et al. 2006; Cavin et al. 1993).

Despite evidence that the MLF can impact compounds involved in the development of red wine color, few studies have focused on its specific impact on red wine color. In a study by Husnik et al. (2007) the capability of the genetically engineered malolactic yeast ML01 to perform the MLF was investigated. It was noted that Cabernet Sauvignon wines produced by ML01 (MLF performed by the yeast) and yeast strain S92 had darker color than wine produced by S92 with a bacterial MLF. The pH of the wines produced were very similar indicating that the loss of color was not due to the change in pH caused by the MLF. The authors speculated the

color change may have been due to the metabolic activity of *O. oeni* impacting negatively on anthocyanins in the wine. However, the impact of *O. oeni* on the concentration of compounds important to color development was not measured in this study. In one of the few other studies to note the impact of MLF on color, (Delaquis et al. 2000) reported that color intensity and redness were enhanced in a Chancellor wine when a particular malolactic culture was used to induce the MLF. Again however, the cause(s) behind this observation were not investigated further. Therefore, the purpose of this research was to quantify the color loss that occurs as a result of the MLF and identify the mechanisms by which MLF bacteria impact red wine color and color stability. The specific objectives of the study were:

- 1.) Investigate the impact of the malolactic fermentation and various *Oenococcus oeni* strains on the color and color stability of red wine
- 2.) Determine the ability of *O. oeni* to degrade the co-pigments caffeic acid and *p*-coumaric acid as well as the vitisin A and B precursors pyruvic acid and acetaldehyde
- 3.) Identify the specific mechanisms by which the MLF impacts color and color stability, degradation of acetaldehyde and/or pyruvic acid, and adsorption of anthocyanins to *O. oeni* cell walls.
- 4.) Explore the use of delaying the MLF to enhance red wine color as a way to mitigate color loss during the MLF.

CHAPTER 2

IMPACT OF MALOLACTIC FERMENTATION ON RED WINE COLOR
AND COLOR STABILITY

ABSTRACT

The impact of MLF on red wine color and the ability of *Oenococcus oeni* to degrade compounds important to the development of stable color were studied. Pinot noir and Merlot wines were produced, where simultaneous alcoholic and malolactic fermentations were induced in half of the wines. At dryness, all wines were pressed prior to sterile-filtration through 0.45 μm membranes. Wines that had not undergone malolactic fermentation (MLF (-)) were then either (a) inoculated with one of three strains of *O. oeni* (MLF (+)) or (b) pH adjusted to the same pH as MLF (+) wines. All wines were sterile-filtered, bottled, and stored at 13°C for analyses. MLF (+) wines had lower concentrations of acetaldehyde, pyruvic acid, and caftaric acid than MLF (-) wines. MLF (+) wines had significantly lower color and polymeric pigments than MLF (-) wines while containing significantly higher monomeric anthocyanins. These differences were consistent throughout 12 months of storage demonstrating that MLF can affect red wine color independent of pH change and that *O. oeni* can impact phenolic and non-phenolic compounds involved in red wine color development. There was no difference in final color and polymeric pigment for the three strains of *O. oeni* tested.

INTRODUCTION

The color of red wine is an important sensory attribute that originates primarily from anthocyanins present in the grape skins (Vivar-Quintana et al. 2002; Fulcrand et al. 2006). These compounds are water-soluble pigments that are extracted into the wine during fermentation (Zoecklein 1995). However, red wine color is due not just to the concentrations of these compounds in the wine. Once anthocyanins have been extracted into the wine they may rapidly form co-pigmentation complexes (Boulton, 2001) where non-colored organic compounds such as *p*-coumaric acid, caffeic acid, catechin, and quercetin associate with anthocyanins (Schwarz et al. 2005; Boulton, 2001; Brouillard et al. 1989) resulting in a hyperchromic shift at 520nm as well as a bathochromic shift towards the blue end of the visible spectrum. Anthocyanins can also polymerize with other anthocyanins and tannins forming pigmented polymers (Fulcrand et al. 1998; Bakker and Timberlake, 1997). A number of anthocyanin derived pigments (pyranoanthocyanins) can be formed by condensation with metabolites, such as pyruvic acid and acetaldehyde, released by fermenting yeast (Morata et al. 2003). For example, pyruvic acid reacts with malvidin-3-*O*-glucoside to form the pyranoanthocyanin vitisin A while acetaldehyde reacts with malvidin-3-*O*-glucoside to form the pyranoanthocyanin vitisin B (Morata et al. 2007; Morata et al. 2003). These compounds are more resistant to SO₂ bleaching than other

anthocyanins and are also resistant to oxidation (Bakker and Timberlake 1997) making them an important component of red wine color and color stability.

Because co-pigmentation and polymeric pigment formation play such an important role in red wine color development and stability, it is important to understand the factors that influence them. For example, while anthocyanin concentration is primarily determined in the vineyard, the types and amounts of other compounds involved in red wine color development can be dramatically influenced by winemaking practices such as increasing fermentation temperature (Girard et al. 1997; Reynolds et al. 2001), extended maceration (Zimman et al. 2002) and saignee (Harbertson et al. 2009). In addition, some recent studies have investigated the impact of wine microorganisms on red wine color. For example, Hayasaka et al. (2007) reported that Cabernet Sauvignon wines produced by two different yeast strains, a *Saccharomyces cerevisiae* strain and a *Saccharomyces bayanus* strain, resulted in significantly different color properties. The authors attributed the differences to the higher production of acetaldehyde by the *S. bayanus* strain leading to greater vitisin B formation. In another study, Morata et al. (2003) demonstrated a strong correlation between pyruvate and acetaldehyde production by yeast and vitisin A and B formation. These and other studies have demonstrated that the action of yeast during alcoholic fermentation can influence the formation of red wine color. However, the development of red wine color is an ongoing process that continues long after the alcoholic fermentation is complete.

The concentration of compounds like pyruvic acid and acetaldehyde do not remain stable and can be affected by other winemaking practices such as the malolactic fermentation (MLF).

The MLF involves the decarboxylation of malic acid to lactic acid by wine lactic acid bacteria, typically *Oenococcus oeni*, resulting in a decrease in acidity that is important for red wines produced in cool climates (Pilone and Kunkee 1972).

Although deacidification is the primary goal of performing the MLF, this process can also result in changes in wine aroma, flavor, and texture (Delaquis-Pascal et al. 2000; Rodriguez et al. 1990; McDaniel et al. 1987; Giannakopoulos et al. 1984). In addition, winemakers have reported that MLF causes a decrease in color, an observation noted in some studies investigating the MLF (Rankine et al. 1970; Husnik et al. 2007) and attributed to pH increase post MLF. Other studies have demonstrated that *O. oeni* can impact the concentration of compounds involved in red wine color such as acetaldehyde (Osborne et al. 2006; Osborne et al. 2000), pyruvic acid (Asentorfer et al. 2003), and the co-pigments *p*-coumaric acid, and caffeic acid (Hernandez et al. 2007; Hernandez et al. 2006; Cavin et al. 1993).

Despite this, few studies have focused on the role MLF may have in the color development of red wines. In a study by Husnik et al. (2007) the capability of the genetically engineered malolactic yeast ML01 to perform the MLF was investigated. It was noted that Cabernet Sauvignon wines produced by ML01 (MLF performed by the yeast) and yeast strain S92 had darker color than wine produced

by S92 with a bacterial MLF. The pH of the wines produced were very similar indicating that the loss of color was not due to the change in pH caused by the MLF. The authors speculated the color change may have been due to the metabolic activity of *O. oeni* impacting negatively on anthocyanins in the wine. However, the impact of *O. oeni* on the concentration of compounds important to color development was not measured. Delaquis et al. (2000) reported that color intensity and redness were enhanced in a Chancellor wine when a particular malolactic culture was used to induce the MLF. Again however, the cause(s) behind this observation were not investigated further. Clearly the malolactic fermentation has the potential to impact color intensity and stability but this phenomenon has not been well characterized. Therefore, the purpose of this research was to investigate the influence of the malolactic fermentation on the color and color stability of red wine and the ability of *O. oeni* to degrade the co-pigments caffeic acid and *p*-coumaric acid as well as the vitisin A and B precursors pyruvic acid and acetaldehyde.

MATERIALS AND METHODS

Grapes

Pinot noir and Merlot grapes were harvested on October 4th and October 14th, 2009, respectively, from Oregon State University's Woodhall Vineyard (Alpine, Oregon, USA). Harvest was determined by sugar levels and fruit ripeness

according to the specifications of the vineyard manager. Upon harvest, grapes were stored for forty-eight hours at 4°C (39.2°F) before being hand-sorted and destemmed with a Velo DPC 40 destemmer/crusher (Altivole, Italy). Pinot noir grapes were not run through the crusher while Merlot grapes were. The grapes were then pooled and divided into 100L stainless steel tanks containing approximately sixty liters of grape must each. An addition of 50mg/L SO₂ (in the form of potassium metabisulfite) was added to each tank and the yeast nutrient Fermaid K® (Lallemand, Montreal, Canada) was added at a rate of 0.125g/L.

Alcoholic Fermentation

Grape must was inoculated with *Saccharomyces cerevisiae* yeast strain VQ-15 (Lallemand) at a rate of 0.25 grams dried yeast per liter of must (approximately 1x10⁶ CFU/mL). Yeast was hydrated according to manufacturer's specifications prior to inoculation. One set of three tanks for both Pinot noir and Merlot was concurrently inoculated with *O. oeni* strain VFO at approximately 1x10⁶ CFU/mL to induce the malolactic fermentation (simultaneous alcoholic and malolactic fermentation).

Fermentations were conducted in a temperature-controlled room held at 26.6°C (80°F). Cap punch downs were performed uniformly twice daily and temperature and °Brix were measured with an Anton-Paar DMA 35N Density Meter (Graz, Austria). Completion of alcoholic fermentation (reducing sugar concentration

below 0.2g/100mL) was confirmed by testing with Bayer Clinitest tablets (Morristown, New Jersey, USA). Malic acid levels were measure by enzymatic assay (R-Biopharm, Darmstadt, Germany) with completion of MLF confirmed once malic acid concentration was below 0.050g/L.

Upon completion of alcoholic fermentation and MLF in all simultaneous treatments, wines were pressed with a Willmes model 6048 pneumatic bladder press (Lorsch, Germany). Each tank of wine was held in the press at a pressure of 0.5 bar for one minute. The press was opened, cake broken up by hand, and run at 1.0 bar for two minutes. Replicates of wine produced by simultaneous ferment were pressed separately while other wines were pooled and mixed after pressing. Wines were then placed in a cold room at 3°C for forty-eight hours.

Filtration

Following cold settling, wines were racked and then filtered through a plate and frame filter fitted with nine 20cmx20cm Beco K-1 3.0µm nominal filter sheets (Langenlonsheim, Germany). Wine was then filtered through 1.0 and .45µm polyethersulfone cartridges (G.W. Kent, Ypsilanti, Michigan, USA) in succession. Filtered wine was then dispensed into sterile one gallon carboys.

Malolactic Fermentation

Three strains of *Oenococcus oeni* were used for the study: Viniflora Oenos (Chr. Hansen, Hørsholm, Denmark), VP41 (Lallemand), and Enoferm® Alpha

(Lallemand). Treatments were inoculated at a rate of approximately 1×10^6 CFU/mL. Malolactic bacteria were hydrated in 0.1% peptone blanks for twenty minutes before inoculation into MLF treatments. Progress of fermentation was monitored by enzymatic assay (Roche Pharmaceuticals, Basel, Switzerland). After completion of MLF, all wines were cold settled at 3°C for forty-eight hours.

Bottling

Upon completion of MLF, all treatments received a sulfite addition equal to 35mg/L SO₂. A portion of the wine that did not undergo MLF was pH adjusted (by addition of 2N NaOH) to match the pH of the wines that had undergone MLF. All wines were then filtered through a 0.45µm polyethersulfone cartridge (GW Kent) and bottled in 350mL brown glass beer bottles and sealed with crown caps. Bottled wines were placed in a cold room at 12.8°C (55°F) until needed for analysis. For purposes of analysis, the day of bottling is considered the day zero time point.

Color Analysis

Wines were analyzed every ninety days for a variety of parameters. All wine samples were adjusted to pH 3.60 prior to testing by addition of 2N NaOH or 25% H₃PO₄. Color was determined by spectrophotometric analysis (Shimadzu UV-3101PC, Kyoto, Japan) at 420nm and 520nm in a quartz 1mm pathlength cuvette. Polymeric pigment and copigmentation were measured by spectrophotometric

analysis (Levengood Joanne and Boulton Roger 2004) (Thermo Scientific Genesys, Madison, Wisconsin, USA) in 10mm polystyrene cuvettes.

High Performace Liquid Chromatography

Anthocyanins and hydroxycinnamic acids were analyzed by high-performance liquid chromatography (HPLC) using a Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA) equipped with HP ChemStation software and photodiode-array detector (DAD). The HPLC was fitted with a LiChroSpher reverse-phase C18 column (4 x 250mm, 5mm particle size) (Merck, Darmstadt, Germany) held at 30°C. All chromatographic solvents were HPLC grade. 98% Formic acid (EMD Chemicals, Darmstadt, Germany) was purchased from Sigma Aldrich (St. Louis, MO). 99.8% Methanol (EMD Chemicals) was purchased from Oregon State University Chemistry Stores (Corvallis, OR). Gradients of solvent A (water/formic acid, 90:10, v/v) and solvent B (methanol) were applied as follows: 5 to 35% B linear (1.0 mL/min) from 0 to 15 min, static at 35% B (1.0 mL/min) from 15 to 20 min, 35 to 80% B linear (1.0 mL/min) from 20 to 25 min, then 5% B (1.0 mL/min) from 25 to 32 min to reequilibrate the column to initial conditions.

Wine samples were centrifuged in 1.5ml microcentrifuge tubes (VWR, Radnor, PA) at 12,000rpm for 10 minutes utilizing an Allegra X-22 centrifuge (Beckman Coulter, Brea, CA) before sampling by HPLC. Wines were sampled in 20µL aliquots. Anthocyanins and hydroxycinnamic acids were detected by scanning from 190 to 700nm. Quantification of anthocyanins was performed against an

external standard of malvidin-3-glucoside (Sigma Aldrich) at 520 nm and expressed as a function of malvidin-3-glucoside concentration. Quantification of hydroxycinnamic acids was performed at 320nm. Caffeic and *p*-coumaric acid external standards were measured while caftaric acid was expressed as caffeic acid equivalents.

Additional Analysis

Acetaldehyde and pyruvic acid concentrations were measured utilizing enzymatic assay (R-Biopharm). Tannin levels were measured according to protein precipitation assay (Adams and Harbertson 1999).

A univariate Analysis of Variance (ANOVA) was used to determine differences between wine treatments. The ANOVA was performed by Minitab® 16.1.1 (State College, Pennsylvania, USA). Tukey's HSD multiple comparison was performed to test least squares means of treatment effects at the 0.05% significance level.

RESULTS

Fermentation

Basic juice parameters of the Pinot noir must after processing were pH 3.35, 25.2 °Brix, and 0.683g/100mL titratable acidity (grams tartaric acid) while the Merlot had parameters of pH 3.55, 24.9 °Brix, and 0.510g/100mL titratable acidity.

Alcoholic fermentations for both the Pinot noir and Merlot proceeded similarly with the Pinot noir fermentations completing in nine days (< 0.5 g/L reducing sugars) while the Merlot alcoholic fermentations were completed in six days.

Pinot noir and Merlot wines undergoing simultaneous alcoholic and malolactic fermentations also completed alcoholic fermentation in nine and six days respectively. In addition, MLF in the Pinot noir wines also completed in nine days (< 0.05 g/L malic acid). However, for the Merlot simultaneous fermentations the MLF proceeded slower and were complete after thirteen days.

Color Analysis

All wines that underwent MLF (MLF+) had reduced color compared to the control (Fig 2.1A & 2.2A). For Pinot noir wines there was approximately an 18% reduction in color @520 nm at the completion of MLF (day 0) no matter which *O. oeni* strain was used or the timing of the MLF (simultaneous or consecutive inoculation) (Fig 2.1A). Over time this reduction in color remained as after 270 days storage, MLF

(+) wines still had approximately 18% less color @520 nm than the control wines (Fig 2A). No difference between the pH adjusted control wine and the control was observed. An even greater loss in color was observed for Merlot wines that had undergone MLF (Fig 2.2A). For example, at the completion of the MLF wines had approximately 22% less color @ 520nm than the control wine and this difference remained during storage (Fig 2.2A). Again, there was no difference in color between the *O. oeni* strains and also no difference between the pH adjusted control and the control wine. There was, however, a significant difference between the color of Merlot wines that had undergone a simultaneous alcoholic and MLF and Merlot wines that underwent MLF at the end of the alcoholic fermentation (Fig 2.2A) with the simultaneous wines having the lowest color @520nm.

Polymeric pigment content was also reduced in MLF (+) wines. For Pinot noir, MLF (+) wines contained about 17% less polymeric pigment content at the completion of MLF (day 0) than the control wine (Fig 2.1B). This difference increased over time with a 20% difference being noted after 180 days storage and an 18% difference after 270 days. For Merlot wines the differences were larger. In Merlot, MLF (+) wines contained approximately 23% less polymeric pigment at the completion of MLF and close to 40% less after 180 days storage (Fig 2.2B). The difference between the control wines and the MLF wines lessened after 270 days storage although there was still a 20% reduction in polymeric pigment content. As was seen with color @ 520nm there was no difference between *O. oeni* strains with

regards to polymeric pigment content or between the pH adjusted control and the control wine. However, Merlot wine produced by a simultaneous fermentation consistently contained the lowest amount of polymeric pigment. For both Pinot noir and Merlot, all wines whether MLF (-) or MLF (+), polymeric pigment content was highest at day 180 and had declined by day 270.

In addition to spectrophotometric analysis of color, the monomeric anthocyanin content of the wines was analyzed. As was seen for color @ 520nm and polymeric content there were significant differences between the control wines and MLF (+) for both Pinot noir (Fig 2.1C) and Merlot (Fig 2.2C). Overall, monomeric anthocyanin concentrations were highest at day 0 and decreased over time. However, MLF (+) wines contained significantly higher concentrations of monomeric anothcyanins than the control wines at every sampling point during storage (Fig 2.1C & 2.2C). Again, no differences were noted between the *O. oeni* strains used or between the pH adjusted control and the control wine.

Chemical Analysis

Wines were also analyzed for compounds that are involved in polymeric pigment formation and copigmentation reactions. Acetaldehyde and pyruvic acid levels measured after the completion of malolactic fermentation are shown in tables 2.1 and 2.2. For Pinot noir and Merlot MLF (+) treatments there was a significant reduction in the concentrations of acetaldehyde and pyruvic acid compared

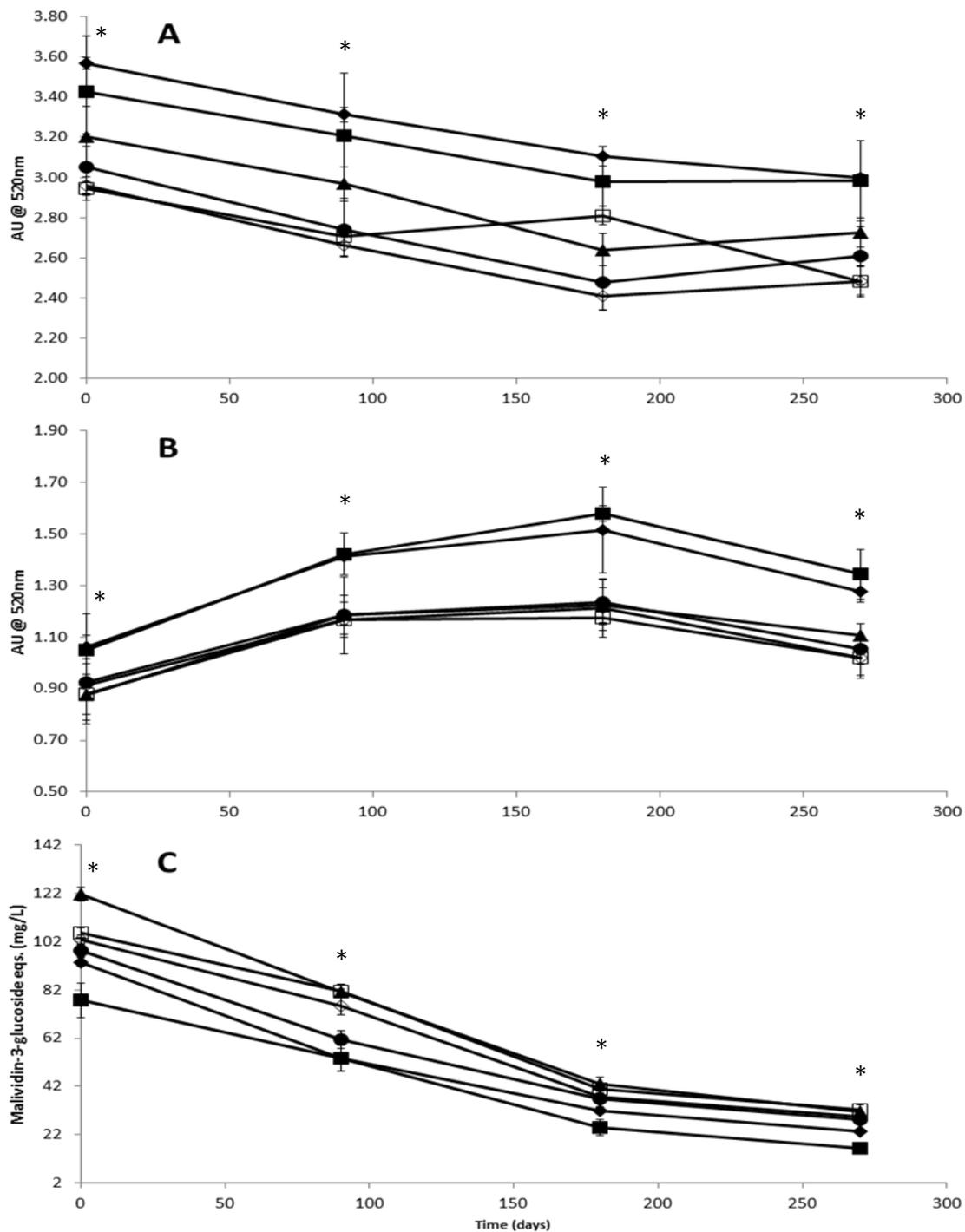


Figure 2.1: Color (A), polymeric pigment (B), and monomeric anthocyanins (C) of Pinot noir wines that have or have not undergone malolactic fermentation during storage at 13°C. (◆) Control (no MLF), (■) pH adjusted control, (▲) simultaneous alcoholic and malolactic fermentation, (●) *O. oeni* Alpha, (◇) VP41, (□), VFO. Error bars represent \pm one standard deviation.(n=3). * indicates significant differences at the $p < 0.05$ level

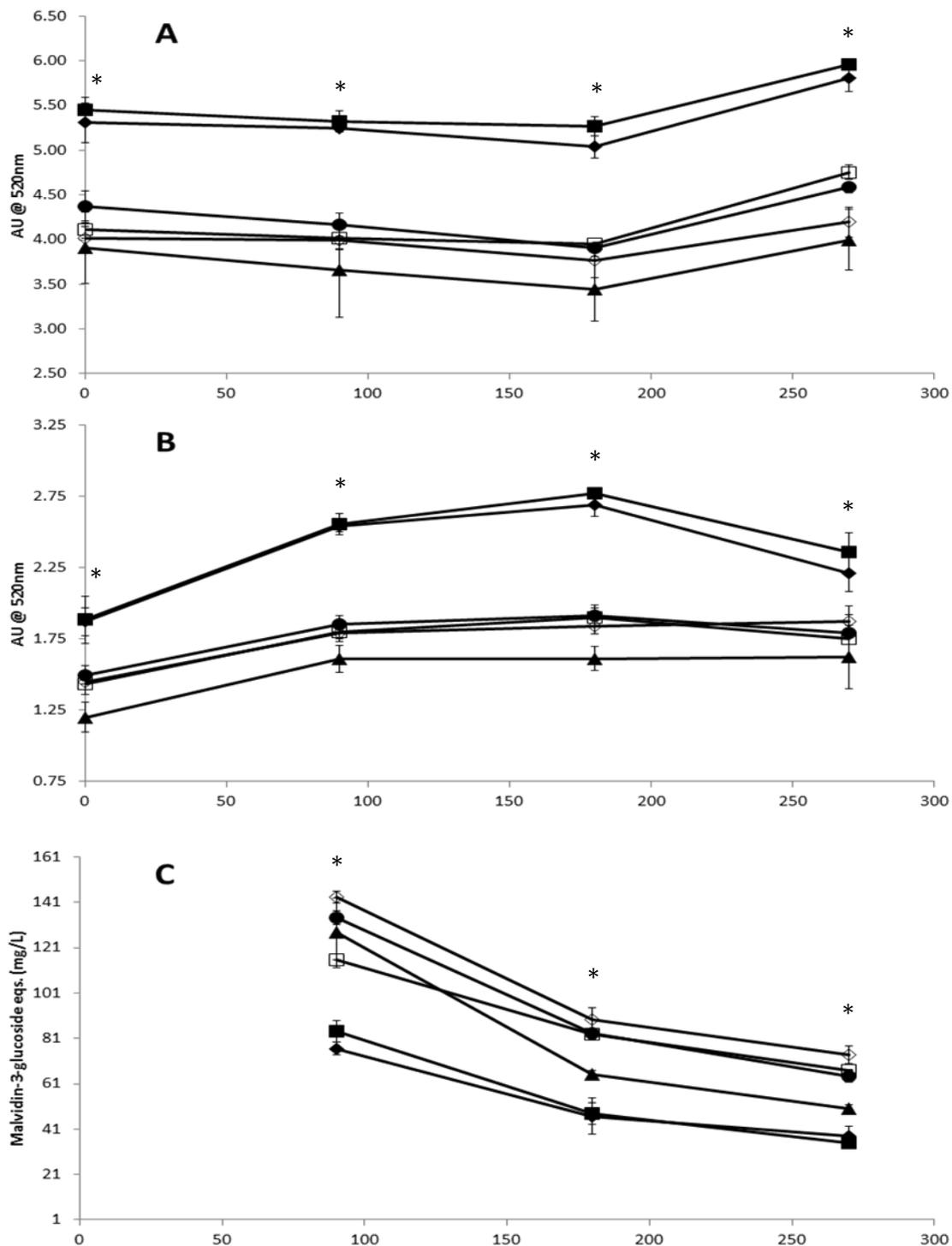


Figure 2.2: Color (A), polymeric pigment (B), and monomeric anthocyanins (C) of Merlot wines that have or have not undergone malolactic fermentation during storage at 13°C. (◆) Control (no MLF), (■) pH adjusted control, (▲) simultaneous alcoholic and malolactic fermentation, (●) *O. oeni* Alpha, (◇) VP41, (□), VFO. Error bars represent \pm one standard deviation (n=3). * indicates significant differences at the $p < 0.05$ level

to the control. Of the three strains of *O. oeni* used, VFO demonstrated the largest reduction in both acetaldehyde and pyruvic acid. VFO was also used in the simultaneous fermentation treatment which showed a similar reduction of acetaldehyde and pyruvic acid.

Table 2.1. Acetaldehyde and pyruvic acid concentrations in Pinot noir wines that did or did not undergo malolactic fermentation¹.

Treatment	Acetaldehyde Concentration (mg/L)	Pyruvic Acid Concentration (mg/L)
Control	18.3 ± 1.7	29.7 ± 5.2
Control pH	19.0 ± 2.5	29.5 ± 3.0
Simultaneous	8.8 ± 5.0	10.5 ± 1.2
VP41	10.4 ± 4.4	18.8 ± 2.2
Alpha	6.9 ± 1.6	17.8 ± 1.2
VFO	5.0 ± 3.1	8.4 ± 4.5

¹values are means of three replicates ± one standard deviation

Table 2.2. Acetaldehyde and pyruvic acid concentrations in Merlot wines that did or did not undergo malolactic fermentation¹

Treatment	Acetaldehyde Concentration (mg/L)	Pyruvic Acid Concentration (mg/L)
Control	9.4 ± 0.7	35.2 ± 1.6
Control pH	9.5 ± 1.7	39.0 ± 0.7
Simultaneous	1.5 ± 0.7	7.7 ± 0.6
VP41	1.2 ± 2.3	11.5 ± 5.1
Alpha	1.6 ± 1.5	8.9 ± 1.3
VFO	0.6 ± 0.6	7.2 ± 1.2

¹values are means of three replicates ± one standard deviation

Hydroxycinnamic acids were also quantified at the completion of malolactic fermentation. There was no statistically significant difference in concentrations of caftaric, caffeic, and *trans*-*p*-coumaric acids between MLF (+) and control wines except for wines that had undergone MLF with *O. oeni* VFO (Table 2.3). In the case of VFO MLF (+) and simultaneous (VFO) wines there was a significant reduction in caftaric acid concentration and an increase in caffeic acid concentration. In addition, although concentrations of *trans*-*p*-coumaric acid were low in all Pinot noir wines it was higher in both the VFO MLF (+) and simultaneous (VFO) wines.

Table 2.3: Concentrations (mg/L) of caftaric, caffeic, and *trans p*-coumaric acids in Pinot noir wines that did or did not undergo malolactic fermentation¹.

Treatment	Caftaric acid	Caffeic acid	<i>trans</i> - <i>p</i> -coumaric acid
Control	25.0 ± 0.3	3.8 ± 0.5	1.0 ± 0.1
Control pH	26.8 ± 0.4	1.3 ± 0.3	1.0 ± 0.1
Simultaneous (VFO)	10.3 ± 2.2	18.6 ± 4.0	1.9 ± 0.4
VFO	5.5 ± 1.9	23.1 ± 1.4	4.7 ± 0.1
Alpha	25.7 ± 0.7	5.4 ± 0.3	1.0 ± 0.1
VP41	22.4 ± 1.4	6.8 ± 0.4	0.8 ± 0.1

¹values are means of three replicates ± one standard deviation

DISCUSSION

The malolactic fermentation caused a significant decrease in color in both Pinot noir and Merlot wines that remained even after nine months. While others have mentioned that malolactic fermentation could result in a decrease in wine color (Rankine et al. 1970; Husnik et al. 2007), to our knowledge this is the first study that has documented the loss of color due to MLF and the reasons for this loss. Often the loss of color due to MLF has been explained as being a result of pH change due to the conversion of malic acid to lactic acid (Pilone and Kunkee 1972). However, in this study pH was equilibrated between wines that had or had not undergone MLF and the drop in color was still observed demonstrating that color loss due to MLF was not due solely to pH increase.

Timing of the MLF inoculation did not seem to impact color loss as wines that underwent MLF either consecutively (after completion of alcoholic fermentation) or simultaneously still demonstrated the same decrease in color. Simultaneous fermentations are sometimes performed during white winemaking as a way to accelerate the winemaking process and allow earlier addition of SO₂ to protect wine from spoilage and oxidation (Pan et al. 2011). However, simultaneous fermentations are not commonly performed in the production of red wines as winemakers are concerned about possible color loss and other sensory impacts. This present study suggests that simultaneous MLF in Pinot noir and Merlot wines

will not result in a greater loss of color compared to conducting MLF after alcoholic fermentation. Further studies regarding the sensory effects of simultaneous red wine fermentations should be conducted to determine if this practice should be utilized for red winemaking.

Commercial manufacturers' of ML bacterial strains often claim differences between the sensory impacts of various strains with regards to flavor, aroma, and color. In this study, the three *O. oeni* strains used did not impact the color of the wines differently. Additional strains should be evaluated to determine if there is any variability between *O. oeni* strains with regard to their impact on red wine color and whether color loss can be minimized by strain selection.

The color loss due to MLF seemed to be driven by decreased polymeric pigment content compared to control wines. While previous research has measured chemical changes in wine as a result of MLF (Delaquis et al. 2000; Boido et al. 2009), this is the first study to report the change in polymeric pigment content due to MLF. Furthermore, the lower polymeric pigment content corresponded with a significantly higher concentration of monomeric anthocyanins. This is in contrast to suggestions that loss of color by MLF is probably due to bacterial metabolism of monomeric anthocyanins or results in no change to the anthocyanin profile (Mangani et al. 2011). In the present study, the higher monomeric anthocyanin content was probably due to lower incorporation of monomeric anthocyanins into polymeric pigment complex versus the control wines (Morata et al. 2007).

The MLF may have impacted polymeric pigment formation by the degradation of pyruvic acid and acetaldehyde. These compounds are known to be involved in the formation of polymeric pigments. Acetaldehyde plays an important role in stable pigment formation by providing an ethyl bridge to link flavanols to anthocyanins or anthocyanins to other anthocyanins (Cheynier et al. 2006), reactions favored at wine pH (Dallas et al. 2003). Reduction of acetaldehyde may have reduced the formation of these ethyl-linked compounds compared to the control wines. In addition, acetaldehyde and pyruvic acid can also be incorporated into vitisins A and B, compounds with increased absorbance at 520nm and resistance to bleaching by SO₂ (Schwarz et al. 2003). Vitisin A and B concentrations, while highly variable, can be important contributors to stable red wine color, particularly in some red wines such as tempranillo (Rentzsch et al. 2010). In this present study polymeric pigments were measured using the sulfite bleaching method that does not differentiate between the different types of polymeric pigment complexes such as vitisins or ethyl-linked flavonal and anthocyanins. Therefore, it is not possible to say whether or not MLF impacted vitisin A or B formation. Future research in this area should be conducted utilizing HPLC-MS techniques that will allow quantification of vitisins (Vivar-Quintana et al. 2002). This will determine whether acetaldehyde and pyruvic acid metabolism by ML bacteria decreases formation of vitisins.

One class of stable color compounds that did not seem to be impacted by the MLF were the copigmentation compounds as no difference was found in the copigmentation content of any of the wines. This was surprising given that *O. oeni* VFO had higher levels of the co-factors caffeic and coumaric acids compared to wines which did not undergo MLF or were inoculated with different *O. oeni* strains. The increased caffeic and *p*-coumaric acid concentrations were likely due to hydroxycinnamic esterase activity converting caftaric and coutaric acid respectively. This conversion by *O. oeni* has been reported previously (Cabrita et al. 2008) but this is the first report of *O. oeni* strain variability for this property. Schwarz et al. (2005) reported that caffeic acid copigmentation may actually result in color loss, a result not seen here in wine with higher concentration of caffeic acid. Conversely, the same study found copigmentation of coumaric acid and anthocyanin to increase red wine color at 520nm but the VFO inoculated wine showed no improvement in color with higher concentrations of coumaric acid. In general, the copigmentation values for all wines were low compared to values reported in other studies (Gutiérrez et al. 2005) and so even if changes in the concentrations of co-factors were important the overall impact on color would have been very small. As the anthocyanin profile (acylated vs. non-acylated forms and percentage of malvidin-3-glucoside) of red wine varieties is quite different (Romero-Cascales et al. 2005), and the contribution to total red wine color by copigmentation complexes can also vary widely across varieties (Boulton 2001) future research should be conducted on a range of red wines to determine if *O.*

oeni hydroxycinnamic esterase activity is beneficial or detrimental to copigmentation and red wine color development. Given that some strains do not have this activity, strain selection could again be a tool a winemaker could use to promote better color stability.

CONCLUSIONS

Malolactic fermentation resulted in a reduction in red color and stable polymeric pigment formation in Pinot noir and Merlot wines and a corresponding higher concentration of monomeric anthocyanins. Color loss was observed when alcoholic and malolactic fermentations were performed simultaneously and also when the MLF was conducted after the alcoholic fermentation. Color loss was independent of the pH change caused by MLF and no differences between three strains of *O. oeni* were observed. All strains degraded acetaldehyde and pyruvic acid during the MLF while VFO demonstrated hydroxycinnamic acid esterase activity by converting caftaric acid to caffeic acid. This conversion did not impact color due to copigmentation as no differences were noted for this value between any of the wines that had or had not undergone MLF. Future studies should focus on the mechanisms by which MLF decrease color in red wine and in particular why polymeric pigment formation is reduced. This should include identification of the specific wine pigments impacted by MLF, including vitisins A and B and ethyl linked

compounds. These findings will aid in determining ways that color loss and reduced polymeric pigment formation due to MLF can be prevented or minimized in the winery.

CHAPTER 3

IMPACT OF TIMING OF MALOLACTIC FERMENTATION AND ACETALDEHYDE METABOLISM ON RED WINE COLOR AND POLYMERIC PIGMENT FORMATION

ABSTRACT

The mechanisms by which MLF reduces red wine color and practices to mitigate this reduction were investigated. MLF was delayed in Pinot noir wines to allow greater polymeric pigment formation prior to MLF. Wines were held at 13°C for 0, 14, 28, 100, and 200 days before inoculation with *O. oeni* VFO to induce MLF. Delaying MLF did not impact loss of color at 520nm but delaying MLF for increasing time periods resulted in wines containing similar polymeric pigment content and monomeric anthocyanin concentrations to the control. Loss of polymeric pigment formation due to degradation of acetaldehyde and/or pyruvic acid by *O. oeni* was investigated where wines that had undergone MLF were supplemented with acetaldehyde and/or pyruvic acid to the levels measured in control wines. Wines with acetaldehyde additions showed higher color and polymeric pigment than MLF wines with no additions while addition of pyruvic acid showed no improvement in color or polymeric pigment. However, acetaldehyde additions did not completely prevent loss of color at 520nm after MLF and the possibility that this color loss was due to fining by *O. oeni* was explored. Wines that did not undergo MLF but were exposed to live or inactivated

O. oeni cells showed no difference in color, polymeric pigment, and monomeric anthocyanin compared to the control suggesting that loss of color during MLF was not due to fining by *O. oeni* cells.

INTRODUCTION

Red wine color is an important sensory component of wine that is impacted primarily by anthocyanins present in the grapes and their extraction during the winemaking process (Fulcrand et al. 2006). Although there has been extensive research on winemaking practices that impact red wine color (Girard et al. 1997; Reynolds et al. 2001; Harbertson et al. 2009), these have generally focused on physical and chemical parameters such as temperature, maceration length, and anthocyanin concentration, rather than the role of wine microorganisms such as *Saccharomyces* and *Oenococcus oeni* that conduct the alcoholic and malolactic fermentations respectively. Studies that have investigated the role of microorganisms on red wine color have reported that yeast can impact color through the adsorption of anthocyanins to their cell walls (Bautista-Ortín et al. 2007; Pérez-Serradilla & de Castro 2008) or through production of acetaldehyde and pyruvic acid (Morata et al. 2003). For example, Morata et al. (2006) reported that yeast strains that produced higher concentrations of acetaldehyde resulted in wines with increased vitisin B content, a compound that can play an important role

in red wine color (Alcalde-Eon et al. 2006). These and other studies have demonstrated that the action of yeast during alcoholic fermentation can influence the formation of red wine color. However, the development of red wine color continues after alcoholic fermentation and the action of *Oenococcus oeni* during the malolactic fermentation (MLF) may also impact red wine color.

The MLF results in deacidification of a wine due to the decarboxylation of malic acid to lactic acid (Pilone & Kunkee 1972). Additional wine aroma, flavor, and textural changes may also result from the MLF (Delaquis et al. 2000; Rodriguez et al. 1990; McDaniel et al. 1987; Giannakopoulos et al. 1984) and winemakers have reported a loss of color occurring also. The observed loss of color has often been attributed to the increase in pH that occurs after MLF (Rankine et al. 1970) but more recent studies have noted changes in color after MLF that could not be explained by pH changes (Delaquis et al. 2000; Husnik et al. 2007). *O. oeni* can degrade compounds important for red wine color such as acetaldehyde (Osborne et al. 2006; Osborne et al. 2000), pyruvic acid (Asentorfer et al. 2003) and may also impact the copigments *p*-coumaric acid, and caffeic acid (Hernandez et al. 2007; Hernandez et al. 2006; Cavin et al. 1993). However, whether the degradation of these compounds is responsible for any color changes during the MLF is unknown. In addition, it is not known if *O. oeni* can impact red wine color through adsorption of anthocyanins to their cell walls as is documented to occur with yeast (Bautista-Ortín et al. 2007; Pérez-Serradilla & de Castro 2008). Therefore, the objectives of

this study were to determine if the degradation of acetaldehyde and/or pyruvic acid contributed to the loss of color caused by the MLF as well as whether color loss could be explained by adsorption of anthocyanins to *O. oeni* cell walls. Finally, the winemaking practice of delaying the MLF to purportedly enhance red wine color would be explored as a way to mitigate color loss during the MLF.

MATERIALS AND METHODS

Grapes

Pinot noir grapes were harvested on October 16th, 2010, from Oregon State University's Woodhall Vineyard (Alpine, Oregon, USA). Harvest was determined by sugar levels and fruit ripeness according to the specifications of the vineyard manager. Upon harvest, grapes were stored for forty-eight hours at 4°C (39.2°F) before being hand-sorted and destemmed with a Velo DPC 40 destemmer/crusher (Altivole, Italy). The grapes were then pooled and divided into 100L stainless steel tanks containing approximately sixty liters of grape must each. An addition of 50mg/L SO₂ (in the form of potassium metabisulfite) was added to each tank and the yeast nutrient Fermaid K® (Lallemand, Montreal, Canada) was added at a rate of 0.125g/L.

Alcoholic Fermentation

Grape must was inoculated with *Saccharomyces cerevisiae* yeast strain VQ-15 (Lallemand) at a rate of 0.25 grams dried yeast per liter of must (approximately 1×10^6 CFU/mL). Yeast was hydrated according to manufacturer's specifications prior to inoculation.

Fermentations were conducted in a temperature-controlled room held at 26.6°C (80°F). Cap punch downs were performed uniformly twice daily and temperature and °Brix were measured with an Anton-Paar DMA 35N Density Meter (Graz, Austria). Completion of alcoholic fermentation (reducing sugar concentration below 0.2g/100mL) was confirmed by testing with Bayer Clinitest tablets (Morristown, New Jersey, USA). Upon completion of alcoholic fermentation, wines were pressed with a Willmes model 6048 pneumatic bladder press (Lorsch, Germany). Wines were pressed firstly at 0.5 bar for one minute before the cake was manually broken up and pressed again at 1.0 bar for two minutes. All wine was then pooled and mixed after pressing. Wines were placed in a cold room at 3°C for forty-eight hours.

Filtration

Following cold settling, wines were racked and then pad filtered (Beco K-1 3.0µm nominal filter sheets (Langenlonsheim, Germany)) before being filtered through 1.0µm and 0.45µm polyethersulfone cartridges (G.W. Kent, Ypsilanti, Michigan,

USA) in succession. Filtered wine was dispensed into sterile one gallon and half gallon carboys and utilized in the following trials.

Acetaldehyde and Pyruvic Acid Addition Trial

Wines were inoculated for MLF with *O. oeni* VFO (Chr. Hansen, Hørsholm, Denmark) at approximately 1×10^6 CFU/mL. Malolactic bacteria were in direct inoculum freeze-dried form and were hydrated in 0.1% peptone blanks for twenty minutes prior to inoculation. All treatments, including an uninoculated control, were performed in triplicate and kept at 25°C until MLF was completed. Progress of the MLF was monitored by enzymatic assay (R-Biopharm, Darmstadt, Germany). At the completion of MLF, acetaldehyde and pyruvic acid concentration was measured in all wines by enzymatic assay (R-Biopharm, Darmstadt, Germany). Wines were then sterile filtered (0.45µm polyethersulfone cartridge (GW Kent)) and dispensed into 350mL brown glass beer bottles. Prior to capping, the following treatments to wines that had undergone MLF were made: 1) Addition of acetaldehyde (Sigma Aldrich, St. Louis, MO, USA) to match concentration of control wine, 2) addition of pyruvic acid (Sigma Aldrich) to match concentration of control wine, 3) addition of both acetaldehyde and pyruvic acid to match concentration of control wine, and 4) MLF wine with no additions. To minimize the binding of added acetaldehyde and pyruvic acid by SO₂ (Larsen et al. 2003), (Zoecklein et al. 1995) no SO₂ was added to the wines. All wines were placed in a cellar room at

13°C until needed for analysis. For purposes of analysis, the day of bottling was considered the day zero time point.

Delayed Malolactic Fermentation Trial

Carboys of Pinot noir wine were kept at 13°C until required. After 0, 14, 28, 100, and 200 days two sets of three carboys were removed from the cellar and brought to room temperature (25°C). At each time point three of the carboys were inoculated with *O. oeni* strain VFO while the remaining three carboys remained as controls. At the conclusion of MLF, a 35mg/L SO₂ addition was made to all wines before sterile filtration (0.45µm polyethersulfone cartridge (GW Kent)) and bottling (350mL brown glass beer bottles sealed with crown caps). Bottled wines were stored at 13°C until required for analysis.

Bacterial Fining Trial

Four hundred mLs of sterile filtered Pinot noir wine (pH 3.5) was dispensed into sterile 500mL Erlenmeyer flasks fitted with air locks. The following treatments were prepared in triplicate: 1) Control wine no MLF; 2) MLF wine (*O. oeni* inoculated at 1 x 10⁶ CFU/mL; 3) Addition of *O. oeni* at approximately 1 x 10⁷ CFU/mL and removal of bacteria by sterile filtration after 24 hrs; 4) Addition of inactivated *O. oeni* at approximately 1x10⁸ CFU/mL. *O. oeni* for use in bacterial fining trials were prepared as follows. Single colonies of *O. oeni* strain VFO were

obtained by streaking on Man, Rogosa, and Shapre (MRS) agar plates (20g/L Tryptone, 5g/L peptone, 5g/L yeast extract, 5g/L glucose, 200mL apple juice, 800mL distilled water, 1mL Tween 80 [5% w/w solution], 20g/L agar, pH 4.5). Plates were incubated aerobically at 25°C for seven days. Colonies were inoculated in 250mL acidic grape juice broth. After seven days of aerobic growth at 25°C, cells were harvested by centrifugation (4000g for 15 minutes), washed twice with 0.2M phosphate buffer (27.80g/L NaH₂PO₄•H₂O, 28.38 g/L Na₂HPO₄, pH 7.0), and resuspended in 0.2M phosphate buffer. This suspension was used to inoculate wines where appropriate while the control wine had an equal volume of sterile buffer added. Inactivated *O. oeni* were prepared by autoclaving the remaining cell suspension at 120°C for 30 minutes. After cooling, this suspension was added to the wine at approximately 1x10⁸ CFU/mL. After 24 hours, samples from treatments 1 and 3 were sterile filtered (0.45µm disposable Nalgene PES membrane filter units (NalgeNuno International, Rochester, NY, USA)), adjusted to pH 3.60 and stored at -80°C until needed for analysis. MLF was monitored by enzymatic assay and when complete all wines were sterile filtered through (0.45µm disposable Nalgene PES membrane) filter units, pH adjusted to 3.60, and stored at -80°C until needed for analysis.

Color Analysis

Prior to analysis wine samples were adjusted to pH 3.60 by addition of 2N NaOH or 25% H₃PO₄. Color was determined by spectrophotometric analysis (Shimadzu UV-

3101PC, Kyoto, Japan) at 420nm and 520nm in a quartz 1mm pathlength cuvette. Polymeric pigment and copigmentation were measured by spectrophotometric analysis (Levengood Joanne and Boulton Roger 2004) (Thermo Scientific Genesys, Madison, Wisconsin, USA) in 10mm polystyrene cuvettes.

High Performance Liquid Chromatography

Monomeric anthocyanins were analyzed by high-performance liquid chromatography (HPLC) using a Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA) equipped with HP ChemStation software and photodiode-array detector (DAD). The HPLC was fitted with a LiChroSpher reverse-phase C18 column (4 x 250mm, 5mm particle size) (Merck, Darmstadt, Germany) held at 30°C. All chromatographic solvents were HPLC grade. 98% Formic acid (EMD Chemicals, Darmstadt, Germany) was purchased from Sigma Aldrich (St. Louis, MO). 99.8% Methanol (EMD Chemicals) was purchased from Oregon State University Chemistry Stores (Corvallis, OR). Gradients of solvent A (water/formic acid, 90:10, v/v) and solvent B (methanol) were applied as follows: 5 to 35% B linear (1.0 mL/min) from 0 to 15 min, static at 35% B (1.0 mL/min) from 15 to 20 min, 35 to 80% B linear (1.0 mL/min) from 20 to 25 min, then 5% B (1.0 mL/min) from 25 to 32 min to re-equilibrate the column to initial conditions. Wine samples were prepared for analysis by centrifugation at 24,000 *g* for 10 minutes. Injection volume was 20µL. Anthocyanins were detected by scanning from 190 to 700nm.

Quantification of anthocyanins was performed against an external standard at 520 nm and expressed as a function of malvidin-3-glucoside concentration.

Statistical Analysis

A univariate Analysis of Variance (ANOVA) was used to determine differences between wine treatments. The ANOVA was performed by Minitab® 16.1.1 (State College, Pennsylvania, USA). Tukey's HSD multiple comparison was performed to test least squares means of treatment effects at the 0.05% significance level.

RESULTS

Fermentation

Basic juice parameters of the Pinot noir must after processing were pH 3.35, 23.5 °Brix, and 0.724g/100mL titratable acidity (grams tartaric acid). Alcoholic fermentation proceeded rapidly and was completed in all tanks after nine days (data not shown).

Acetaldehyde and Pyruvic Acid Addition Trial

After the completion of MLF, acetaldehyde and pyruvic acid concentrations decreased compared to the control wines (data not shown). Additions of acetaldehyde, pyruvic acid, or acetaldehyde and pyruvic acid were made to wines to match the concentrations present in the control wine. Color and chemical

analysis of the wines was then performed 0 and 90 days later. At day 0, all wines that had undergone MLF had significantly reduced color at 520nm in comparison to the control wine (Figure 3.1). MLF wine with no additions had approximately 15% less red color at 520nm compared to the control while wines with an acetaldehyde addition had approximately 11% less color. However, there was no statistically significant difference in color between wines that had undergone MLF and had or had not had additions of acetaldehyde and/or pyruvic acid. After 90 days of aging, all wines that had undergone MLF still had significantly reduced

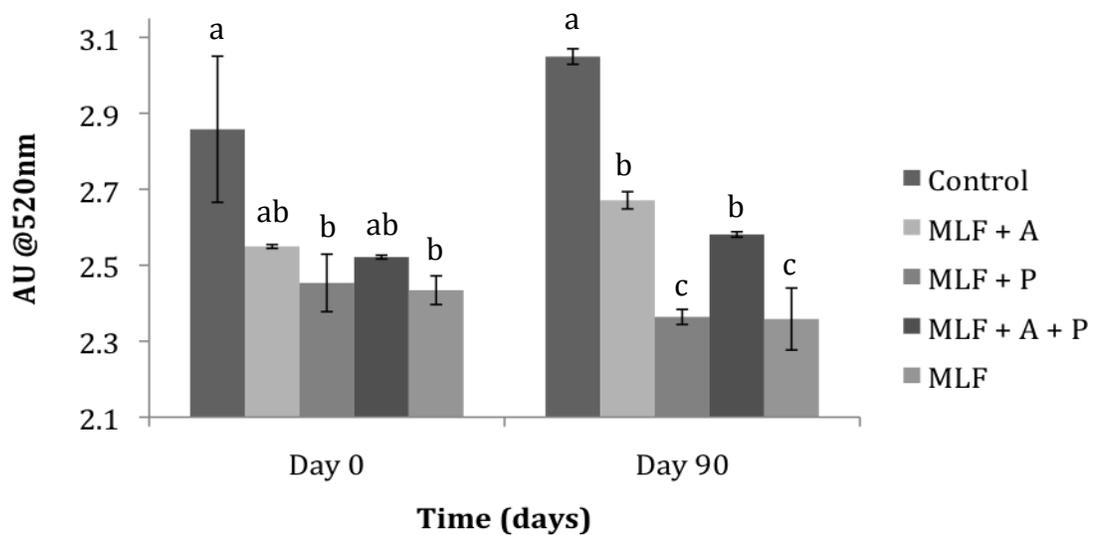


Figure 3.1: Color at 520nm of Pinot noir wine with restoration of acetaldehyde (A) and/or pyruvic acid (P) after malolactic fermentation at bottling (day 0) and 90 days after bottling. Error bars represent \pm one standard deviation ($n=3$). $a-b$ values with different subscript letters within a time point are significantly different at $p<0.05$

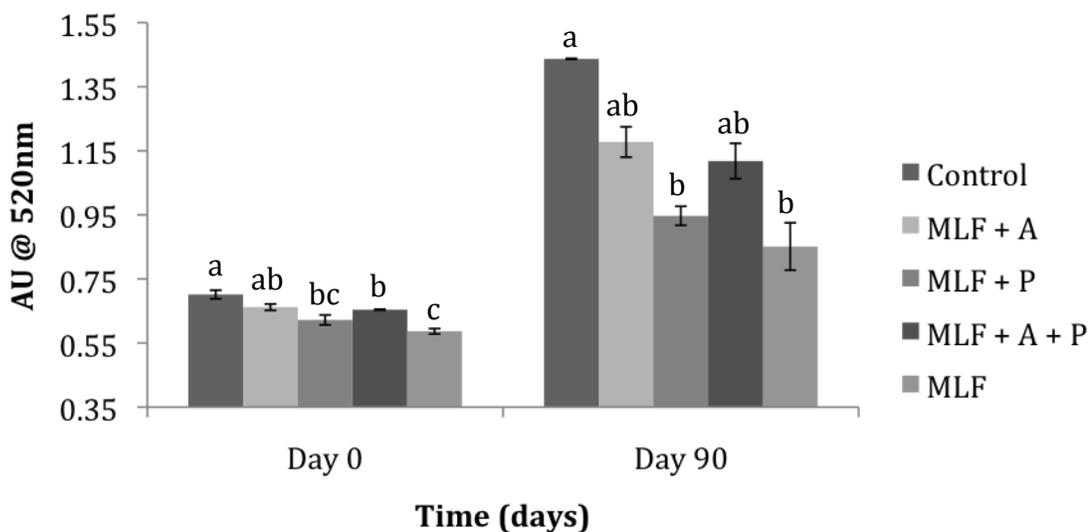


Figure 3.2: Polymeric pigment in Pinot noir with restoration of acetaldehyde (A) and/or pyruvic acid (P) after malolactic fermentation at bottling (day 0) and 90 days after bottling. Error bars represent \pm one standard deviation ($n=3$). ^{a-c} values with different subscript letters within a time point are significantly different at $p<0.05$

color at 520nm in comparison to the control (Figure 3.1). However, wines to which acetaldehyde or acetaldehyde and pyruvic acid had been added had significantly more color than MLF wine that had either no additions or just an addition of pyruvic acid. The acetaldehyde addition wine had just half the loss in color experienced by the standard MLF treatment.

Polymeric pigment content demonstrated the same trend as color at 520nm. At day 0 polymeric pigment was reduced in all wines that had undergone MLF compared to the control wine (Figure 3.2) although the differences were minor. However, after 90 days the difference in polymeric pigment content between the

control wine and wines that underwent MLF had increased considerably (Figure 3.2). MLF wines to which acetaldehyde or acetaldehyde and pyruvic acid had been added had significantly higher polymeric pigment than treatments that did not. MLF wine with no additions had approximately 41% less polymeric pigment compared to the control while the acetaldehyde addition treatment only had an 18% reduction. Wine with the acetaldehyde and pyruvic acid addition had 22% less polymeric pigment content in comparison to the control while the pyruvic acid addition treatment had 34% less polymeric pigment.

At day 0 there was no difference between the monomeric anthocyanin content of the control wine and MLF wines with acetaldehyde, pyruvic acid, acetaldehyde and pyruvic acid additions, or MLF with no additions (Figure 3.3). At 90 days, MLF wines with acetaldehyde or acetaldehyde and pyruvic acid additions contained statistically the same concentration of monomeric anthocyanins as the control. In contrast, MLF wines that had an addition of pyruvic acid or no additions contained significantly higher concentrations of monomeric anthocyanins.

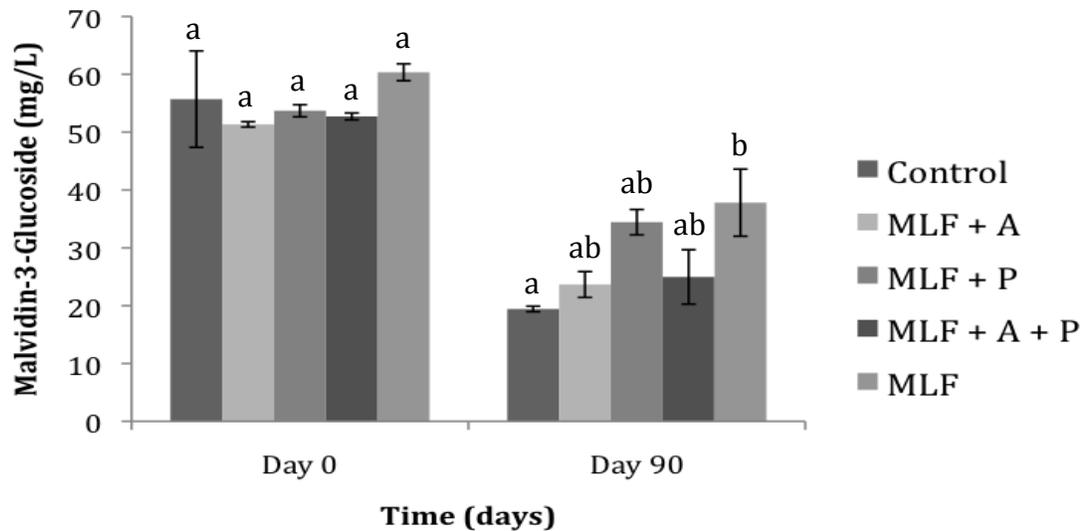


Figure 3.3: Concentration of monomeric anthocyanins in Pinot noir with restoration of acetaldehyde (A) and/or pyruvic acid (P) after malolactic fermentation at bottling (day 0) and 90 days after bottling. Error bars represent \pm one standard deviation (n=3). ^{a-b} values with different subscript letters within a time point are significantly different at $p < 0.05$

Delayed Malolactic Fermentation Trial

Wines that had or had not undergone MLF after set storage times (0, 14, 28, 100, and 200 days) were analyzed for various color parameters. Compared to the control, color at 520nm was reduced for wines that underwent MLF no matter how long the MLF had been delayed (Figure 3.4). For the day 0 time point, the MLF treatment had approximately 18% less color at 520nm than its control while after delaying MLF inoculation for 200 days, the MLF treatment still had 17% less color than its control. While color at 520nm did not seem to change due to delaying MLF

there were some differences in polymeric pigment content. Delaying the MLF for greater periods of time resulted in a decrease in the polymeric pigment content

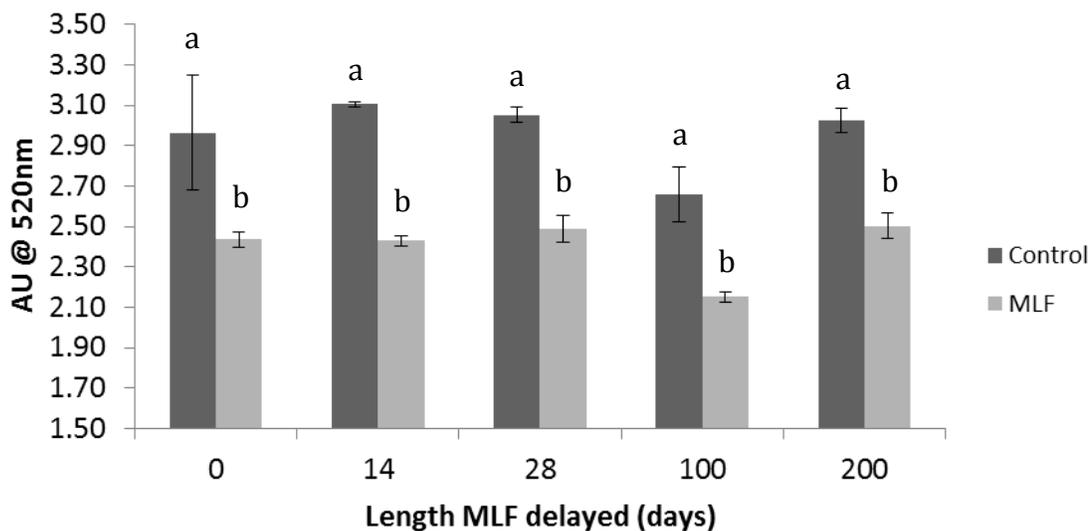


Figure 3.4: Color at 520nm of Pinot noir Wines that underwent malolactic fermentation delayed for set period of time. Error bars represent \pm one standard deviation ($n=3$). ^{a-b} values with different subscript letters within a time point are significantly different at $p<0.05$

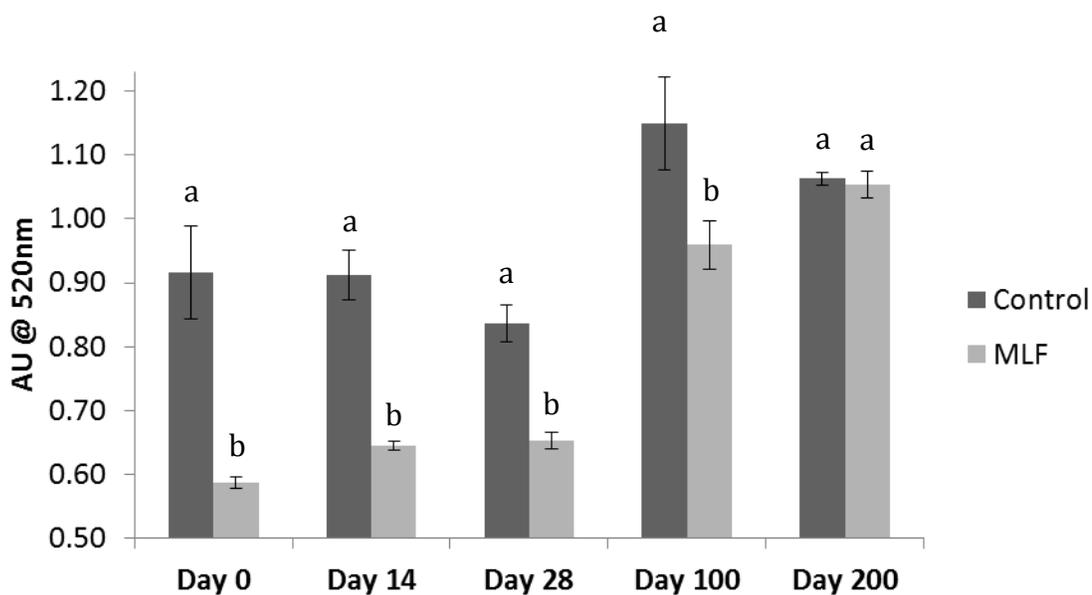


Figure 3.5: Polymeric pigment content of Pinot noir Wines that underwent malolactic fermentation delayed for set period of time. Error bars represent \pm one standard deviation ($n=3$). ^{a-b} values with different subscript letters within a time point are significantly different at $p<0.05$

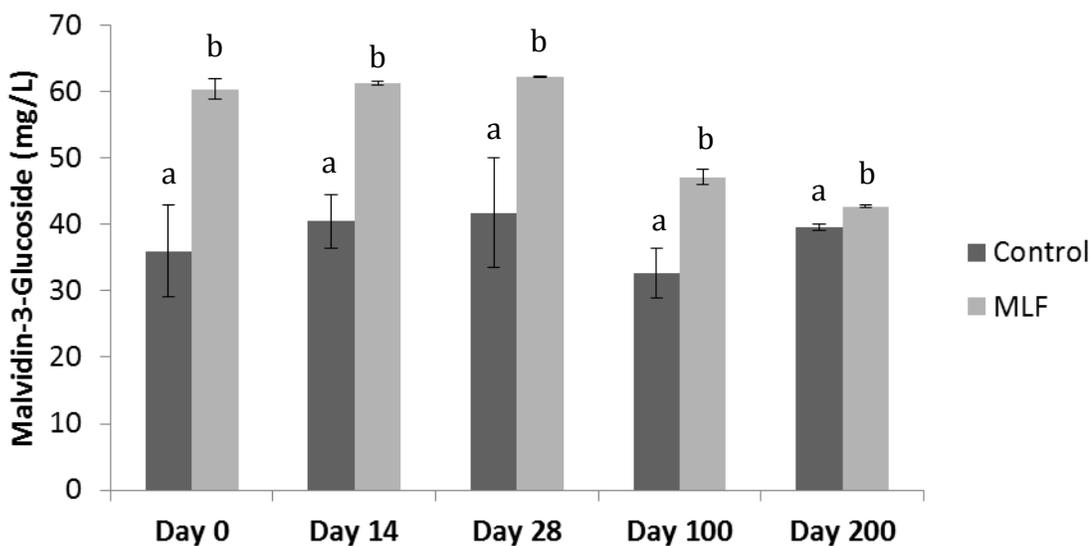


Figure 3.6: Monomeric anthocyanin concentration of Pinot noir Wines that underwent malolactic fermentation delayed for set period of time. Error bars represent \pm one standard deviation ($n=3$). ^{a-b} values with different subscript letters within a time point are significantly different at $p<0.05$

differences of wines that had or had not undergone MLF (Figure 3.5). For example, with no delay of MLF (day 0) wines had 36% less polymeric pigment than the control. After delaying MLF 28 days, MLF wine had just a 22% difference in polymeric pigment in comparison to its control while delaying the MLF for 200 days resulted in wine that contained the same polymeric pigment content as the control. Monomeric anthocyanins content followed the same trend as polymeric pigments and were significantly higher in wines that had undergone MLF compared to the control (Figure 3.6). With no delay in MLF, the wine had approximately 68% more monomeric anthocyanins than the control while delaying MLF 28 days resulted in 49% greater monomeric anthocyanins. Again, delaying the MLF by 200 days largely removed the differences between wines that had or had not undergone MLF as only an 8% difference was noted although this difference was still statistically significant.

Bacterial Fining Trial

To determine if loss of color was due to fining by bacterial cells, Pinot noir wine that had not undergone MLF was exposed to either live or dead *O. oeni* cells for different periods of time. After 24 hrs, wine to which approximately 1×10^7 CFU/ml of live *O. oeni* had been added showed no difference in color at 520nm or polymeric pigment content compared to the control (Table 3.1). There was however a slight decrease in monomeric anthocyanin content. After 14 days, wines

inoculated with *O. oeni* at approximately 1×10^6 CFU/mL had completed MLF and these wines had significantly lower color at 520nm and polymeric pigment content than the control wine. The MLF wine also had significantly higher levels of total monomeric anthocyanins compared to the control. In contrast, wines exposed to approximately 1×10^8 CFU/mL inactivated *O. oeni* for the same 14 day time period were statistically the same as the control for color at 520nm, polymeric pigments, and monomeric anthocyanin content. Polymeric pigment content increased significantly in the control between testing at 1 and 14 days.

Table 3.1: Color at 520nm, total monomeric anthocyanins, and polymeric pigment for Pinot noir wine exposed to *O. oeni* bacteria.

	*Color (A520nm)	*Monomeric Anthocyanin (mg/L m-3-g eqs.)	*Polymeric Pigment (A520nm)
Control ¹	3.15 ^a (0.06)	45.1 ^a (0.2)	1.32 ^a (0.02)
MLF ²	2.94 ^b (0.01)	52.3 ^b (0.2)	1.17 ^b (0.01)
Dead Cells ³	3.12 ^a (0.05)	44.9 ^a (0.8)	1.28 ^a (0.03)
Control 24 ⁴	3.13 ^a (0.07)	68.3 ^c (0.3)	1.03 ^c (0.02)
Live 24 ⁵	3.03 ^a (0.01)	65.3 ^d (0.2)	0.99 ^c (0.00)

*Values are means from triplicate fermentations \pm SD

^{a-c} Means values with different subscript letters within a column are significantly different at $p < 0.05$

¹Control held for 14 days

²Malolactic fermentation completed in 14 days

³Exposure to approx. 1×10^8 CFU/mL inactivated *O. oeni* for 14 days

⁴Control sampled after 24 hours

⁵Exposure to approx. 1×10^7 CFU/mL *O. oeni* for 24 hours

DISCUSSION

Previous work in our laboratory had demonstrated that malolactic fermentation resulted in a significant decrease in color at 520 nm, lower polymeric pigment content, and higher concentrations of the more unstable monomeric anthocyanins. This present study confirmed these results but also investigated possible reasons for the color loss caused by MLF as well as practical strategies to minimize these losses. One strategy that winemakers have adopted to improve Pinot noir color is to delay the MLF. Anecdotally, winemakers have reported that long or delayed MLFs tend to give wines with greater color. In this present study this observation was investigated where MLF was delayed in wines for up to 6 months. Delaying the MLF did not appear to have a significant impact on color loss at 520nm but did impact the formation of polymeric pigments. As MLF was delayed for longer periods of time the polymeric pigment content and monomeric anthocyanin concentration of MLF (+) wines became more similar to those of the control. In fact, after 200 days delayed MLF wines that had or had not undergone MLF contained the same polymeric pigment content and monomeric anthocyanin concentrations. Given that it took up to 200 days of delaying MLF to mitigate loss of polymeric pigment due to MLF, it seems that the reactions involved were occurring quite slowly at cellar temperatures (13°C). Furthermore, the practical concern of leaving wine for 200 days without the protection of SO₂ would have to be considered as this would leave the wine vulnerable to microbial spoilage.

Improved polymeric pigment formation in wines where MLF was delayed was most likely due to acetaldehyde being present in the wine for an extended period of time. A number of studies have reported the ability of *O. oeni* to degrade acetaldehyde in wine (Osborne et al. 2000; Osborne et al. 2006) and acetaldehyde is known to play a role in the formation of polymeric pigments. For example, acetaldehyde forms ethyl-linked bridges for flavanol and anthocyanin polymeric pigment reactions as well as links between anthocyanins (Cheynier et al. 2006). Additionally, acetaldehyde and pyruvic acid can form vitisins A and B respectively through reactions directly with the anthocyanin (Schwarz et al. 2003). Removal of acetaldehyde by *O. oeni* may have reduced the formation of these compounds. However, in this present study polymeric pigments were measured using the sulfite bleaching method that does not differentiate between the different types of polymeric pigment complexes such as vitisins or ethyl-linked flavonal and anthocyanins. Therefore, it is not possible to say whether the delay of MLF favored the formation of one class of compounds over the other.

The effect of acetaldehyde and pyruvic acid degradation during MLF was studied by restoring these compounds back to concentrations present in the MLF (-) control wine. Because acetaldehyde is involved in both vitisin B and ethyl-linked bridging reactions the degradation of acetaldehyde impacted color loss to a greater extent than just the loss of pyruvic acid did. Pyruvic acid additions did not reduce color or polymeric pigment loss due to MLF and so it is likely that vitisin A

formation played little to no role in the color of the Pinot noir wine. In contrast, addition of acetaldehyde improved color and polymeric pigment content compared to the standard MLF (+) treatment. For example, the addition of acetaldehyde post-MLF resulted in only a 12% reduction in color and 18% lower polymeric pigment compared to the control while the standard MLF (+) wine had 23% less color and 41% less polymeric pigment. The impact of acetaldehyde on color compared to pyruvic acid is probably related to the fact that acetaldehyde is involved in both the formation of ethyl-linked bridges as well as vitisin B while pyruvic acid is only involved in vitisin A formation. Furthermore, the importance of vitisins to red wine color has not been well established with Morata et al (2006) reporting that vitisin A and B levels in Tempranillo, a highly pigmented wine, were very low measuring between 0.5 and 1.9 mg/L.

While metabolism of acetaldehyde by *O. oeni* explained some color loss and particularly the reduction in polymeric pigment content, it did not explain it all. Therefore, the adsorption of anthocyanins to *O. oeni* cell walls was examined to determine whether this could account for the remainder of the observed color loss. Two treatments were used where in the first live cells were added to the wine (and removed after 24 hrs) while in a second treatment inactivated cells were added. The rates of addition mimicked what the cell concentration at the end of the MLF would have been. No matter whether live or dead cells were added there was no significant loss of color @ 520 nm or polymeric pigment content compared to the

control. Interestingly, a statistically significant reduction in monomeric anthocyanins was found between wines with an addition of live cells for 24 hrs and the control although this difference was very small (2 mg/L). Previous experiments had shown that MLF (+) wines contained much higher concentrations of monomeric anthocyanins suggesting that any small reduction in anthocyanins due to adsorption to *O. oeni* cells is quickly outweighed by reduced formation of polymeric pigments relative to MLF (-) wines.

O. oeni cells inactivated by autoclaving should have provided the greatest opportunity for the effects of cell wall fining to be seen due to cell rupture and increased surface area. However, no significant difference in color, polymeric pigment, and total monomeric anthocyanins compared to the MLF (-) control was observed while wine that underwent MLF contained lower color and polymeric pigment and higher monomeric anthocyanins compared to the control. The lack of color loss due to fining is in contrast to what has been reported for yeast (Morata et al 2005) and may be due to the reduced surface area of bacteria compared to yeast. For example, Morata et al. (2005) reported that yeast reached a maximum total cell surface area of 10 m²/L at a population of 1x10⁸ CFU/mL and adsorbed 2-6% of total anthocyanins during alcoholic fermentation. At the same population, *O. oeni* cells have a much lower surface area given a cell size several orders of magnitude smaller than *S. cerevisiae* (Fugelsang and Edwards, 2005). The difference may also be due to the electrostatic nature of the cell walls. In a study

conducted in a model red grape juice medium, Medina et. al (2005) found that anthocyanins with higher polarity, delphinidin and petunidin, were more likely to be adsorbed than anthocyanins with less polarity, peonidin and malvidin, on yeast cell walls. *O. oeni* is a Gram + bacteria with a peptidoglycan cell wall accounting for approximately 90% of the dry cell mass (Dicks et al. 1995). Because of the relatively low level of charged moieties in the peptidoglycan network (Silveira et al. 2004), gram + cell walls will likely adsorb anthocyanins at a much different rate than the cell wall of *S. cerevisiae*.

CONCLUSIONS

The mechanisms by which *O. oeni* bacteria causes loss of red wine color and ways to mitigate this color loss were studied. Delaying the MLF for up to three months resulted in wines containing similar polymeric pigment and monomeric anthocyanin concentrations as the control wine. These wines however still showed a loss of color at 520 nm compared to the control. Addition of acetaldehyde that had been metabolized by *O. onei* during the MLF restored approximately half the color and polymeric pigment lost during MLF while no improvement in color or polymeric pigment was found with restoration of pyruvic acid. Bacterial fining of color could not account for color loss or reduced polymeric pigment formation caused by the MLF. Monomeric anthocyanin concentrations also indicated that

adsorption of anthocyanins by MLF bacteria cell walls was minimal and had no impact on wine color.

Results from this study suggest that winemakers can improve the polymeric pigment content of their wines by delaying MLF while storing wine at cool cellar temperatures to prevent microbial spoilage. Additionally, selection of high acetaldehyde producing yeast or use of *O. oeni* strains that do not metabolize acetaldehyde may minimize color loss due to MLF.

OVERALL SUMMARY AND FUTURE WORK

Wine color is an important component of wine quality. Techniques which maximize color extraction or stabilize color compounds in wine are prized by winemakers and the industry. It has been established through previous research that yeast can interact directly with pigments via adsorption or indirectly through the production of cofactors. However, little research has been conducted to measure the impact malolactic fermentation has on red wine color. This lack of research may be attributed to assumptions that the pH shift induced by MLF was responsible for the color change. This study attempted to quantify the color loss that is a result of MLF, determine if pH increase was responsible for the color change, identify color compounds impacted by the presence of MLF bacteria, and test winemaking techniques which may mitigate color change from MLF.

Pinot noir and Merlot wines were inoculated for simultaneous and consecutive alcoholic and malolactic fermentations. Wines which underwent MLF at any time had reduced color at 520nm, lower levels of polymeric pigment, and reduced concentrations of acetaldehyde and pyruvic acid. Minor variations in concentrations of hydroxycinnamic acids were observed and copigmentation levels did not account for color loss from MLF. The bacteria strains tested showed no differences in affect on color indicating strain selection may not be a tool the winemaker can use to minimize color loss.

As concentrations of acetaldehyde and pyruvic acid were greatly reduced after MLF, subsequent experiments tested the restoration of these compounds after the completion of MLF. Wines that received acetaldehyde had significantly better color than treatments which received pyruvic acid or no additions. However, addition of acetaldehyde could not fully restore the color lost due to MLF.

The effect of delaying MLF was tested by storing wines at cellar temperature for extended periods of time prior to inoculation for MLF. MLF was delayed for up to 200 days and all wines that underwent the fermentation had significant reductions in color. However, delaying MLF 200 days resulted in a wine with the same polymeric pigment content and monomeric anthocyanin concentrations as the control wine. To test whether the loss in color was due to bacterial fining, wines were exposed to live and inactivated MLF bacteria for varying time periods. Wines exposed to bacterial cells had no reduction in color, polymeric pigment, or changes in monomeric anthocyanins in comparison to the control wine. These results suggest that bacterial fining is not responsible for the loss of color during MLF.

As the metabolism of acetaldehyde does not account for the total loss of color after MLF, future research should focus on specific quantification of color compounds such as vitisin A and B to determine their relative importance. Additionally, other strains of *O. oeni* should be tested to determine whether winemakers can select for low color impact by MLF bacteria. Testing of MLF delay and acetaldehyde

additions should be performed on a commercial scale to verify that this study's results are repeatable for the wine industry.

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