The Impact of Commercial Tannin Additions and Addition Timing on Finger Lakes Red Wine Color

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Master of Science

by

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Abstract

In winemaking, the addition of enological tannins is intended to improve color extraction, color stability during aging, or various sensory properties. Many different commercial tannin preparations are available to winemakers, but caution in selection and use are called for, as product composition can vary by batch, manufacturer, plant source and extraction method used. Likewise, little definitive guidance on use or addition rate is available to winemakers beyond manufacturer recommendations or anecdotes from other winemakers. This experiment was designed to determine whether the same relative dose of tannin material added at different times—before, during or after fermentation—affected wine color development and stability after two years of bottle aging. Red wines were made from Finger Lakes Pinot Noir and Lemberger, two cultivars with relatively low tannin content, and treated with two different types of commercial tannins. Samples were analyzed by direct spectrophotometric readings, protein precipitation assays and high performance liquid chromatography (HPLC) to assess differences in phenolic fractions, co-pigmentation factors and visible color characteristics. Analysis revealed few significant differences between treatments. Variations occurred during fermentation, and some significant differences were noted after three months in the bottle; but few significant differences were apparent after two years.

Keywords: tannin, enological tannin, additives, anthocyanin, copigmentation, red wine color, Pinot Noir, Lemberger, wine aging, polymeric pigments, LPP, SPP

Biographical Sketch

Major Robert G. Gallasch, USAF Retired, earned his Bachelor of Arts degree in History in 1984 from the University of Rochester, Rochester NY, and a Masters of Arts degree in International Relations from Creighton University, Omaha NE, in 1999. After twenty years of service, he retired from the United States Air Force and then attended Cornell University to study viticulture and wine making. He lives with his family on a small farm in Moravia, New York.

Dedication

This work is dedicated to my family. My beloved wife and partner in life, Gail; and my wonderful children, Robert and Samantha. Without their patience, love and support this project could never have been undertaken, much less completed.

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Introduction

Cool climate viticulture presents several challenges to vintners who want to produce quality red wines. Shorter growing seasons, abundant rainfall, and cooler summer temperatures affect fruit maturity and promote higher levels of fungal infection at harvest. As a result, winemakers are concerned about maximizing color extraction from their grapes, and especially for lighter colored cultivars like Pinot Noir, want to do their utmost to stabilize the color of the wine after bottling. Other winemakers want to modify the wine's mouth-feel or reduce green, unripe characteristics. One of the techniques available to winemakers is adding enological tannins to the wine. Enological tannins may be derived from several different sources, either from grape skins and seeds, or from a variety of other plants, including wood from oak trees (*Quercus)* or extracts from the quebracho tree (*Schinopsis lorentzii,* or *Aspidosperma quebracho-blanco)*. There are many different enological tannin preparations available on the market and researchers have found that the chemical composition and concentration of useable tannins in these products varies with the source of the tannin material and the extraction method used (Laghi and others 2010). Until recently there has been little guidance for winemakers beyond manufacturer-recommended usage and doses, or anecdotal traditions from other winemaker's results (Harbertson 2010). Currently, however, more research, using increasingly sophisticated methods, is being focused on the composition of enological tannins. While early work on wine color evolution and wine aging was performed in France (Ribereau-Gayon and others 2006), research on the effects of enological tannin additives began initially in warm viticultural regions,

including Australia, California and Spain (Bautista-Ortin 2005; de Gaulejac and others 2001; Harbertson and others 2012; Laghi and others 2010; Mitropoulou, Hatzidimitriou, Paraskevopoulou 2011; Neves and others 2010; Obradovic 2006; Rinaldi and others 2010; Soto Vázquez, Río Segade, Orriols Fernández 2010).

A growing demand for red wines in cool climate viticultural regions, like the Finger Lakes of New York, is driving research interest in tannin additives. In cooler climates, winemakers often have trouble with poor color, lower brix and higher acidity (Harbertson 2010). Tannin additions are often marketed as a possible solution for poor color extraction or stability, especially in low colored varietals (e.g. Pinot Noir), or to reduce the loss of color during aging (Harbertson 2010; Neves and others 2010; Obradovic 2006; Obreque-Slíer 2009; Soto Vázquez, Río Segade, Orriols Fernández 2010), but their effectiveness has been called into question by various research efforts (Bautista-Ortín and others 2007; Cíchová M., Petříček J., Fiala J. 2008; Harbertson 2010; Harbertson and others 2012; Main and Morris 2007; Neves and others 2010; Obradovic 2006; Obreque-Slíer 2009; Parker and others 2007). Commercial tannin additives may have some usefulness for color extraction and stabilization only under a limited range of circumstances; for example, when the grape variety is low in polyphenols ((Neves and others 2010). Several authors have also suggested that enological tannins can be useful when fruit is carrying a high fungal disease load, by inhibiting laccase activity (Bautista-Ortín and others 2007; Keulder 2006; Neves and others 2010; Obradovic 2006; Obreque-Slíer 2009; Parker and others 2007), although this assertion needs further examination (Keulder 2006).

Wine Color Chemistry

Wine is a complex matrix composed of water, ethanol, organic and inorganic acids, sugars, and numerous phenolic and aromatic compounds. In terms of overall amounts, wines are composed primarily of water (~85-90%), and ethanol (~10-15%, or ~72-120 g/L), followed by glycerol (~5-10 g/L), and various acids (e.g. tartaric, malic, lactic, succinic) in amounts ranging from a few grams per liter to milligrams per liter (Peynaud 1984). Many other compounds are present in smaller amounts, milligrams per liter and below. Specific wine composition can vary significantly between cultivars used and even by vintage. This study focuses on the phenolic compounds – tannins, flavonoids (e.g. anthocyanins), and the various co-factors that can affect color formation. The word "tannin" is a functional term, originally denoting the plant-derived, high molecular weight polyphenolic compounds used to "tan" animal hides into leather (Margalit 2004; Waterhouse 2002). Winemakers have long associated the tannins from grapes, or from the wood used for barrels, with red wine quality and aging potential(Ribereau-Gayon and others 2006). Grape tannins themselves are either colorless or yellow. Wine's red color comes from anthocyanins, present in glycosylated forms (bound to sugars) in the skins of grapes, and extracted during the maceration (skin contact) phase of winemaking (Singleton and Esau 1969). Anthocyanins are not stable in wine over time, however, and it is the reaction between anthocyanins and tannins that produce the long-term stable color desired by winemakers (Ribereau-Gayon and others 2006).

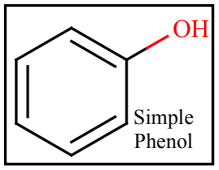

Figure 1-1: Simple Phenolics

Phenolics

Phenolic compounds are plant-derived compounds containing an aromatic ring structure, with one or more hydroxyl groups. The primary form is the simple phenol, which has a single

aromatic ring and is the building block of for many compounds, including wine color and tannins (Figure 1-1). These compounds are found in plants as part of the biosynthesis pathway for building lignin (Jackson 2008).

In wines, the phenolic compounds are generally grouped into two categories- the non-flavanoids and the flavonoids. Non-flavanoids include simple phenols like caffeic acid, one of the hydroxycinnamic acids (Figure 1-2). Hydroxycinnamic acids, particularly caffeic acid and its derivatives, are easily oxidized, and contribute to yellow coloration and browning in white wines, as well as the formation of various aroma compounds (Ribereau-Gayon and others 2006). Other non-flavanoids include benzoic acids, stilbenes and various hydrolyzable tannins (oligomers of either gallic or ellagic acid linked to sugars). Hydrolyzable tannins are not found in grapes, but rather are extracted from wood (e.g. oak barrels) in contact with the wine. These compounds are hydrolysed by the acidic conditions in wine or by enzymatic action, and rapidly esterify with ethanol (Waterhouse 2002). More important for red wine color is a class of phenols called anthocyanidins (Figure 1-3), which are extracted from both grape skins and seeds. They make up the majority of the phenols present in red wines (Zoecklein and others 1999). Flavonoids are categorized by a specific three-ring structure consisting of a central oxygen-containing pyran ring bound to two aromatic rings with a specific bonding structure. In wine, all flavonoids have the same hydroxyl groups on the A-ring, but different classes will be defined by configurations on the C-ring

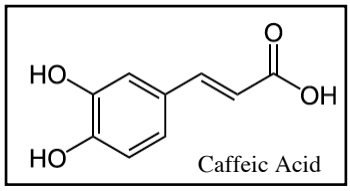

Figure 1-3: Hydroxycinnamic Acid

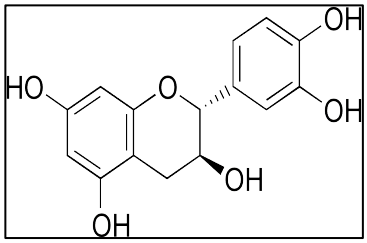

Figure 1-4: Anthocyanidins

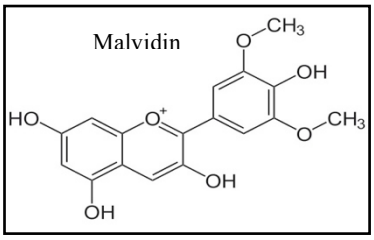

Figure 1-2: Malvidin

(Waterhouse 2002). The simple flavonoid structure has a positively charged, fully aromatic C-ring, but is highly unstable. *V. vinifera* cultivars contain five different forms (malvidin, cyanidin, peonidin, petunidin, delphnidin), with the malvidin form (Figure 1-4) being most abundant(Boulton 2001). Flavans have a fully saturated C-ring. Flavan-3-ols (e.g. catechin and its isomer epicatechin), the most abundant class of flavonoids, have an alcohol attached at the 3-position of the C-ring (Figure 1-5) (Waterhouse 2002). Classes of flavonoids are further subdivided by B-ring attachments. This is commonly a hydroxyl (-OH) group on the 4-position and methoxy group on the 3- and/or 5-positions. Many other compounds can be formed by conjugation with sugars or other compounds to these oxygen molecules (Waterhouse 2002).

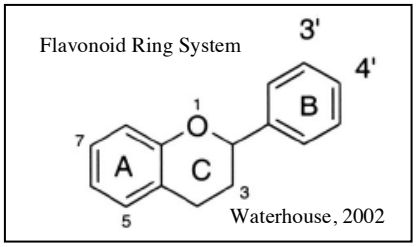

Figure 1-5: Flavonoid Ring System

Anthocyanins

Anthocyanins are the glycosylated form (i.e. bound to a sugar molecule) of the anthocyanidin and are responsible most of the color in red wine (Figure 1-6). Anthocyanin color is pH dependent, and shifts from red to violet-blue at pH > 3.5, and to colorless forms at higher pHs. Maximum color loss has been observed between pH 3.2-3.5 (Ribereau-Gayon and others 2006); at typical wine pH (between pH 3.0 and 4.0) only about 50% of potential red color for the most common anthocyanin (malvidin-3-glucoside) is visible. This is due to the dynamic equilibrium between its red-colored flavylium ion, and its colorless pseudo base form (Zoecklein and others 1999). Anthocyanins are also readily oxidized and subject to bisulfite bleaching. Chira (2011) found a strong correlation

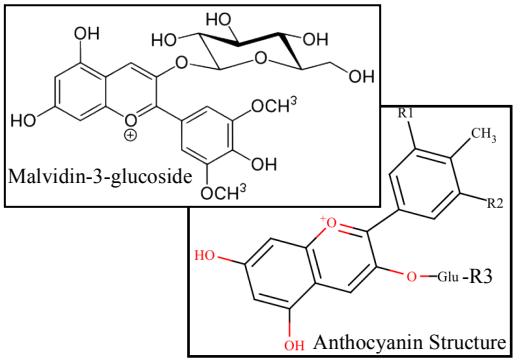

Figure 1-6: Anthocyanins

between aging and hue, with color shifting towards orange and appearing as hue augmentation by spectrophotometry. This was presumed to be due to anthocyanin losses during aging. The combination of these losses and rearrangement reactions between phenolic compounds were evident in a lower mean degree of polymerization with aging (Chira and others 2011).

Co-pigmentation

Anthocyanins can have non-covalent interactions with other phenolic compounds to produce the copigmentation effect, which results in an enhancement of color (Boulton 2001). This effect is important because it may account for 30% to 50% of the color of young red wines, which are typically darker than can be explained solely by the amount colored compounds present (Boulton 2001). According to Waterhouse (2002) copigmentation is a temporary association between anthocyanins and colorless cofactors; in this transient interaction, no chemical bonds are formed. A phenomenon called charge transfer complex formation, or π–π interactions, is responsible for these interactions (Waterhouse 2002), which produces an impact on both wine color and color intensity (Ribereau-Gayon and others 2006). Color changes arising from copigmentation are the result of two phenomena. The first is a bathochromatic shift, where the wavelength (λ) at which absorption occurs increases, causing the perceived wine color to shift towards the blue-violet, even at pH values where anthocyanin color would normally appear red. The second effect is a hyperchromic shift, which is perceived as an increase in color intensity (representing the amount of visible color), which is due to an increase in the amount of electromagnetic energy the stack can absorb (Waterhouse 2002). There is general agreement that co-pigmentation effects are pH dependent; however, research since Boulton (2001) (Asenstorfer and others 2003b; Lambert and others 2011; Waterhouse 2002) indicates that anthocyanins can and do participate in both copigmentation stacking and in self-associations, rather than exclusively one or the other. At

typical wine pH (pH 3.6), the intermolecular copigmentation effects may not be as significant as quinoidal base anthocyanin self-association, for color enhancement or stable polymeric pigment formation (Lambert and others 2011). Asenstorfer (2003) and Lambert (2011) found that typical wine conditions (pH 3.6) favored quinoidal base anthocyanin self-associations over flavylium ion intermolecular copigmentation interactions. The extent of the co-pigmentation effect in a wine depends on not only the pH of the solution, but also on the availability and type of co-factors present, their ratio to free anthocyanins, storage temperature, presence or absence of metal cations, and the amount of solvent present (e.g. ethanol) (Birse 2007; Boulton 2001; Brouillard and Dangles 1994a; Lambert and others 2011; Waterhouse 2002). Illustrating the impact of ethanol as the solvent, Gennari (Gennari and others 1992), reported that at pH 3.5, the color enhancement (as measured by absorbance at 520nm) due to copigmentation effects was only 5% in water, but 18% in an ethanol solution. There also is a minimum anthocyanin concentration needed before significant copigmentation effects become apparent. Boulton (Boulton 2001) reports this minimum as approximately 35μM (~18.5 mg/L malvidin-3-glucoside equivalents), so that the effects are visible in most red wines (300-500 mg/L) but not blush or rosé wines (5-50 mg/L). In addition, Boulton (Boulton 2001) found that specific cofactors have different degrees of interaction. Some flavones (e.g. quercetin) are strong cofactors, resulting in stronger copigmentation interactions, and also appear to increase their own solubility in young red wines. For example, quercetin can be found at concentrations of 20-50 mg/L in young red wines, while Boulton found it difficult to dissolve more than 5 mg/L in his model wine solutions. He concluded that the copigmentation complexes allowed these strong cofactors to be held in wines at multiples of their normal solubility (Boulton 2001).

Boulton (Boulton 2010) reports that co-pigmentation factors are usually monomeric flavonoids and cinnamates, and that co-pigmentation may be limited by either the availability of these co-factors or anthocyanins. Monomeric phenols (instead of dimers, trimmers or larger tannin complexes) are the most common cofactors, accounting for approximately 95% of the color variation observed from copigmentation (Boulton 2001). Numerous possible co-factors have also been observed to produce a co-pigmentation effect, including other anthocyanins, catechins, gallic acid, coumarins (derivatives of cinnamic acids), phenolic acids, flavonols, flavanols (Boulton 2001; Lambert and others 2011; Ribereau-Gayon and others 2006).

According to Boulton (Boulton 2001) and Jackson (Jackson 2008), the colored anthocyanin forms are almost planar in structural orientation, making loose stacking interactions between anthocyanins or co-pigments both easier and more probable. This stacking effect also physically prevents water molecules from hydrating the colored flavylium and bluish quinoidal forms and converting them to colorless forms (Brouillard and Dangles 1994a; Goto and Kondo 1991; Lambert and others 2011; Waterhouse 2002). On the other hand, other authors argue that the neutral colorless quinoidal base forms either predominate or are equally associated with copigmentation stacking (Asen, Stewart, Norris 1972; Asenstorfer and others 2003a; Lambert and others 2011). Associative co-pigmentation complexes are formed via several reinforcing mechanisms: hydrophilic attractions between glucose components, hydrophobic repulsion of water molecules, and co-pigment compounds, apparently slowing covalent bond formation (Birse 2007; Brouillard and Dangles 1994a; Brouillard and others 2010; Goto and Kondo 1991; Jackson 2008). The acylated forms of non-malvidin pigments, where the glucoside sugar molecules also have attached acid groups, like acetic, *p*-coumaric, or caffeic acids (Birse 2007; Boulton 2001; Brouillard and others 2010; Lambert and others 2011), are especially strong

cofactors. Grapes with predominantly malvidin-3-glucoside anthocyanins (e.g. Pinot Noir) will tend to produce wines with poorer color because their anthocyanidins lack these acylated structures (Birse 2007; Boulton 2001), or because the malvidin-3-glucodised form is thermodynamically favored for self-association over association with cofactors (Asenstorfer and others 2003b; Lambert and others 2011) . Lambert (2011) indicates that cofactors tend to enhance color only when they are available in excess, and believes that self-association between malvidin-3-glucosides is the primary contributor to copigmentation; the dilution effect Boulton (2001) described was due primarily to a loss of self-association, rather than simply a disruption of copigmentation stacks (Lambert and others 2011).

Copigmentation effects are dynamic phenomena (Brouillard and Dangles 1994a), which help protect anthocyanins until the slower polymerization reactions can occur; these effects can be considered a "storage-form" for anthocyanins (Sacchi, Bisson, Adams 2005; Soto Vázquez, Río Segade, Orriols Fernández 2010). Red and purple colors may be lost due to several factors, including oxidation (which shifts colors towards more yellow-brown), thermal degradation and other chemical breakdown reactions (Ribereau-Gayon and others 2006). Losses due to precipitation occur as solids aggregate, capture anthocyanins, and settle out of solution. Anthocyanins also bind with proteins, acetaldehyde-tannin complexes, or fining agents, and precipitate out of solution. This leads to a reduction in total color. Overall, color losses due to oxidative degradation and precipitation are more significant than those due to thermal or chemical breakdown (Zoecklein and others 1999). Boulton argues that copigmentation complexes also serve to protect anthocyanins from oxidation (Boulton 2001). He found that oxidation reaction rates were related to the concentration of free monomeric anthocyanins, and the rates were slowed in wines with higher levels of copigmentation. The same holds true for

polymerization reactions, where the reduction in the pool of free monomeric anthocyanins due to copigmentation also slowed the rate of polymerization reactions; it was also speculated that the copigmentation complexes themselves limit the rate of polymeric pigment formation (Boulton 2001).

In young red wine there is a race underway between factors causing the loss of color and those which produce stable colored compounds. Stable color results from slower, but longer lasting, polymerization reactions. The products of these reactions, the fraction of colored compounds which resist SO_2 bleaching, have been described as polymeric pigments (Somers 1971), although not all the compounds defined by Somers as polymeric pigments are actually polymeric, colored or stable (Harbertson and Spayd 2006). However, as a generalization, the term polymeric pigment is adequate to describe the primary colored compounds present in aged red wines. They result from a polymerization process whereby stable chemical bonds form between tannins and the colored anthocyanins (Somers and Evans 1977). Copigmentation and related co-factors also have implications for color extraction and stability. According to Boulton (Boulton 2001; Mazza 1999; Mazza and Brouillard 1990), the concentration and availability of cofactors during fermentation is critical to maximizing color extraction and color retention. Maximum anthocyanin extraction, which occurs early in fermentation, is equilibrium dependent. Once saturation level is reached, no further anthocyanins will be extracted from the grape skins, regardless of the mechanical or thermal vinification techniques employed (Boulton 2001). Boulton argued that thermovinification techniques were a double-edged sword; on the one hand, higher temperatures increased the solubility of most anthocyanin and cofactor species, which potentially improves extraction from the skins. Thermodynamically, however, higher temperatures also favor the dissociation of copigmentation complexes that more quickly

saturated the pool of free anthocyanins, stopping extraction and reducing both visible and total color (Boulton 2001; Brouillard and Dangles 1994b; Brouillard and others 2010; Mansfield and Zoecklein 2003; Mazza and Brouillard 1990; Sacchi, Bisson, Adams 2005; Schwarz and others 2005). To extract more of the available colored compounds from the skins, the pool of free anthocyanins in the wine must be reduced. This has been demonstrated by adding thoroughly macerated grape skins from a red wine (i.e. maximum anthocyanin extraction) to a white juice; additional color was extracted from the skins (Boulton 2001). If co-factors are present in sufficient quantities, they will rapidly form stacked copigmentation complexes with free anthocyanins(Brouillard and Dangles 1994b), reducing anthocyanin concentration and potentially enabling further extraction from the skins (Bautista-Ortín and others 2007; Boulton 2001; Mazza and Brouillard 1990). The formation of copigmentation complexes also helps keep the available anthocyanins in solution by preventing interaction with other reactive compounds, including proteins and polysaccharides (Birse 2007; Boulton 2001; Darias-Martín and others 2006; De Beer, Dalene et al. 2006; Del Pozo-Insfran 2006). This reduces the amount of anthocyanins "captured" by adsorption to solids (e.g. yeast lees, grape skins, pulp, and seeds) that settle out of solution during fermentation. Without cofactors, copigmentation complexes cannot form, and more colored anthocyanin material will be lost. Boulton concluded that the availability of cofactors during the early stages of fermentation would have a significant effect on color formation by not only increasing color temporarily through the copigmentation effect, but also by increasing the total amount of anthocyanins extracted from the macerating grape skins. In contrast, grape varieties low in both color and cofactors (e.g. Pinot Noir, low in acylated forms of non-malvidin pigments) would produce wines with less color because of saturation; equilibrium limits anthocyanin extraction (Boulton 2001; Darias-Martín and others

2006; Mazza 1999). Other authors have related improvements in monomeric pigment extraction in Pinot Noir fermentations with elevated copigmentation or cofactor extraction (Fischer, Strasser, Gutzler 2000; Mansfield and Zoecklein 2003; Sacchi, Bisson, Adams 2005; Schwarz and others 2005; Soto Vázquez, Río Segade, Orriols Fernández 2010)

Tannins

The term "tannin" is a functional term describing higher molecular weight phenolics composed of multiple simple phenols. It is a complex category of phenolic compounds, defined by the ability to produce stable combinations with either proteins or polysaccharides (Ribereau-Gayon and others 2006). Tannins also contribute to perceived astringency in wine (and other food products) by precipitating salivary proteins (Zoecklein and others 1999). Tannins, like the anthocyanins, are produced by the plant and accumulate in grape skins, seeds, stems and other woody parts of the grapevine. Unlike anthocyanins, which are almost completely extracted during the first two or three days of maceration, the higher molecular weight tannins require extended skin/seed contact of at least five to six days (Boulton and others 1999); tannin extraction continues with increased contact time, until a maximum level of extraction is reached, based on the solubility of the tannin material at the temperature, pH, SO_2, and ethanol levels of the fermenting must (Ribéreau-Gayon 1974; Sacchi, Bisson, Adams 2005). In addition to equilibrium effects and the solubility of individual components, the total percentage of available phenolic material extracted from the grapes depends on the extent to which grape cells are perforated or degraded by mechanical, chemical or thermal means (Ough and Amerine 1961; Ribéreau-Gayon 1974; Singleton and Rossi 1965),

Role of Tannins in Wine

Many researchers have examined the role of wine tannins in color stabilization during fermentation and wine aging (Adams and Harbertson 1999; Bautista-Ortin 2005; Bautista-Ortín and others 2007; Boulton 2010; Boulton 2001; Boulton and others 1999; Brouillard and Dangles 1994b; Cíchová M., Petříček J., Fiala J. 2008; Darias-Martín and others 2006; Darias-Martín and others 2001; Fulcrand and others 2006; Harbertson, Picciotto, Adams 2003; Harbertson 2010; Harbertson and others 2012; Kennedy and Waterhouse 2000; Kennedy 2008; Keulder 2006; Sacchi, Bisson, Adams 2005; Singleton and Rossi 1965; Singleton and Esau 1969; Singleton, Sullivan, Kramer 1971; Singleton, Orthofer, Lamuela-Raventós 1999; Somers and Evans 1977; Somers and Evans 1979; Somers 1971), as well as their impact on other sensory characteristics like bitterness, astringency, and aroma contributions or aroma masking (Bautista-Ortin 2005; Chira and others 2011; Mitropoulou, Hatzidimitriou, Paraskevopoulou 2011; Peynaud 1980; Ribéreau-Gayon, Boidron, Terrier 1975).

Hydrolysable Tannins

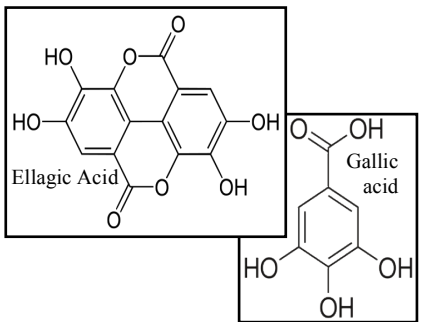

Figure 1-7. Hydrolysable Tannin sub-units

Tannins are broadly grouped into two classifications, hydrolysable and condensed, based on their origin and basic sub-unit. Hydrolysable tannins are built from non-flavonoid phenols, with sub-units of gallic acid (forming gallotannins) or ellagic acid (forming ellagitannins) bound to a sugar (hexose) core by esterification (Figure 1-7) (Obradovic 2006; Ribéreau-Gayon 1974; Waterhouse 2002). This ester bond is water and ethanol soluble, giving rise to the name for this class of tannins (Waterhouse 2002). Gallic acid is found in *V. vinifera* seeds and skins, and is also a component of hydrolysable tannins. Neither ellagic acid nor its

hydrolysable tannin form (Figure 1-8) is found in *V. vinifera* grapes; it originates instead from other fruits or various woody plants like oak (genus *Quercus)* used for cooperage, or quebracho (*Aspidosperma quebracho-blanco* or *Schinopsis lorentzii*), used as a tannin source for industrial or enological additives. These hydrolysable tannins normally enter wine via contact with oak barrels, wood chips or commercial enological tannin additives (Ribereau-Gayon and others 2006). Two common ellagitannin isomers found in oak barrels are vescalagin and castalagin (Ribereau-Gayon and others 2006). Different monomeric and dimeric ellagitannins are found in different species of oak used for cooperage, or oak and chestnut (*Castanea sativa*) used in the production of tannin additives (Ribereau-Gayon and others 2006). Hydrolysable tannins are important to winemakers due to their perceived impact on the sensory properties—aroma, bitterness and astringency—of wine.

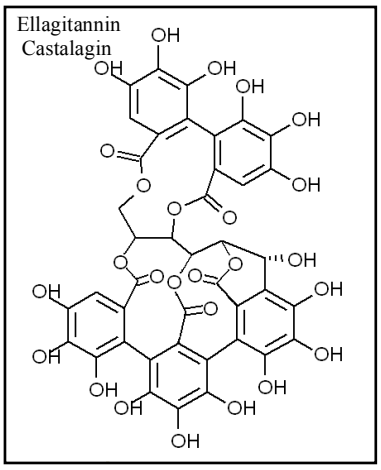

Figure 1-8. An Ellagitannin

Condensed Tannins

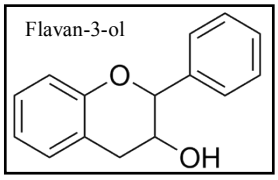

Figure 1-9. Flavan-3-ol Structure

Condensed tannins, are grape-derived, high molecular weight compounds, also known as proanthocyanidins (Birse 2007; Kennedy, Saucier, Glories 2006; Obradovic 2006; Waterhouse 2002). They are colorless polymers in the visible spectrum (although they can be detected in the UV-range, at 280 nm), and are composed of linked flavan-3-ol (e.g. catechin or epicatechin) monomer sub-units (Figure 1-9) (Birse 2007). Proanthocyanidins range in size from dimers (two sub-units), oligomers (with a small number of subunits; Birse, 2007) to hundreds of subunits, with an almost endless variety of configurations possible (Ribereau-Gayon, 2006). Proanthocyanidin oligomers of up to 16-subunits have been found in *V. vinifera* (Garrido and

Borges 2011). Condensed tannins vary in size (expressed as mean degree of polymerization, mDP) and subunit composition by where they are located in the grape berry. Seed tannins, which average ~10 mDP, are composed of catechin, epicatechin and epicatechin with a gallate terminal unit. Those found in grape skins tend to be larger (~32 mDP) and are primarily composed of epicatechin and epigallocatechin (Harbertson, Kennedy, Adams 2002; Harbertson and others 2008; Harbertson 2010) . Proanthocyanidin oligomers appear to polymeric phenols during fermentation, and appear to play a significant role in the initial formation of stable polymeric pigments in young red wines (Boulton and others 1999). One of the most abundant small condensed tannins, procyanidins, which hydrolyze to cyanidin (Garrido and Borges 2011), are found in the seeds and skins of most red grape cultivars, except Pinot Noir (Thorngate 1993), and are extracted during maceration. During fermentation and the first year of aging, these small condensed tannin polymers form chemical bonds with each other and also with anthocyanins; either directly or through intermediaries like acetaldehyde. The gradual disappearance of anthocyanins, with a concurrent transformation into the rather broad classification of "polymeric pigments" during aging has been attributed to various condensation, polymerization and oxidative reactions (Birse 2007; Fulcrand and others 2006; Garrido and Borges 2011; Jackson 2008; Schwarz and others 2005; Vivas and others 2004), including copigmentation stacking interactions and anthocyanin self-association (Boulton 2001; Brouillard and Dangles 1994b; Lambert and others 2011; Schwarz and others 2005). Low initial color and long term color stability issues in Pinot Noir wines, for example, may be caused by a lack of procyanidins, preventing anthocyanin stabilization during the early stages of wine making and aging (Jackson 2008), although Boulton has argued that the lack of copigmentation is also significant (Boulton 2010). If not stabilized early on, a greater percentage of available anthocyanins will be lost to

precipitation, oxidation and the various mechanisms of chemical degradation. Jackson (2008) also notes another color loss phenomenon occurring during fermentation; after several days, ethanol levels rise to a concentration that affects co-pigmentation. Ethanol destabilizes the hydrogen bonding of the anthocyanin complexes (Schwarz and others 2005; Soto Vázquez, Río Segade, Orriols Fernández 2010), causing a disruption of the co-pigmentation stacking effect. This appears as a dramatic loss of color even though the level of anthocyanins has not decreased significantly.

Analysis of Phenolic Compounds

Associative assessments of wine

It has long been known that tannins are an important component of red wine (Peynaud and Ribereau-Gayon 1971; Peynaud 1984; Ribéreau-Gayon, Pontallier, Glories 1983; Ribéreau-Gayon 1960; Singleton and Rossi 1965). Even before the advent of modern analytical methods, winemakers and researchers were keenly aware that wine was not a static beverage; it changed over time, not only during the fermentation process, but also during aging in barrels, and even in the bottle. As the wine aged, precipitates formed in the bottle, color changes were observed, and the sensory properties of the wine changed. A whole lexicon of wine-specific terminology developed to explain what constituted a good wine, and what was happening as it got older. This terminology was of necessity descriptive – a young wine might be "simple" or "bold", whereas an older wine might be considered "complex." Tannins "mellowed" or became "softer" or "rounded." Flavors "blended" and aromas became a distinctive "bouquet." Terms for wine faults and for desired characteristics were associative in nature – what the researcher or winemaker observed could be described as being similar to something else, be it an aroma, a flavor, or any

one of a number of other wine properties. However, associative description provides only limited analytical clarity. Producers wanted to know more about wine and winemaking, in order to improve their products, reduce waste and increase profits. In response, scientists began applying new tools – optical microscopes, chemistry, advances in materials technology – to the study of issues related to wine making, quality and aging. Wine started being described in terms both traditional (from sensory evaluation) and scientific, utilizing the language of microbiology, chemistry, and other physical properties (i.e. color). Even sensory analysis has moved beyond the descriptive into the realm of instrument-mediated quantitative measurement, as the biochemical mechanisms underlying human perception are decoded (Lawless and Heymann 1999; Lawless 2010). Wine is a complex beverage and improvements in the sensitivity and selectivity of analytical methods have enabled researchers to both appreciate wine's complexity and provide useful quality control inputs to the wine industry.

Early instrumental analysis of wine tannin and color

Foundational work on wine chemistry and microbiology began after World War II at the University of Bordeaux, by enologists Emile Peynaud and Jean Ribereau-Gayon (Peynaud 1984). The systematic analysis of wine tannins and color, still in its infancy in the 1960s (Ribereau-Gayon and others 2006), began to move the science of wine color beyond descriptions of what humans could see. Researchers started identifying the components of wine color and began explaining what was happening to these components during fermentation and aging (Glories 1974; Ribereau-Gayon and Stonestreet 1965; Singleton and Rossi 1965). Although the basic chemical composition of wines had been known for some time, understanding both the biological origins of different components of wine (with the contributions of the yeast and other microorganisms), as well as the specific chemistry of changes to the components of wines during

aging required the development of new methodologies (Adams and Harbertson 1999; Birse 2007; Harbertson, Picciotto, Adams 2003; Ribéreau-Gayon, Pontallier, Glories 1983; Singleton, Orthofer, Lamuela-Raventós 1999; Somers and Evans 1977; Timberlake and Bridle 1985; Williams and others 1984).

Direct Spectrophotometric Methods

Photometric methods (direct measurement of color)

Early efforts to measure wine color were based on simple colorimeters or other methods that compared wine samples to a color reference (Somers and Evans 1977). Photometric methods, which first became available in the 1930s, allowed measurement of "relative luminosity" and use of the trichromatic coordinate system, improving precision and repeatability (Peynaud 1984; Somers and Evans 1977; Steinberger July 30, 2004). In the 1960s, building on the methods developed to analyze agricultural produce, processed foods and industrial chemicals, photometry became the international standard for measuring wine color, but these methods did not provide information about the composition of wine color (Somers and Evans 1977).

Oxidation-Precipitation methods

Indirect measurement of phenolic fractions

Refinements to spectrophotometer methods, using oxidation-precipitation reactions to measure the tannin content of a wine, have proven important for progress in this area. Initially, the standard methods used by industry had problems when applied to wine phenolics – unless conditions of experiments were duplicated precisely, the methods suffered from precipitate formation, unexplained variation of results, and subsequent difficulty in comparing results between researchers (Adams and Harbertson 1999; Singleton and Rossi 1965). Researchers needed better understanding of the nature of these reactions and their relationship to the phenolic

content of grapes and wine. Early methods involved titrimetiric oxidation of dealcoholized wine with permanganate solution, before and after treatment with carbon (Singleton and Rossi 1965). The difference between the two measurements was described as "tannin plus coloring matter," but without special fractionating procedures it was not possible to determine the breakdown between tannins, color, and molecules responsible for astringency (Ribereau-Gayon and others 2006; Singleton and Rossi 1965).

Folin-Denis method (total phenolics)

The Folin-Denis method (1912) improved on oxidation-precipitation methods, using reagents that produced color-forming reactions with monohydric-phenols, polyphenols, flavonoids, tannins and other readily oxidized substances, thus yielding a measurement of "total phenolics" instead of total tannins. The goal of enology researchers (e.g. Bakker, Brouillard; Burroughs, Glories, Singleton, Somers, Timberlake, Ribereau-Gayon, Nagel, Wulf, and Peynaud) was to find or adapt a method that would produce a complete reaction with all the phenols present, provide reproducible results and suffer from little or no interference from other substances like ascorbic acid. The Folin reagent made this possible because it survived at typical wine pH (between 3.0 and 4.0) long enough to react with the phenol ions and cause a stable color change. At higher pHs, the alkaline conditions destroyed the blue-colored formation faster than the reagent could react with the phenol ions, rendering colorimetric reading unreliable (Singleton and Rossi 1965).

Folin-Ciocalteu method (total phenolic content)

The Folin-Ciocalteu method (1927) improved on the earlier Folin-Denis method, giving a better estimate of total phenolic content through more complete oxidation, yielding better measurements with lower interference and lower precipitate formation. One advantage over the

direct spectrophotometric method is that it forms "a colored species with a standard molar absorptivity. By comparison, absorbance at 280 nm for different phenolics results in very different molar absorptivities" (Sacks, G.L., personal communication, 2012), In addition, gallic acid was found to be a better standard than tannic acid because of its low cost, purity, solubility and availability (Singleton and Rossi 1965). However, preparation of the reagents and standards remained challenging, until high quality standardized commercial preparations could be purchased (Singleton and Rossi 1965). Singleton (1999) also thought that Folin-Ciocalteau results, although numerically different from other methods, could be generally correlated with results from other methods, if the samples were similar. Harbertson (2006) found that results from Folin-Ciocalteau and ferric chloride methods were highly correlated. Keulder (2006) cautioned that the Folin-Ciocalteau method can over-estimate phenols because all hydroxyl groups are oxidized; Mullen (2007) found that Folin-Ciocalteau estimates did not correlate well with HPLC results. More recently, Rinaldi found that the Folin-Ciocalteu index (total phenolics) was a poor predictor of wine astringency (Rinaldi and others 2010).

Current spectrophotometric methods

Current approaches to measuring wine color via spectrophotometry utilize equipment capable of taking measurements at different wavelengths throughout the UV-visible light spectrum (i.e. multiple wavelengths, λ), ranging from approximately 280 nm through 700 nm (Table 1). In addition, various wet chemistry techniques, involving pH changes, buffering solutions and different reagents, are employed to create color changes and/or precipitants (Adams and Harbertson 1999; Iland and others 2004; Jacobson 2006; Kennedy 2008; Singleton and Rossi 1965; Somers and Evans 1977; Somers 1971). By measuring the absorption at wavelengths known to be the wavelength of maximum absorbance for different compounds (λ_{max}), relative

levels of those compounds, or groups of compounds, can be made (Jacobson 2006). Furthermore, by taking advantage of known selectivity (e.g. pH dependent color, bisulfite bleaching or acetaldehyde binding), approximations of different phenolic fractions can be obtained by use of simple arithmetic computations (Adams and Harbertson 1999; Bakker, Preston, Timberlake 1986; Harbertson, Kennedy, Adams 2002; Harbertson, Picciotto, Adams 2003; Jackson 2008; Jacobson 2006; Nagel and Wulf 1979; Ribereau-Gayon and others 2006; Somers and Evans 1977; Timberlake and Bridle 1976). Some assumptions are inherent in these calculations of colored fractions. The total phenolics measurement applies a correction factor ($A_{280} - 4$) to account for absorbance at 280 nm by non-phenolic compounds (Somers and Evans 1977). Other assumptions include complete bleaching by SO_2, complete liberation of monomeric anthocyanins with acetaldehyde addition, and that the wine buffer dilution will disassociate all copigmentation complexes (Birse 2007). Somers concluded that although the chemical definition of color could not be defined, useful data on young red wine phenolic composition could be gained by spectral readings at three different wavelengths (280nm, 420nm and 520nm), which gave measurements of non-visible phenolics (280nm), visible red color (520nm) and visible yellow-brown color (420nm). Later work (Tsanova-Savova, Dimov, Ribarova 2002), added measurements of the visible blue color component (620nm) as well. Somers compared the ratio of monomeric anthocyanins to polymeric pigments, based on the tendency of monomeric forms to polymerize as wine ages, in order to provide objective guidelines about wine quality and aging potential (Somers and Evans 1977). Later work by Boulton (2001) challenged the precision of the Somers method for assessing free anthocyanins by bleaching, finding that because Somers combined measurements of free and copigmented anthocyanins, the levels of ionized (colored) anthocyanins predicted (without accounting for copigmentation effects) yielded inflated

anthocyanin levels. A modification of Somers' method, which included a measurement of wine diluted in a buffer solution to break apart copigmentation complexes, yielded improved resolution of color due to ionized anthocyanins (Boulton 2001). The measurement of polymeric pigments by protein precipitation can also be combined with bisulfite bleaching into one assay, enabling differentiation between monomeric anthocyanins and polymeric pigments. Performing the measurements at the same time, with the same standard curve, enables direct comparison of results, something that is otherwise problematic. Polymeric pigments were found to continue to absorb at 520 nm with bisulfite present (Harbertson, Picciotto, Adams 2003). Monomeric anthocyanins are removed by the bisulfite-bleaching step, allowing for direct measurement of polymeric pigments (Harbertson, Picciotto, Adams 2003).

However, observations noted less than half of the polymeric pigments precipitated with protein addition, leading to the conclusion that there were two classes of polymeric pigments – one that precipitated with protein (large polymeric pigment, LPP), and one that did not (small polymeric pigment, SPP) (Harbertson, Picciotto, Adams 2003). Fruit contained mostly SPPs, while most LPPs appeared to be formed during winemaking. LPPs also formed a much higher percentage of color when the pH was raised to pH 4.9. This proved an easy way to directly measure the large polymeric pigment component in wine samples (Harbertson, Picciotto, Adams 2003). Not all anthocyanin-derived "pigments" included in the original Somers definition of polymeric pigments (i.e. colored compounds which are not bisulfite bleachable) are either stable or polymeric - some colored compounds are polymeric, others (e.g. vitisins) are not, and not all are completely resistant to bisulfite bleaching (Harbertson and Spayd 2006). Copigmentation is believed to occur primarily between monomeric anthocyanins and other colorless wine phenolics (e.g. monomeric flavan-3-ols, flavonols, procyanidins, hydroxycinnamates, hydroxybenzoates;

Lambert, 2011) decreasing as wine ages due to polymerization of these monomeric anthocyanins (Boulton 2001). Anthocyanin self-association is also important for copigmentation (Lambert, 2011). Some cofactors associate more strongly than others; Lambert (2011) found that quercetin was a strong cofactor, while catechin, caffeic acid and oligomeric procyanidins were not strong copigments. Because these free monomeric anthocyanins and the polymeric pigments have different spectral characteristics, they can be monitored by spectrophotometry; absorbance characteristics may also change depending on the specific cofactors involved in copigmentation stacking (Boulton 2001; Schwarz and others 2005; Singleton, Orthofer, Lamuela-Raventós 1999; Versari, A, R Boulton, and G Parpinello. 2008).

High Performance Liquid Chromatography (HPLC)

High performance Liquid Chromatography (HPLC) can be used for both identifying individual chemical components of wine and for validating the results of other methods (Harbertson, Kennedy, Adams 2002; Harbertson, Picciotto, Adams 2003; Peng 2001), or for establishing comparisons across methods. A limiting factor for any of the methods besides direct measurement of absorbance is the selection and availability of suitable laboratory reference standards. Reference standards are used to prepare the standard curves needed for expressing results in terms of standardized units and for identifying individual species detected by HPLC equipment (Chira and others 2011; Cíchová M., Petříček J., Fiala J. 2008; del Rio and Kennedy 2006; Grindlay and others 2011; Harbertson and Spayd 2006; Harbertson and others 2008; Harbertson and others 2012; Laghi and others 2010; Mercurio and Smith 2008; Mullen, Marks, Crozier 2007; Obreque-Slíer 2009; Parker and others 2007; Peng 2001; Rodrigues 2012; Versari, Boulton, Parpinello 2007; Versari, Boulton, Parpinello 2008). Although laboratory-based purification techniques, using expensive specialized equipment, can be used to prepare reference

standards, this is not the cheapest or most efficient solution (Laghi and others 2010; Peng 2001; Souquet and others 2000). Finding a suitable reference standard that is inexpensive, readily available and which produces linear response curves with available methods is important for both research and industry laboratories (Harbertson, Picciotto, Adams 2003; Harbertson and Spayd 2006).

Polymeric nature of wine pigments

Measuring polymeric pigments

Newly made wines contain mostly monomeric anthocyanins, with little polymeric pigment, (Bakker and Timberlake 1985; Birse 2007; Boulton 2001; Fulcrand and others 2006; Ribereau-Gayon and others 2006; Somers and Evans 1977; Somers 1971), while nearly all the color from aged wines is in the form of polymeric pigments (Asen, Stewart, Norris 1972; Birse 2007; Boulton 2001; Fulcrand Helene and others 2004; Schwarz, Jerz, Winterhalter 2003; Somers and Evans 1977; Somers and Evans 1979; Somers 1971; Somers and Ziemelis 1980; Somers and Ziemelis 1985). Research done in the 1970s improved the ability of spectrophotometry methods to identify and discriminate between the different components of wine color. One criticism of previous methods was that they did not look at wine in its natural state. Changes made to the wine sample during preparation also affected the equilibrium chemistry of the various components of the wine (Adams and Harbertson 1999; Bakker, Preston, Timberlake 1986; Harbertson, Picciotto, Adams 2003; Jackson 2008; Singleton and Rossi 1965; Somers and Evans 1977; Somers 1971).

Chris Somers and Michael Evans, researchers at the Australian Wine Research Institute, developed an assay for directly measuring the polymeric pigment fraction in wine (Somers 1971). The Somers-Evans method has been widely adopted (Birse 2007; Boulton 2001;

Harbertson, Picciotto, Adams 2003; Iland and others 2004; Jackson 2008; Jacobson 2006; Jensen and others 2008; Zoecklein and others 1999) and has been the basis for much subsequent research into wine color and phenolic evolution. Their method takes advantage of the bleaching effect SO_2 has on wine color. Somers found that anthocyanins were instantly decolorized by the addition of excessive amounts of SO_2 at wine pH, and concluded that residual color (measured at 520 nm) must be due to polymeric pigments (Somers 1971). Bisulfite bleaching has since become a staple of wine color analysis (Birse 2007). The original Somers-Evans method has also been modified since then to allow direct measurement of other phenolic fractions not assessed by the original method. These include an acetaldehyde addition step to measure monomeric anthocyanins (Boulton 2001; Burroughs 1975; Hagerman and Butler 1981; Hagerman and Butler 1989) and the addition of a buffer solution to allow for measurement of color from copigmentation complexes (Boulton 2010; Boulton 2001; Harbertson, Picciotto, Adams 2003; Harbertson 2010).

Measuring anthocyanins (monomeric pigments and copigmentation)

Anthocyanins play a more important role in young wine color than polymeric pigments (Boulton 2001; Somers and Evans 1977), but decline with aging. In older wines, complex polymeric pigments have greater impact on color (Somers and Evans 1977). Furthermore, the fact that acetaldehyde binds more strongly to SO_2 than to anthocyanins (Burroughs 1975) means that the addition of excess amounts of acetaldehyde allows the researcher to determine (via changes in optical density) what proportion of total anthocyanins were bleached in the original wine sample. The addition frees the bisulfite-bound anthocyanins in the sample, allowing direct spectral measurement of the total amount of colored SO_2-bleachable pigments, which are primarily free anthocyanins in their colored form (Birse 2007; Jacobson 2006; Singleton,

Orthofer, Lamuela-Raventós 1999). Furthermore, when the wine is acidified (~ pH≤ 1), all the anthocyanins are forced into their colored forms, allowing estimation of the total anthocyanin content (both colored and colorless at wine pH). Since polymeric pigments are much less affected by pH changes than are monomeric pigments, the monomeric contribution to color can be estimated. This has proven especially useful for analyzing the color of young red wines (Boulton 2001; Burroughs 1975; Singleton, Orthofer, Lamuela-Raventós 1999). The calculations needed to achieve the estimates are simple. Different properties (i.e. color density, hue, total anthocyanins and phenolics, bleachable vs. non-bleachable anthocyanins, etc.) can be calculated by simple arithmetic computations using the different spectral readings. However, comparisons of results between Somers original methodology (Somers and Evans 1977; Somers 1971) and those of later modified methods (Boulton 2001), highlights the complexity of wine color chemistry and the problems of indirect assessments when interpreting results. The two commonly used methods for assessing SO_2 bleachable colored anthocyanins illustrate this challenge – The Somers assay returned consistently higher values than the Boulton method (Darias-Martín and others 2006). This is because they are measuring different, but overlapping, subsets of the colored compounds affecting spectral absorption. Boulton's method gives both co-pigmented and ionized or "free" anthocyanins, while the Somers assay doesn't allow analysis of different fractions, and only makes a broad assessment of total "colored" anthocyanins (Birse 2007; Boulton 2001; Darias-Martín and others 2006; Harbertson, Picciotto, Adams 2003). Improvements in HPLC resolution have also aided analysis of SO_2-resistant polymeric pigments in red wine. By combining traditional spectrophotometric color analysis with HPLC analysis of specific components, researchers have been able to improve characterization of unbleached polymeric pigments and identify small anthocyanin derivatives, which are also not sulfite

bleachable. Comparative analysis determined that the levels of monomeric pigments, rather than tannin content, provided a better correlation for the level of copigmentation. Total wine color could be described by a combination of three components: copigmentation (8%-30%), free anthocyanins (24%-35%), and polymeric pigments (35%-63%) (Boulton and others 1999; Versari, Boulton, Parpinello 2008).

Pyranoanthocyanin color compounds

Research has demonstrated that other pigments found in red wine are derived from anthocyanin reactions with yeast metabolites (Bakker and Timberlake 1997; Birse 2007; Fulcrand and others 1996; Kennedy and Waterhouse 2000; Romero and Bakker 2000; Timberlake and Bridle 1976; Waterhouse 2002). These pyranoanthocyanin-type pigments (Figure 1-11) are monomeric or low molecular weight colored compounds that, like the larger polymeric pigments, resist bisulfite bleaching. Pyranoanthocyanin-type pigments resist bisulfite bleaching because the anthocyanin molecule is protected at the C4 position, where the bisulfite ion would normally attach (Birse 2007). The most common pyranoanthocyanin pigment found in red wine is Vitisin-A, a reaction product of malvidin-3-glucoside and pyruvic acid, which at wine pH typically contributes a red-orange color. Pyranoanthocyanin color is also pH dependent, but is much more strongly colored at lower pHs than malvidin-3-glucoside and retains more of its color at higher pHs (e.g. pH 7) (Bakker and Timberlake 1997; Romero and Bakker 2000), potentially contributing a higher percentage of apparent color (relative to monomeric or "free" anthocyanins) than its concentration might indicate. Bakker and Timberlake (1997) found that pyranoanthocyanins were even more

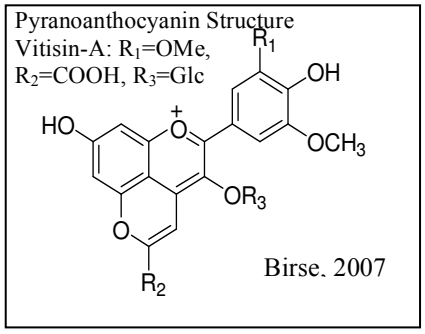

Figure 1-10: Pyranoanthocyanins

abundant in Port wines, and that pyranoanthocyanin concentration increased with age (Bakker and others 1998), indicating that it is one of the stable color compounds contributing to the color of aged wine. Along with other ethyl- or acetaldehyde-linked (e.g. Vitisin-B) anthocyanin-flavanols (Birse 2007; Romero and Bakker 2000), and its acetyl- and *p*-coumaryl derivatives, pyranoanthocyanins typically account for 1-4%, and sometimes as much as 10%, of the SO_2-resistant pigment (Asenstorfer, Hayasaka, Jones 2001; Bakker and others 1998). This may skew color fraction results, depending on the wet chemistry method used. Pyranoanthocyanins therefore contribute to the bisulfite-bleaching resistant color fraction, which is typically described as "polymeric pigments" in the literature (Adams and Harbertson 1999; Bautista-Ortin 2005; Birse 2007; Harbertson, Picciotto, Adams 2003), but not the protein precipitable fraction, since these compounds are too small to precipitate with proteins and will therefore not show up directly in assays like Adams-Harbertson (Adams and Harbertson 1999; Birse 2007; Harbertson, Picciotto, Adams 2003). Harbertson speculated that the vitisins would likely be included in the small polymeric pigment (SPP) fraction, along with other small, bleaching-resistant colored compounds (Harbertson, Picciotto, Adams 2003). Atanasova (Atanasova and others 2002) found that vitisins could form polymers with tannins. Vitisin-A, and other pyranoanthocyanins, also have a lower spectrophotometric absorbance maximum (λ_{max} at 501 nm) than malvidin-3-glucoside (λ_{max} at 528 nm), so that spectrophotometric measurements at the higher wavelength will underestimate its contribution (Birse 2007).

Yeast also plays a major role in the final phenolic profile of wines, including the adsorption (binding) of anthocyanins to yeast cells, acetaldehyde mediated bridging between anthocyanins and flavan-3-ols, and interactions between various degradation products and tannins. German researchers from the Geisenheim Research Center demonstrated that grape variety and yeast

microbiology played a role in the development of specific phenolic profiles in grapes and wines. Exploration of the biochemical pathways of phenolic production (polyphenol biosynthesis) in grapevines found that specific anthocyanin content and composition were variety dependent, and as suspected, linked to plant anti-fungal defenses (Dietrich and Pour-Nikfardjam 2009). Polyphenolics showed both positive and inhibitory anti-microbial effects too. High levels of polyphenolics inhibited malolactic and spoilage bacteria, while *Saccharomyces cerevisiae* could tolerate most polyphenols. Wine polyphenols helped protect cell walls from hydrolytic enzymes at the end of fermentation, possibly slowing the rate of cell autolysis (Dietrich and Pour-Nikfardjam 2009).

Protein Precipitation methods

Assessing total tannins and tannin fractions

The Somers-Evans assay demonstrated the polymeric nature of wine color, but the complexity of the colored components in wine led to the development of more descriptive analytical methodologies. Analysis of the more complex, heterogeneous tannins, polymeric pigments and larger molecular weight compounds required greater selectivity. Colorimetric methods, by themselves, are unable to distinguish between individual tannins. However, by exploiting the known affinity for tannins to bind to proteins and precipitate out of a solution, different tannin fractions could be assessed (Adams and Harbertson 1999; Hagerman and Butler 1978; Hagerman and Butler 1981). Researchers also wanted a fast, cheap analytical tool for use at wineries, as well as by research laboratories. Towards that end, Adams and Harbertson (1999) evaluated several commonly available proteins, including bovine serum albumin (BSA), gelatin and casein. These proteins, which were already used in wineries as fining agents, were appropriate for use with protein precipitation assays. However, the accuracy of the results

depends on the ranges of tannin species precipitated by the different proteins, and as well as by the presence of other compounds (e.g. sugars) which can interfere with the protein-tannin binding reactions. Furthermore, none of these commonly available proteins bind with lower molecular weight tannins, i.e. those composed of four or fewer flavan-3-ol subunits (Adams and Harbertson 1999). BSA, in particular, has become widely used for simple solution-based protein precipitation assays (Adams and Harbertson 1999; Birse 2007; Hagerman and Butler 1978; Harbertson, Kennedy, Adams 2002; Harbertson, Picciotto, Adams 2003; Harbertson and Spayd 2006; Jackson 2008; Jensen and others 2008; Keulder 2006; Mercurio and others 2007; Rinaldi and others 2010), although other proteins have been used as well, including the Glories gelatin index (Glories 1984) and ovalbumin proteins (Zamora 2004)

Protein precipitation assays are also useful for examining the tannins in grape berries. James Harbertson at U.C. Davis analyzed grape skin and seed tannins with regard to their impact on astringency. Harbertson scaled down the BSA protein precipitation assay developed by Hagerman and Butler (Hagerman and Butler 1978), for use with berry sized samples (in 1.5 mL microfuge tubes) (Harbertson, Kennedy, Adams 2002). Tannins can be measured in both the berries at harvest, and in the finished wines.

Sample dilution has also been shown to have an impact on the results from protein precipitation assays (Jensen and others 2008). Tannin response results proved non-linear for some dilution ranges, especially for highly concentrated (i.e. low dilution) samples, probably due to insufficient protein available to precipitate all the tannin. It has also been noted that a minimum threshold of tannin concentration is needed for precipitation to occur (Jensen and others 2008). Highly diluted samples might not reach sufficient concentrations for precipitation to occur, yielding inaccurate results. To ensure reliable tannin measurement, sample dilutions

should be managed so the tannin response falls between 0.3-0.75 absorbance units when taking spectrophotometric measurements (Jensen and others 2008).

Iron-reactive phenolics method

Results from protein precipitation methods can also be compared against results using iron-reactive phenolics methods, which use a ferric chloride reagent instead of the Folin-Ciocalteu reagent. This approach is useful because it allows for the measurement of both tannins and total phenolics in the same standardized units, and is not subject to interference from bisulfite or reducing sugars, simplifying standardization of results. The Ferric Chloride reagent reacts with caffeic acid, caftaric acid, catechins, quercetin, gallic acid and all phenolics containing highly oxidizable "vicinal dihydroxyls" (i.e. two functional groups bonded to adjacent carbon atoms) (March, Advanced Organic Chemistry, 1985; (Harbertson and Spayd 2006). There are some limitations—iron-reactive methods have very different responses for different phenolic classes, and do not measure monomeric (monohydroxylated) phenolics or anthocyanins (Harbertson and Spayd 2006; Harbertson and others 2012).

Polysaccharide (methyl cellulose) precipitation methods

Researchers in Australia are also exploring new assays using a polysaccharide (methyl cellulose) precipitation (MCP) instead of the protein precipitation assay used in the Adams-Harbertson method. Their goal was to develop approaches geared towards higher volume, faster throughput methods needed by the Australian wine industry. Because MCP method does not have multiple incubation steps, it can be adapted to use with microplate readers; subsequently, 96 samples can be analyzed in 90 minutes with MCP, compared to 10-15 samples via Adams-Harbertson in the same period. However, results are not directly comparable between the two methods. The BSA protein precipitation used in Adams-Harbertson correlated better to wine

astringency than MCP (Mercurio and others 2007). Both methods removed about the same volume of tannin material from the sample, but the precipitation mechanisms differ, in that the protein only precipitates large polymeric pigments and the ferric chloride reagent doesn't react with anthocyanins, while MCP precipitates all tannins, including small polymeric pigments and anthocyanins (Mercurio and others 2010). Collaborations between Australian and American researchers pointed to the inhibitory effect of polysaccharides on tannin aggregation, and demonstrated that polysaccharides in wine interfere with tannin-anthocyanin pigmented polymer formation (Hanlin and others 2010). Cell wall polysaccharides could conceivably be used as selective fining agents for tannin removal, without stripping off flavor, aroma or color enhancing compounds. However, it also highlighted that any tannin additions be made after polysaccharide removal, to keep additives from binding to polysaccharides during maceration (Hanlin and others 2010).

Improving phenolic extraction from grapes

Phenolic concentration by cultivar

Cultivar differences in phenolic concentration has been found to be much higher than previously thought, emphasizing the relative importance of winemaking practices (i.e. maceration or other extraction techniques) over viticultural practices for determining a wine's final tannin concentration (Harbertson and others 2008). Harbertson (2002) found no relationship between total tannin per berry and total tannins found in the resulting wines. Instead, the major factor contributing to varietal differences in seed tannin content seems to due to the number of seeds per berry, rather than differences in the amounts of tannin per seed (Harbertson, Kennedy, Adams 2002). Fruit maturity has also been shown to influence phenolic extraction ratios. Wines made from increasingly mature fruit exhibited a higher proportion of

seed-derived tannins, with skin tannins extracted early in the fermentation process, and proportionally more seed tannins extracted as maceration continued (Kennedy 2008).

Maceration effects

During the maceration phase of the wine making process, anthocyanins are extracted largely from grape skins into the fermenting must. Seeds and stems also contribute various tannins if present during maceration. Studies of the tannin content and composition in grape stems, for example, found that the condensed tannins from grape stems were 60% epicatechin, with additional epigallocatechin; catechin was the predominant free monomer (Harbertson, Kennedy, Adams 2002; Souquet and others 2000). Anthocyanin extraction reaches its peak early in fermentation, but tannin extraction continues with prolonged skin and seed contact (Ginjom and others 2010; Harbertson, Kennedy, Adams 2002; Harbertson and others 2008; Harbertson 2010; Kennedy 2008; Ribéreau-Gayon and Lucia 1968; Sacchi, Bisson, Adams 2005; Soto Vázquez, Río Segade, Orriols Fernández 2010; Zanoni and others 2010). In a similar study, over half the polymeric pigment formation occurred during fermentation, rather than from polymerization during aging, highlighting the importance of the maceration phase for color formation (Parker and others 2007). An Italian research team following anthocyanin extraction kinetics during fermentation reported that anthocyanin concentrations resulted from dynamic interplay between solid-to-liquid extraction dynamics and oxidative degradation, and concluded that the polymeric pigment formation noted during fermentation was not the result of extraction, but of condensation reactions between anthocyanins and tannins (Zanoni and others 2010).

Aging

Finally, one examination of the contribution of copigmentation in the color of red wines during aging showed the decline of both copigmentation and free anthocyanins, while polymeric pigments increased over a two year period (Darias-Martín and others 2006).

Cap management

Different maceration techniques, including mechanized punch-down, pump-over and thermovinification have been demonstrated to effect different rates of extraction for both anthocyanins and tannins (del Rio and Kennedy 2006; Fischer, Strasser, Gutzler 2000; Harbertson, Kennedy, Adams 2002; Harbertson and others 2008; Sacchi, Bisson, Adams 2005). In one comparative study, thermovinification extracted the maximum amount of phenolics, while mechanical and pump-over techniques extracted significantly more phenolics than traditional methods (Fischer, Strasser, Gutzler 2000). Mechanical cap punching has been shown to be more effective at increasing phenolic extraction than cold traditional macerations, while cold soaking and automated pump-over techniques performed poorly, producing either no impact, or a negative impact, on wine color (Soto Vázquez, Río Segade, Orriols Fernández 2010). Size also matters; researchers have found repeatable differences between commercial and micro-scale fermentations (Zanoni and others 2010). Micro scale vinifications showed higher rates (i.e. faster) of extraction for malvidin and total phenolics (measured by absorbance at 280 nm) while industrial scale vinifications extracted greater total amounts of phenolic material, and also had lower rates of anthocyanin degradation, likely due to lower oxygen exposure in industrial settings (Zanoni and others 2010).

In addition, post-fermentation factors (especially temperature) also have a significant impact on wine color. Factors that decreased anthocyanin content tended to increase polymeric pigment content (Sacchi, Bisson, Adams 2005).

Combination techniques

In an effort to improve the phenolic and chromatic properties of red wines for aging, European researchers have compared a number of different wine making techniques, including different maceration protocols, cold soaking, automated pump-over and tannin plus enzyme additions (Bautista-Ortín and others 2007; Soto Vázquez, Río Segade, Orriols Fernández 2010). In one study, researchers found that the greatest color intensity values resulted from enzyme plus tannin treatments (tannins added at 10 g/hL, pre-fermentation). Tannin + enzyme treatment resulted in significantly higher red and yellow color components, as well as higher concentrations of gallic acid, catechin and quercetin. Sensory testing also gave the best marks to the tannin + enzyme treatments, despite the higher addition rates, for color, aroma, flavor and "structural" properties (Soto Vázquez, Río Segade, Orriols Fernández 2010). Bautista (2007) found that to the contrary, the pump-over techniques improved the extraction of polymeric phenols, but also that the tannin treated wines retained higher levels of anthocyanins and smaller pigmented compounds (Bautista-Ortín and others 2007).

Enological tannin additions

Use and availability of enological tannins

Enological tannins have been used in winemaking both directly (as an additive) and indirectly (through use of oak barrels) since ancient times (Fleming 2001; McGovern 2003). Winemakers have added other grape materials (skins or seeds), as well as other herbal or woody plant derived substances, to fermenting musts or finished wines in an attempt to improve wine

quality (Fleming 2001; Robinson 2006). Likewise, winemakers have also tried adding other grape varieties to improve color or wine quality; French winemakers in the Côte Rôtie, for example, have a long tradition of co-fermenting different types of grapes, including Viognier with Syrah (Robinson 2006). Italian Chianti was traditionally a co-fermentation of primarily Sangiovese, with small amounts of Canaiolo Nero, Trebbiono and Malvasia Bianca (Beazley, Johnson, Robinson 2007; Robinson 2006). Additions therefore, regardless of form, have a long history of use. The availability of commercially prepared additives, however, is a fairly recent development resulting from a number of factors, which have driven winemakers to seek less expensive ways to produce traditional wines or to create new and innovative wine styles. Some of these trends include the growth of the global wine industry, with large scale commercialization and increasing international competition, challenges to traditional wine styles, changing mass market preferences and regulatory changes in several winemaking regions which have allowed use of new additives, or new uses for old additives (Del Pozo-Insfran 2006; Galpin 2006; Mitchell 2006; Robinson 2006). For example, prior to 1999, European winemakers faced regulatory restrictions limiting the use of tannin products. In North America, Australia and South Africa, in contrast, regulations were somewhat more liberal regarding the use of tannin products for other quality improvement purposes (Mitchell 2006). Since then, there has been a general liberalization of the regulations regarding use of tannin additives and oak wood (dust, chips, slats or boards), to allow their use for other purposes beyond fining—color stabilization, color extraction during maceration, elimination reductive odors, improving wine "structure" and mouthfeel, inhibiting laccase activity in grapes with high fungal loads, replacing oak barrels for aging (i.e. impacting flavor or aroma maturation) (Bautista-Ortín and others 2007; Mitchell 2006; Obreque-Slíer 2009; Robinson 2006).

Types of commercial enological tannin products

There are a tremendous number of commercial tannin preparations available to winemakers (Bautista-Ortín and others 2007; Harbertson 2010; Harbertson and others 2012; Mitropoulou, Hatzidimitriou, Paraskevopoulou 2011; Neves and others 2010; Obreque-Slíer 2009; Rinaldi and others 2010; Soto Vázquez, Río Segade, Orriols Fernández 2010). Enological tannins can be classified by their composition, the source of the tannin material, or even by purpose. In general, the source of the tannin material is the primary reference; tannins can be derived from grapes, or more specifically from grape skins or seeds. Products may also be derived from oak or quebracho wood, from chestnut or other woody plant sources. In addition, products may be formulated blends of tannins from multiple sources. Chilean researchers analyzed ten different commercial tannin preparations and found no relationship between tannin content or purity of tannin content, and the price of the product (Obreque-Slíer 2009). Other researchers have also found wide variation in tannin content, batch-to-batch variations, and discrepancies between label descriptions and tannin content (Bautista-Ortin 2005; Bautista-Ortín and others 2007; Del Pozo-Insfran 2006; Galpin 2006; Harbertson 2010; Harbertson and others 2012; Keulder 2006; Mitchell 2006; Mitropoulou, Hatzidimitriou, Paraskevopoulou 2011; Neves and others 2010; Obradovic 2006; Obreque-Slíer 2009; Rinaldi and others 2010; Soto Vázquez, Río Segade, Orriols Fernández 2010). The upshot of these findings is that in the marketplace, commercial tannin additives remain very much in the *caveat emptor* category. Winemaker beware!

The effect of tannin additions on color formation and stability

Tannin addition trials are often made in conjunction with other treatment options, like enzyme additions, different maceration techniques, or temperature regimes (e.g. cold or hot treatments). In one trial conducted to determine if a combination of commercial tannin extracts

and enzyme treatment could improve color extraction and stability in red Monastrell wines color differences were observed at the beginning of winemaking, but diminished by eight months post-bottling, suggesting that the peaks of color intensity that occurred early in fermentation were due to copigmentation effects from the added tannin material (Bautista-Ortin 2005). In particular, wines with tannin additions were found to be more yellow in color, with higher bitterness, dryness, and astringency ratings, and overall lower sensory scores for color and aroma. The addition of tannin material may have shifted the anthocyanin/tannin equilibrium to favor the formation of polymerized tannins, resulting in more yellow coloration (Bautista-Ortin 2005). In contrast, later work by the same researchers showed that wines with tannin additions showed the best chromatic characteristics at bottling and during eight months of bottle aging (Bautista-Ortín and others 2007).

Such contradictory results are one of the challenges in understanding the impact of tannin additions on the evolution of wine color. In Syrah, pre- or post-fermentation additions of either grape seed or grape skin tannins, at 200-400 ppm, showed higher spectral values than control wines, suggesting more stable color formation (Obradovic 2006). In contrast, commercial tannin additions to a red wines at the start of fermentation (100mg/L dose rate) increased the total phenol content of the finished wines, with effects were still apparent after one year of bottle aging (Keulder 2006). However, those tannin additions did not appear to stabilize the wine color, and both positive and negative sensory impacts to "structure" and mouth feel were noted. Further, the amount of tannin provided by the additions was insignificant in comparison to the amount of tannin extracted from the grapes, and the tannin additions did not appear to stabilize wine color, relative to the control wine (Keulder 2006), with one exception – a Cabernet Sauvignon with high levels of rot.

The efficacy of tannin additions seems to be based in, part, on phenolic concentrations prior to addition. In one study, tannin additions of 200 mg/L to Shiraz, followed for up to two years in the bottle, showed no significant differences in wine color or pigmentation. It appeared to the authors that the Shiraz fruit used had sufficient native tannins present to react with all available anthocyanins, so the tannin additions didn't improve polymeric pigment formation (Parker and others 2007). Similarly, a study in which two doses of tannins were added before or immediately after fermentation, and combined with different maceration lengths, resulted in increased color intensity only in wines that were otherwise low in polyphenols (i.e. the short maceration treatments) (Neves and others 2010). Low dose rate additions did impact color, but the high dose rate trials, in the short maceration period (i.e. low polyphenol) wines did show significant color increases – most likely due to the addition of proanthocyanidins, compensating for the low levels in the must (Neves and others 2010). In Cynthiana wines, a study comparing use of macerating enzymes and post-fermentation tannin additions (20 g/hL purified white grape seed tannins) found only minor differences between the two approaches (Main and Morris 2007). Tannin additions increased browning, total anthocyanins, and percentages of both polymeric pigments and ionized color, but no increase in total phenolics was observed. Further, while tannin-treated wines had no color differences at eleven months aging, by twenty-two months, they were darker and more yellow, suggesting that total storage time had a far greater impact than either treatment. More recently, a study of the interaction between tannin additions and mannoprotein (polysaccharide) additions on wine color evolution and tannin stability showed that one of the tannin treatments, composed of small molecular weight compounds (~8-14 mDP), showed some color stabilization effects, with no additive effects from mannoprotein treatments (Rodrigues 2012).

Impact of fruit quality on the tannin addition efficacy

The quality of the fruit being fermented may be important for the effectiveness of tannin additions. As mentioned above, Keulder (2006) found only one instance where tannin additions increased red color relative to the control wine, in Cabernet Sauvignon fruit with high levels of rot. In that case, it was possible that the greater impact was due to high laccase activity caused by fungal infection, and that the tannin additions may have helped inhibit oxidation enzymes. Bautista-Ortin (2007) found that after eight months of aging, the color intensity, anthocyanin and tannin contents decreased due to condensation, oxidation, and polymerization reactions, but that the levels of flavan-3-ols, flavonols and monomeric anthocyanins remained higher in the tannin treated wines. One conclusion from this study was that tannin additions had better impact on stable color formation in wines made from less ripe fruit (Bautista-Ortín and others 2007). With riper fruit, they observed higher anthocyanin oxidation and precipitation, not buffered by the tannin additions. This finding, that fruit quality (level of molds and rots) may influence the effectiveness of tannin additions deserves further study and if valid, would have useful implications for cool climate winemakers, who often suffer from higher levels fungal infection and rots in their fruit, and might provide better guidance about when they should use enological tannins.

Composition of enological tannins

One common finding in studies of enological tannins speaks to the variable nature of commercial products. Several groups analyzed enological tannin products by HPLC and found considerable differences, highlighting the impact of tannin source, composition and extraction methodology and the propensity for negative sensory properties of the tannin products carrying over into the wines (Keulder 2006; Mansfield and Zoecklein 2003; Obradovic 2006). Parker

(2007) also used HPLC analysis to demonstrate the variability between phenolics and non-phenolic compounds in these commercial tannin products. In one product, for example, most of the tannins present were short chain molecules and only about 50% depolymerized in wine, reducing the actual amount of tannin extracted into the wine after addition. More recently, a survey of commercial products showed that the total amount of tannins present were much less than predicted by the composition analysis of the additives (Harbertson and others 2012). Neves (2010) found significantly increased levels of gallic acid in the finished wines after tannin additions, even though only one of the commercial preparations purported to contain gallic acid. In at least one of the products, higher levels of galloylated polymeric proanthocyanins degraded under wine pH and ethanol conditions, releasing gallic acid. In another study, HPLC analysis of ten commercial tannin products found different types of mainly hydrolysable gallotannins, ellagitannins, condensed or proanthocyanidic tannins or blends. Gallic acid was the only non-flavonoid present in all extracts, and concentrations of total phenols varied considerably between products, with significant labeling discrepancies noted. Pricing for these products was highly variable, with vegetable and oak blends being generally cheaper than grape derived tannins , and the extraction process used in manufacturing was found to be important – some purportedly grape-derived products showed the presence of wood-derived ellagic acid, indicating the presence of oak chips or slabs during processing (Obradovic 2006). These findings, in conjunction with similar warnings (Bautista-Ortín and others 2007; Harbertson 2010; Harbertson and others 2012; Neves and others 2010; Parker and others 2007; Seddon and Downey 2008) about the chemical composition of different commercial products, highlights the need to exercise caution when choosing additives and for manufacturers to improve quality controls (Obreque-Slíer 2009).

Compositional variability is of particular importance, as research suggests that stable color is produced only from grape-derived tannins, and not from hydrolysable (i.e. oak derived) tannins (Harbertson 2010; Harbertson and others 2012). In a study exploring the impact condensed vs. hydrolysable tannin additions Harbertson found discrepancies between the amounts of tannin expected (based on an analysis of the additives) and what was assessed in the finished wines, suggesting solubility limits with commercial tannin additives (Harbertson and others 2012). When the results for iron-reactive and protein precipitation assays were compared, significant differences with the shape of the precipitation curve were noted; the iron precipitable tannin assay produced linear results (allowing better estimation of desired addition rate), while the protein precipitation method results were non-linear, making predictions less useful (Harbertson and others 2012). In particular the researchers found that products with a higher proportion of the smaller iron-reactive tannins showed lower levels of extraction than were predicted by their model (data not shown, Harbertson, 2012). Extraction was also limited by the total amount of tannins present in each formulation. The tannin content of the products tested ranged from 12% to 48%, and more than 50% of those tannins present were not protein precipitable, indicating that low molecular weight tannins were used in these additives (Harbertson and others 2012). In the trials, the manufacturer-recommended doses had little to no impact on final wine tannin content. Higher dose rates (e.g. ≥300 mg/L) were required to achieve any changes to wine polyphenol levels, and increases in tannins, iron-reactive phenolics, and large polymeric pigments were only noted in the two highest treatment rates (600-800 mg/L catechin eq.). Regardless of tannin type, only the highest dose rates proved significantly different from the control. Parker (2007) also found solubility issues with commercial tannin products. In one his trials, less than 50% of the tannin material present in the product depolymerized in acid, indicating it would probably not be

extracted into the wine (Parker and others 2007). These findings echo earlier work by Australian researchers (Seddon and Downey 2008), highlighting continuing labeling problems, variability of composition and batch-to-batch quality control issues with commercial tannin products.

European researchers, reacting to regulatory changes allowing the addition of enological tannins, began work on "fingerprinting" the absorption spectra of different compounds in order to enable rapid characterization of commercial preparations. Oak, chestnut, and quebracho tannin species could be characterized by the pattern of specific absorption peaks between 1500-1044 nm. The commercial preparations tested did not show any color impact in the visible spectrum (Laghi and others 2010). Follow-on projects in Portugal also considered the chemical "fingerprint" of varietal specific anthocyanins, as well as the impact of storage temperature on anthocyanin degradation during aging (Garrido and Borges 2011).

Sensory impact of tannin additions

Negative impact on wine color

Although one of the primary goals for tannin addition use is to improve color characteristics, researchers have also found some potential negative impacts on wine color. Harbertson (2012) found that high dose rate treatments increased brown colors. Bautista-Ortin (2005) found that wines treated with tannin additions appeared more yellow than the control. French researchers found a negative correlation between astringency and hue (Chira and others 2011). Other researchers have noted similar increases in yellow/brown colors due to tannin additions (Keulder 2006; Main and Morris 2007; Neves and others 2010; Rodrigues 2012; Soto Vázquez, Río Segade, Orriols Fernández 2010; Vivas and others 2004)

Impact on taste (sweetness, sourness, bitterness, and astringency)

Researchers examining Bordeaux wines (Merlot and Cabernet Sauvignon), from vintages dating back to 1978, for aging properties found correlation between the mean-degree-of-polymerization (mDP) and astringency (Chira and others 2011). Astringency increased as mDP increased, but the mDP also decreased significantly as the wine aged; Chira (2011) concluded that the higher mDP proanthocyanidins are hydrolyzed or subject to other rearrangement reactions, or precipitate, over time. Oxygen exposure in particular, which leads to acetaldehyde formation, reduces mDP (Chira and others 2011). Harbertson (2010) found that traditional wine maker descriptors for "hard" or "soft" tannin appeared to be related to the amount of tannin present rather than different chemical structures. Likewise, grape derived tannins proved more effective at precipitating salivary proteins, indicating that grape derived (i.e. condensed) tannins have a greater impact on perception of astringency. Smaller sized tannins increased bitterness, while larger tannins yielded "harsher" astringency, and higher tannin content equaled higher overall astringency. However, very large additions were required to affect wine astringency, but these high dose rate treatments also had other, negative, sensory impacts, increasing bitter, earthy flavors (Harbertson and others 2008; Harbertson 2010). In sensory testing of wines with enological tannin additions conducted in 2012, the wines with high rate tannin additions could be discriminated from those with low rate additions. Wines with high rate tannin additions had more negative sensory scores. Increased bitterness and earthy flavors were seen in all the higher dose-rate treatments, yielding generally lower (negative) sensory scores (Harbertson and others 2012). Significantly, Harbertson's sensory panel was consistently able to discriminate between the control and the tannin-treated wines. Judges found "red fruit" descriptors to be the common attribute among tannin-treated wines, and perceptions of sweetness and viscosity were lowered though only minor increases to astringency were noted (Harbertson and others 2012). Harbertson

concluded that tannin additions were unjustified and may have limited or negative impacts on quality (Harbertson and others 2012).

Though rich in phenols, commercial tannins do not necessarily participate in astringency-inducing reactions with salivary proteins. In one study on the subject, researchers found the composition of the wine, rather than the type of commercial tannin product, had the largest impact on astringency, and that increasing complexity in polyphenolic structures resulted in lower levels of astringency response (Rinaldi and others 2010). In a study of pre- and post-fermentation grape-derived tannin additions to South Australian Shiraz wines, Parker (2007) found that despite significantly higher tannin concentrations during aging, the sensory impact on astringency was small and not significantly different from the control after one year (Parker and others 2007). Bautista-Ortin (2005) noted that sensory panelists found wines treated with tannin additions to have higher bitterness, dryness, and astringency ratings, with overall lower sensory scores for color and aroma (Bautista-Ortin 2005).

Impact on wine aroma

Tannin additions have also been shown to have an impact on wine aroma. In an examination of the impact of grape seed and skin tannins on the headspace volatility of aroma compounds in a model wine system, both origin of tannin (i.e. seed or skin) and concentration of addition affected aroma volatility (Mitropoulou, Hatzidimitriou, Paraskevopoulou 2011). Mitropoulou (2011) found that ester volatility generally increased with additions of skin tannins (1 g/L), but then decreased significantly (for ethyl esters, isobutanol, linalool) as the dose rates increased (5 g/L and 10 g/L). Some hydrophilic compounds, like isoamyl acetate, increased their volatility as skin tannin concentrations went up. At the highest dose rate (10 g/L), the volatility decreased a further 60%. Grape seed tannins showed similar, although less pronounced, trends regarding

volatility, increasing slightly for most aroma compounds at the lower dose rates, while declining with higher dose rates—although the rate of decline indicated that seed tannins reduced aromatic volatility less strongly than skin tannins did. The seed tannins, in particular, appeared to favor aggregative interactions and formation of precipitants (Mitropoulou, Hatzidimitriou, Paraskevopoulou 2011). Bautista-Ortin (2005) noted decreases sensory panel aroma scores for tannin treated wines. Mitropoulou (2011) found that the tannin additions affected aroma compound volatility in model wines; the volatility of hydrophobic compounds generally decreased with tannin additions, although some hydrophilic compounds increased their volatility as tannin dose rates increased. Decreases in the volatility of different compounds depended on both the dose rates and source of the tannin additives (i.e. seed or skin tannins). Although the specific composition of the tannin additives were not analyzed, Mitropoulou attributed these results to hydrophobic interactions; lowered solubility of aroma compounds due the prevention of hydrophobic binding for structural reasons, including the presence of procyanidins. Mitropoulou also suggested that attractive interactions and aggregations were favored as mDP increased, increasing the ability of the tannins to form colloidal-sized particles (Mitropoulou, Hatzidimitriou, Paraskevopoulou 2011). Harbertson (2012) also acknowledged that tannin additions could have an impact on wine aroma, suggesting that although the dose thresholds might be quite large with respect to changes in perceived astringency, the thresholds needed to alter aroma perceptions seemed quite small (data not given). He also suggested that aroma impact might be due to other compounds in the tannin product, rather than to the tannins themselves (Harbertson and others 2012).

Chapter 2

Introduction

Research efforts to date have demonstrated the complex nature of red wine color evolution; color development begins during maceration and continues during aging. The work of Adams, Harbertson, Bautista-Ortin, Boulton, Versari, and others has provided an appreciation of the dynamics of wine color formation, polymerization and stability (or lack thereof) during aging. Industry-focused research over the past decade has helped winemakers to understand the limitations they face when trying to employ commercially available tannin additives in wines in order to improve color or other sensory properties ("structure," "body," mouthfeel, bitterness, or astringency). It is now understood that the maceration phase of winemaking is critical for color extraction from the grapes and polymeric pigment formation in more ways than just simple extraction. Colorless cofactors and copigmentation stacking effects are known to be important not only for short term increases in wine color, but also for increased anthocyanin extractability and stabilization in the wine matrix, so that these colored compounds are not lost before they participate in polymerization reactions, forming the long-term stable polymeric pigments which are the dominant form of aged wine color (Rodrigues 2012; Zanoni and others 2010). The type of tannins extracted from the grapes (or added via a commercial product) are important for this process (Laghi and others 2010; Obreque-Slíer 2009); the size and composition of polyphenolic compounds, whether they are hydrolyzed or condensed tannins, the ratio of polyphenolics to anthocyanins, and the availability of colorless cofactors all play important roles in color formation and stabilization (Laghi and others 2010; Zanoni and others 2010). From recent research (Bautista-Ortín and others 2007; Boulton 2010; Cíchová M., Petříček J., Fiala J. 2008;

Harbertson and others 2012; Keulder 2006; Laghi and others 2010) it is apparent that enological tannin products only improve wine color characteristics under certain conditions, principally when the grapes being fermented are low in phenolics (Neves and others 2010;), low in copigmentation cofactors (which also contribute to polymerization reactions), or when the grapes suffer from high levels of laccase activity from fungal infection ((Bautista-Ortín and others 2007; Keulder 2006; Mitchell 2006; Obreque-Slíer 2009). Timing of enological tannin additions and the doses used also seem to play a role, albeit a secondary one; under some conditions, additions at the start of maceration have a greater impact on stable color formation, while in other studies, positive effects have also been noted for post-fermentation additions (Bautista-Ortín and others 2007; Harbertson and others 2012; Keulder 2006; Neves and others 2010; Soto Vázquez, Río Segade, Orriols Fernández 2010). Dose rates, in particular, seem to have both lower limit and high range effects. Doses which are too low do not appear to have any significant impact on wine color (Boulton 2010; Harbertson 2010), although there may be some temporary improvements in some of the characteristics, depending on the experiment profile and which elements of color are being measured (Neves and others 2010; Soto Vázquez, Río Segade, Orriols Fernández 2010). At the other extreme, higher dose rates, which may indeed produce more stable changes in wine color, also come with high risk of introducing unwanted sensory attributes to the wine, including increased yellow/brown colors (Bautista-Ortín and others 2007; Soto Vázquez, Río Segade, Orriols Fernández 2010), increased bitterness and earthy characteristics (Harbertson and others 2012), and possible masking of fruity or floral aromas (Cíchová M., Petříček J., Fiala J. 2008; Mitropoulou, Hatzidimitriou, Paraskevopoulou 2011).

This experiment examined the impact of commercial tannin addition timing on red wine color development and stability, and enhances understanding of the potential benefits and

limitations of commercial tannin additives in several ways. To begin with, low dose rates were confirmed as largely ineffectual. Next, there were some clear short-term (e.g. after three months) impacts from tannin additions and noticeable differences between different formulations of tannin additives. It was also apparent that, after two years of aging, only some significant differences could still be seen, primarily from the initial treatments, but only for certain parameters which may not be relevant to consumers. Understanding these differences and limitations will help winemakers determine when and if tannin additions are appropriate for their winemaking objectives, and will also help manufacturers improve their labeling and guidance for winemakers.

Materials and Methods

Enological tannins

The two commercial tannin additives used for this experiment, *Institute Oenologique De Champagne (IOE) Volutan* and *IOE Tannin SR-Terroir* (Epernay, France), were donated by Lallemand (Montreal, Canada). *Volutan* is a liquid formulation derived from white grapes. The manufacturer recommended dose rate for red wine additions was 15-40 mL/hL, and it was added at a dose rate equivalent to 8.9 mL/hL. *Tannin SR-Terroir* is a powdered formulation which according to the manufacturer is composed of unspecified "catechins", suplemented with unspecified "grape seed tannins." The recommended dose rate for red wine additions was 5 to 15 g/hL, and it was added at a dose rate equivalent to 3.3 g/hL (Table 2-1). For both products, a low dose rate was chosen to minimize risk of bitter, earthy flavors affecting planned sensory trials, as reported in other studies (Bautista-Ortín and others 2007; Cíchová M., Petříček J., Fiala J. 2008; Harbertson and others 2012).

Grapes

One ton of Pinot Noir grapes was sourced from Sheldrake Point Vineyard on the western shore of Cayuga Lake (Ovid, NY), and one ton of Lemberger grapes was received from the Cornell Lansing vineyard (Lansing, N.Y.) (Figure 2-1). Fruit was harvested by hand, packed in standard 40-pound grape lugs and transported to the Vinification and Brewing Laboratory (V&B) at Cornell's New York State Agricultural Experiment Station (NYSAES) in Geneva, NY. Harvested grapes were received at the facility on the afternoon of harvest and stored in a cooler overnight, before crushing the following morning (Table 2-2).

Wine production

Crushing/destemming operations were performed using a horizontal auger screw-type destemmer-crusher (Gestione Rossi e Camma, Prospero Inc., Pleasantville NY). For each variety, the must from the crusher was divided evenly between fourteen 114 L (28 total), temperature-controlled, open-topped stainless steel fermentation vessels (Vance Metal Fabricators, Geneva, NY) for maceration and fermentation. In order to ensure a vigorous fermentation, a complex yeast nutrient was added to the must prior to pitching the yeast (0.25 g/L Fermaid® K, Lallemand Inc.). Standard amendments for microbial suppression (50mg/L SO_2, potassium metabisulfite, Fisher Scientific), were also made to the must (Table 2-3, 2-4) prior to inoculation with a *Saccharomyces cerevisiae* yeast, Lalvin ICV-D254® (0.264 g/L, Lallemand Inc. Montreal QC, Canada). Dry yeast were stored in a sealed, refrigerated container until use; rehydration followed manufacturer recommendations, using warm, non-chlorinated water (40° C) mixed with a yeast rehydration aid (0.3 g/L Go-Ferm®, Lallemand Inc.).

Maceration on the skins continued for seven days, before dejuicing using a stainless steel vertical hydraulic basket press (Mori PZ-82 Hydraulic Press, San Casciano Italy). During maceration, the cap of skins and seeds, which floats to the top of the tank and forms a thick layer,

was punched down manually twice per day. The temperature profile of the fermentation was computer controlled, according to the standard red grape fermentation temperature control protocol (Table 2-5).

After pressing, eight gallons of wine (from each fermenter) was transferred to glass carboys (one 19 L primary, and one 11 L spare), fitted with airlocks, and allowed to complete fermentations at ambient temperature. All carboys were inoculated with *Oenococcus oeni* (Enoferm® ALPHA, Lallemand Inc.) and allowed to undergo malolactic fermentation (MLF) at 20° C. After it had been determined that MLF was complete, all wines were cold stabilized at -4°C for 5 weeks and racked off the lees.

At bottling, copper sulfate solution (0.5 mg/L as Cu) was added to all wines to reduce or eliminate differences due to sulfur off-aromas (H_2S). After being allowed to warm up to ambient temperature overnight, the wines were bottled in 750 mL green glass screw-top bottles (Waterloo Container Corp., Waterloo NY), using a single-head, vacuum pump bottle filler (Enolmatic®, Tenco S.N.C., Avegno, Italy). Bottles were sparged with nitrogen prior to filling to reduce oxygen exposure, filled and then sealed with screw cap closures, using a single head electric capping machine (Prospero Equipment Corp.). After bottling, the wine was put into standard 12-bottle cardboard cases and stored in a temperature-stabilized warehouse (16 °C), until they were opened for analysis.

Treatments

Each variety received three different treatments, plus the control, duplicated for each type of tannin. Control and treated wines were fermented in duplicate. The same addition rate (Tables 2-3, 2-4 and 2-1) was used for each type of tannin, so that the same total amount of the commercial product was added to each fermentation lot, but additions were made at different

times during fermentation: at the beginning of fermentation (after SO_2 addition and yeast addition), incrementally throughout the fermentation period, and at the end of fermentation, immediately after pressing.

Sampling

Aliquots of 250mL were collected in duplicate every second day throughout fermentation and MLF and at bottling. Samples were stored in 250 mL plastic screw-cap narrow-mouth field sample bottles and frozen at -20°C for later analysis. Bottled wine was stored in a temperature-stabilized warehouse at 16 °C, and opened for analysis after approximately two years of bottle aging.

Chemical analysis of the finished wines.

Prior to cold stabilization, wines were analyzed for residual sugars, malic acid, ethanol (%ABV), pH, and total acidity (TA) (Table 2-6). All samples were centrifuged at 20 g for 15 minutes before analysis. Testing for residual sugar (glucose/fructose assay), and malic acid were performed using enzymatic assays on a Chemwell Model 2900 (Awareness Technology, Palm City FL). Total acidity (TA) and pH were measured using an auto-titrator (Metrohm 848 Titrino Plus, with 869 compact sample changer). For ethanol quantification, a wine sample was diluted 1:10 with butanol (2% butanol solution in distilled H_2O), and measured on a gas chromatograph (GC-FID with wax column, 30mm X 0.25mm X 1.0µm, Hewlett Packard 5890 Series II Gas Chromatograph with HP 7673 injector). Results were stated as percent alcohol by volume (%ABV) by comparing a known response ratio from the known ratio of standards (ethanol:butanol), with the ratio of samples (unknown ethanol : known butanol). Calculations for deriving ethanol concentrations (%ABV) were performed in Microsoft Excel. Free and total SO_2 (data not shown) were also measured prior to bottling using a wet chemistry colorimetric

assay on an automated flow injection analysis system (FOSS FIAstar-5000, Hillerod, Denmark, EU); the results (data not shown) indicated that the Pinot Noir wine required an additional SO_2 treatment; 50 ppm SO_2 was added before bottling.

Spectrophotometric analysis.

All spectrophotometric readings were performed using the Spectronic Genesys-2 spectrophotometer (Thermo Electron Corporation, Madison WI), with an eight-slot sled (7-samples plus one reference standard). The unit was zeroed to the blank between readings at different wavelengths, or at the start of a new batch run. Readings were recorded manually. In order to reduce oxidation after removing the aliquot for analysis, each 250 mL sample bottle was sparged with cover gas (HP300 high purity Nitrogen, by Airgas East Inc., Salem NH), before being resealed and stored overnight in a refrigerator. At the end of the day, the readings were transcribed from the handwritten data log (see appendix) to an Excel spreadsheet, where both sorting and further calculations could be performed.

Direct Spectroscopic Measurement

Aliquots (1 mL) were pipetted directly into the disposable 10 mm P/L 1.5 mL plastic cuvettes. A cuvette with HPLC grade distilled deionized H_2O was used as the blank. Cuvettes were placed in the spectrophotometer, and readings were taken of each cuvette at 420nm (A^{420}), 520nm (A^{520}), and 620nm (A^{620}). The 280nm (A^{280}) reading required the use of 1mm path length quartz cuvettes. Readings made at 280nm were corrected to a 10 mm path length for calculations and analysis of results. Between each reading, cuvettes were rinsed with distilled water, then with 100% ethanol, and allowed to dry. In most cases, the aliquot in the 10mm path length cuvettes could also be used for the SO_2 bleaching step of the Somers-Boulton assay. Dilution of the wine samples was not needed for the direct measurements.

Phenolic analysis

Phenolic fractions were analyzed using the Somers-Boulton Assay, the Folin- Ciocalteau Assay, and the Adams-Harbertson Assay as described in Jacobson, (2006), except as noted below.

Somers-Boulton assay

The Somers-Boulton assay was conducted as described in Jacobson (2006) and original publications ((Boulton and others 1999; Jacobson 2006; Somers and Evans 1977). However, the acidification step for assessing total anthocyanins (pH <1, A^{520}_{HCL}) was omitted, and color density was calculated in accordance with Organisation International de la Vigne et du Vin (OIV) (Jacobson 2006) recommendations as the sum of red, yellow and blue measurements ($A^{520}+A^{420}+A^{620}$). The reagents for the bleaching and wine buffer assays were added directly to the 1.5 mL cuvettes, necessitating thorough mixing; each cuvette was covered with a piece of Parafilm and inverted several times. The measurement using the acetaldehyde solution was prepared in a test tube; 50 µL of acetaldehyde solution was added to 5 mL of sample. It was vortexed, but incubated for only 45 minutes. After transferring the solution to a cuvette, readings were taken at 520 nm ($A^{520}_{Acetaldehyde}$).

Folin-Ciocalteau assay.

The Folin-Ciocalteau assay was conducted in accordance with procedures outlined by Singleton (Singleton and Rossi 1965; Singleton, Orthofer, Lamuela-Raventós 1999), but with changes to incubation timing, wavelength, sample dilution, and standard curve preparation. A shorter incubation period was judged to be sufficient for the necessary reactions to occur, while allowing a faster throughput of samples. The range of the standard curve was increased in order to ensure covering the expected response range of the wine samples. Samples produced

spectrophotometric readings within the range of the instrument without the need for sample dilution, so that step was omitted. Liquid volumes were also scaled-down considerably. The gallic acid standard curve solutions (a 1000 mg/L stock solution and 100mg/L working solution) were prepared in 50 mL volumetric flasks. Rather than 100% ethanol, HPLC grade distilled, deionized H_2O was used to prepare gallic acid solutions. A different six-point standard curve (0, 20, 40, 60, 80, 100 ppm) was used (Table 2-7). In order to keep the spectrophotometer readings within the instrument's range, the red wine samples were diluted 10-fold (1 mL wine to 9 mL dH_2O). Using a pipette, a 200µL aliquot of each diluted wine sample was added to a test tube containing 2.6 mL dH_2O. After the addition of 200µL of Folin-Ciocalteau reagent, the test tubes were vortexed and allowed to incubate for 6 minutes at room temperature. Next, 2.0 mL Sodium carbonate solution was added to the test tubes and they were incubated for a further 1.5 hours, after which the solutions were transferred to 1.5 mL 10 mm cuvettes and absorbance was read at 765 nm (A^{765}). The blank from the standard curve preparation was used to zero the spectrophotometer.

This assay required the use of a commercially prepared reagent (Folin-Ciocalteau Reagent, Sigma). All testing used reagent from the same lot number and a calibration curve was prepared for each day's run.

Adams-Harbertson assay

The Adams-Harbertson assay was performed in accordance with the procedures laid out by Harbertson ((Harbertson, Picciotto, Adams 2003; Harbertson and Spayd 2006), with the following exceptions: dilution of the wine samples before running the analyses was not necessary, although the range of the standard curve was extended to accommodate the range of absorption values observed; the catechin standard ranged from 0-350 µl, in 50µl increments

(Table 2-8). The washing step, after removing supernatant from the Eppendorf tube, was omitted. Finally, re-dissolving the pellet (in the Eppendorf tube) for the iron-reactive tannin measurements proved more challenging than anticipated based on the discussion in the literature; at least 10 -15 minutes of alternating vortex and ultrasound bath (Branson Sonicator B-220H) treatment was required.

Reagents

HPLC-grade distilled deionized H_2O (Millipore filtration, HPLC grade) was used throughout as for sample or reagent dilutions, when preparing reagents and as a spectrophotometric blank, per the specific methodology.

Somers-Boulton reagents. Potassium metabisulfite ($K_2S_2O_5$, crystalline), acetaldehyde (C_2H_4O, 99.5%), and potassium bitartrate ($KHC_4H_4O_6$, reagent grade) were sourced from Fisher Scientific (Fair Lawn, NJ) and 100% ethanol (EtOH, 200 proof, ACS/USP grade) from Pharmco-AAPER (Broofield, CT.)

Folin-Ciocalteau reagents. Sodium carbonate (Na_2CO_3, anhydrous, >99.5%, ACS grade) was sourced from Fisher Scientific, gallic acid ($C_7H_6O_5$, 98%) was sourced from Acros Organics (NJ, USA) and Folin-Ciocalteau phenol reagent (F9252, Lot 107K0002) was sourced from Sigma-Aldrich (St. Louis MO).

Adams-Harbertson reagents. Glacial acetic acid (~200mM, $C_2H_4O_2$), sodium chloride (~170mM, NaCl, extra-pure) and tri-ethanolamine (5%, w/v, 99%, C6H15NO3,) were sourced from Acros Organics (NJ). Sodium hydroxide (NaOH), ferric chloride powder (FeCl3, anhydrous, laboratory grade, I89-500), potassium metabisulfite (K2S2O5, crystalline) and potassium bitartrate (0.5%, w/v, KHC4H4O6) were sourced from Fisher Scientific (Fair Lawn, NJ). 100% ethanol (EtOH, 200 proof, ACS/USP grade) was sourced from Pharmco-AAPER

(Broofield, CT). Sodium dodecyl sulfate (5%, w/v, 98%) was sourced from Strem Chemicals (Newburgport, MA). BSA protein (Albumin Bovine, RIA grade, A7888), and (+) catechin hydrate (C15H14O6, 98%), was sourced from Sigma Chemical Corporation (St. Louis MO).

Sample preparation

Individual 250mL samples were removed from the freezer (-20 °C) and allowed to thaw to ambient temperature. Each bottle was shaken by hand, or vortexed (Fisher Scientific Mini-Roto S56), to ensure thorough mixing before samples were removed for testing. Samples were pipetted (Alpha pipette, 10:100 and 100:1000, or Pipette-Man Gilson, Model p20), using Fisher Brand disposable tips (Fisher Scientific) into a 1.5 mL cuvette, or into intermediate containers – 1.5 mL disposable eppendorf tubes (Grad MCT Flat Cap, LPS Inc., Rochester NY), clean 5 mL glass test tubes, or 15 mL screw top plastic sample tubes, depending on the volume of liquid required for each step.

Calculations

Calculations for the direct spectrophotometric, Somers-Boulton and Adams-Harbertson methods are per Jacobson (2006) or the originator's work (Boulton and others 1999; Jacobson 2006; Ribereau-Gayon and others 2006; Somers and Evans 1977) (see Table 2-9).

Statistics

Results from duplicate samples (up to four per lot per date) were averaged before plotting the results. The variability of individual sample measurements is represented by error bars displaying the Standard error of the mean (SE) (Payton, Greenstone, Schenker 2003; Streiner 1996). After plotting the results by method and fraction being assessed, significance was determined by comparing the SE of the treatment and the control. If the error bars did not

overlap, the results were considered to be genuine differences due to treatment, rather than random chance or measurement error. In many cases, apparent differences between the control and the treatments were visible on the graphs (or a percentage difference in tabular form) (Tables 2-9, 2-10, 2-11, 2-12), but overlapping error bars, indicating the variability in the data points, meant that the differences were not significant. Throughout the discussion of the results, those that had only minor differences, with overlapping ranges of error, are described as being not significantly different (NSD). Results that did not exhibit overlapping error bars are described as being significantly different. Results are shown graphically, in the figures, with SE error bars, and are described in the discussion in terms of the treatment's percentage difference from the control average.

Modified Standard Curve Calculation

Given the large numbers of sample runs, the results for the standard curves were averaged for each method to produce one standard curve per method, with the intercept set equal to zero (b=0) (Adams-Harbertson averaged $R^2 = 0.9971$; Folin-Ciocalteau averaged $R^2 = 0.9995$). This averaged standard curve was used to calculate the tannin concentrations (as either catechin or gallic acid equivalents) for the wine samples according to the formula Y=mX+b; where X=tannin concentration, Y= absorbance measurement, b=intercept of standard curve plot, and m=slope of the standard curve plot (where b=0). Results were calculated using Microsoft Excel for Mac 2011©.

High Performance Liquid Chromatography (HPLC)

An HPLC analysis of the wine samples and tannin additives were performed at the Cornell University Enology Extension Laboratory (NYSAES Geneva NY). All wine samples were first centrifuged and then filtered through a syringe filter (PES 0.22 µm, 13mm diameter, Celltreat

Scientific Products), and were run on a Hewlett Packard Series 1100 HPLC, fitted with a reverse phase, Microsorb Metaguard standard c18 style column (Varian LiChrospher 5 RP-18). A 250mmx4.6mm, 5µm particle size, 100Å pore size, end-capped (ECAP) column was used. The method was performed in accordance with Enology Extension Laboratory standard operating procedures with the following properties:

- Mobile phase A-- water:phosphoric acid (99.5:0.5, v/v.).
- Mobile phase B – acetonitrile:water:phosphoric acid (50:49.5:0.5, v/v/v).
- Gradient elution profile 0%B (2 minutes), 20%B (7 minutes), 40%B (25 minutes), 40%B (31 minutes), 80%B (35 minutes), 100%B (40 minutes), 100%B (42 minutes), 0%B (50 minutes).
- Injection – 50 µl with needle wash in ethanol.
- Flow rate of 1.0 mL/min.
- Column temperature 30°C
- Detection wavelengths of 210, 280, 320, 360 and 520 nm (with 210 and 280 nm being primary for the tannin-only analysis).

Sensory Screening

An eight-person panel, consisting of five women and three men, evaluated both the Pinot Noir and Lemberger treatments after two years aging, on 11/02/2011. The panelists ranged from their early twenties to fifty, and included both inexperienced and highly experienced wine tasters. Verbal instructions were given at the beginning of the test, but no training was conducted. Panelists were asked to rate each treatment against the control for perceived color and overall preference, given a centered 9-point scale (-4 to +4, with 0=control). The raw data was tabulated, resultant values calculated, and graphs of changing parameters over time were plotted in Microsoft Excel. The statistical analysis was performed using "SPSS for Windows."

Results and Discussion

Growing conditions for the 2009 vintage were cold and wet, which prevented complete ripening of the grapes and also increased the pressure from fungal diseases. As a result, the grapes came to the crush pad with less ripeness, more rot and lower sugar levels than would be considered optimum by growers. These harvest conditions were a good test for the effectiveness of tannin additions.

Wine chemistry

During the fermentation phase, both the Lemberger and Pinot Noir wines followed predicted trends for sugar degradation, ethanol production, and degradation of malic acid during MLF. All wines fermented to dryness (<0.09 g/L residual sugar), and no differences due to treatment were noted for residual sugars, malic acid, total acidity, pH, or ethanol levels (Table 2-6).

Varietal differences during fermentation

Phenolic differences between the Pinot Noir and Lemberger grapes used for this experiment were apparent during fermentation (Figure 2-2). A reduction in colored phenolics was observed between the fifth and seventh days of fermentation in the Pinot Noir fermentations, but not in the Lemberger. Samples from all the Pinot Noir treatments followed an identical pattern: they all suffered a dip in visible color (Figure 2-P-1), visible anthocyanins (Figure 2-P-4), blueness (Figure 2-P-2), color density (Figure 2-P-5), copigmentation effects (Figure 2-P-6), monomeric pigments (Figure 2-P-13), small (Figure 2-P-11) and large (Figure 2-P-12) polymeric pigments, and total polymeric pigments (Figure 2-P-14). With the exception of color due to copigmentation (Figure 2-L-6), the Lemberger wines did not. Hue and total phenolics remained unchanged in both types of wine (data not shown).

HPLC results

Even HPLC methods, capable of detecting individual compounds at extremely low concentrations, can have difficulty deciphering the complex wine phenolic matrix. There are literally hundreds of thousands of different combinations of polyphenolic compounds, and reference standards for all but a few do not exist. Very often, individual compounds are lost in the general background "noise" – the dreaded "hump-o-gram" familiar to HPLC users (McMaster 2006) - and could not be easily separated for analysis (Figure 2-3). That was the case with this experiment; all samples were subject to a tannin assay, but the high level of background phenolic clutter made it impossible to determine what, if any differences between the samples were due to treatment variations (data not shown).

Phenolic and color development, during fermentation

Pinot Noir, during fermentation.

In general, phenolic extraction during maceration for the Pinot Noir followed the typical pattern described in the literature (Bautista-Ortin 2005; Bautista-Ortín and others 2007; Hanlin and others 2010; Sacchi, Bisson, Adams 2005; Zanoni and others 2010). Levels of total phenolics (Figure 2-P-8, 2-P-9)were predictably low at the start, followed by rapid extraction of anthocyanins (Figure 2-P-4, 2-P-13) and small polymeric pigments (SPP) (Figure 2-P-12) during the first three days of maceration. After three days of maceration, SPP levels declined for all treatments, but were not significantly different from control after the third day of maceration, until after pressing. An initial spike in large polymeric pigments (LPP) (Figure 2-P-11) was also observed for all treatments on the second day of maceration (10/08/09), with peaks for the Volutan initial and incremental treatments 30% and 44% higher than control, respectively (Table 2-9). However, this was a short-lived phenomenon, which disappeared by the third day of

fermentation. After pressing, the levels of SPP, monomeric pigments and anthocyanins increased in both control and treatment wines. For all Pinot Noir treatments after pressing (10/14/09, Table 2-4), levels of SPP were significantly lower than the levels measured in the control wines, while levels for LPP for all treatments were significantly higher than control. LPP levels remained at or below control until after pressing and racking into carboys, at which point all treatments were significantly higher than the controls (63% to 71% higher, Table 2-9), based on standard error of the mean. Total phenolics (Figure 2-P-8, 2-P-9) increased steadily throughout maceration. No significant differences between control and treatments were noted in the Pinot Noir wines during fermentation for total phenolics, visible color, color density, hue, or blueness (except for the temporary effects noted above).

In the Pinot Noir, during fermentation, the only differences noted involved polymeric phenolic aggregations (polymeric pigments, Figure 2-P-7; LPP, Figure 2-P-11; SPP, Figure 2-P-12; and iron-reactive tannins, Figure 2-P-10), and these differences were slight. The smaller phenolic compounds in this fraction (i.e. polymeric phenols) were generally slightly higher than the control for all the treatments before pressing (10/14/09), while the larger compounds were somewhat lower than the control for all treatments, at least until pressing. After pressing (10/16/09), however, the larger phenolics (LPPs and iron-reactive tannins, Figures 2-P-11, 2-P-10) were generally higher than the control, while the smaller ones (SPPs, Figure 2-P-12) were lower than control, for all treatments. This suggests that the tannin additions, regardless of type, or when the tannin was added (initial or incremental additions), accelerated formation of larger phenolic polymers in the Pinot Noir wines during fermentation, at least relative to the levels of the smaller colored phenolic fraction. However, total phenolics level did not vary significantly and this effect has apparently not been observed in other experiments; Harbertson (2012) used

the same measurement techniques, but made measurements later than in this experiment - at 14 to 21 weeks post-treatment - and did not report this pattern of treatment related difference between the LPP and SPP fractions at that point in aging. Other researchers studying tannin additions measured different parameters, making comparisons difficult. It is possible, though not proven, that the effects observed here were the result of the treatments having an impact on phenolic polymerization, via copigmentation complexes and extra copigmentation cofactors provided by the commercial tannin additions (Boulton 2001; Neves and others 2010; Parker and others 2007; Schwarz and others 2005).

Lemberger, during fermentation

The Lemberger wines also exhibited some instances where color due to copigmentation effects (Figure 2-L-6) showed differences from the control before treatments had been made. However, there were no significant differences between the control and any of the treatments for visible color, hue, visible anthocyanins, or total phenolics during fermentation. Color due to copigmentation effects (Figure 2-L-6) exhibited some variability during maceration, with decreases relative to control noted for the Volutan initial, Volutan incremental, and SR-Terroir incremental treatments on 11/02/09, two days before pressing. These had disappeared, however, in samples taken immediately after pressing on 11/05/09 (Table 2-5). Levels of SPP (Figure 2-L-12) and LPP (Figure 2-L-11) did show some treatment-related differences during fermentation. In particular, the SR-Terroir initial treatment was significantly higher than the control for small polymeric pigments (Figure 2-L-12) and somewhat higher in large polymeric pigments (Figure 2-L-11) during the last three days of fermentation (11/02/09 - 11/05/09), although SPP remained significantly higher after pressing while LPP decreased dramatically at pressing. The Volutan initial treatment showed elevated levels of LPP relative to the control by the second half of

fermentation (10/31/09, 11/02/09, 11/05/09) and carried those differences into the aging phase of the experiment.

Overall, however, there were few consistent differences due to the treatments during fermentation, for the Lemberger.. Only the final treatments (both Volutan and SR-Terroir) showed elevated levels of iron reactive tannins (Figure 2-L-10), immediately after pressing (on 11/05/09). Those same treatments also had elevated levels of color due to copigmentation (Figure 2-L-6) on that date. This appears to be consistent with the findings of Boulton (2001), Neves (2010) and Parker (2007) regarding the impact of tannin additions, particularly seed tannins, on copigmentation effects, as well as on gallic acid concentrations (which the IRT assay is particularly sensitive to). This may reflect the composition of the tannin additives - Neves (2010) noted increases in gallic acid (thought to be from high levels of galloylated proanthocyanidins in the additive) after tannin additions.

Phenolic and color development, post-fermentation

Pinot Noir, post-fermentation.

Post-fermentation, there were very few persistent differences between the control wines and the treatments. No significant differences were noted between the control wines and treated wines for most characteristics measured; visible color at 520 nm (Figure 2-P-1), blueness (Figure 2-P-2), hue (Figure 2-P-3), visible anthocyanins (Figure 2-P-4), color density (Figure 2-P-5), total phenolics by direct spectrophotometry (Figure 2-P-8), and iron-reactive tannins (i.e. total polymeric tannins) (Figure 2-P-10).

Color due to copigmentation (Figure 2-P-6) was elevated for only one treatment, SR-Terroir initial, and only at three months (+312%, Table 2-10). Total phenolics by Folin-Ciocalteau

decreased relative to control at three months (on 02/05/10) only for the Volutan initial treatment. No significant differences were evident after two years (sample date 10/20/11).

Levels of iron reactive tannins decreased relative to control for all treatments (with the possible exception of the Volutan initial treatment, which showed a slight overlap in error bars with control) at the three month point. Large polymeric pigments (Figure 2-P-11) were elevated for all treatments shortly after pressing (10/16/09), while the SPP level (Figure 2-P-12) was slightly lower for only the Volutan final treatment. At three months, levels of both LPP and SPP had fallen significantly relative to control for all treatments. Monomeric pigments (Figure 2-P-13) and total color (Figure 2-P-14) measurements at three months also showed significant declines for the Volutan incremental and all the SR-Terroir treatments. After two years, however, there were no significant differences in polymeric phenolic levels.

Volutan initial treatment, Pinot Noir, post-fermentation.

No significant differences from control were noted during the aging period for visible color (Figure 2-P-1), blueness (Figure 2-P-2), hue (Figure 2-P-3), visible anthocyanins (Figure 2-P-4), color density (Figure 2-P-5), color due to copigmentation (Figure 2-P-6), total phenolics by direct spectrophotometry (Figure 2-P-8), total monomeric pigments by Adams-Harbertson (Figure 2-P-13), or total polymeric pigments (total color at pH 4.9) (Figure 2-P-14).

Total phenolics, as measured by the Folin-Ciocalteau method (Figure 2-P-9), exhibited a decline after three months aging (-20%, Table 2-9) relative to the control wines, but the differences had disappeared after two years. Iron-reactive tannins (i.e. total polymeric phenolics) (Figure 2-P-10) appeared to be lower than control at three-months, but not two years. Small and large polymeric pigments (Figure 2-P-12, 2-P-11) exhibited an inverse relationship during aging. Immediately after fermentation, SPP were somewhat lower (-27%), while LPP were slightly

higher (+68%) from control (Table 2-9). At the three-month point, SPP showed no significant differences from the control wines, while the LPP did show significant differences (-56%), lower than control. Differences disappeared by the two year measurement.

Volutan incremental treatment, Pinot Noir, post-fermentation.

The Volutan incremental treatment did not show any significant differences from control during aging for visible color (Figure 2-P-1), blueness (Figure 2-P-2), hue (Figure 2-P-3), visible anthocyanins (Figure 2-P-4), color density (Figure 2-P-5), color due to copigmentation effects (Figure 2-P-6), polymeric pigments (Figure 2-P-7), total phenolics by direct spectrophotometry (Figure 2-P-8) or Folin-Ciocalteau (Figure 2-P-9).

The polymeric pigment fractions measured by the Adams-Harbertson assay did show several differences from control for this treatment. Iron-reactive tannins (Figure 2-P-10) were significantly lower than control after three months (-98%, Table 2-9), but no significant differences after two years. LPPs (Figure 2-P-11) were slightly elevated (+64%,Table 2-9) after pressing, but then dropped significantly relative to control after three months (-100%, Table 2-9). SPPs (Figure 2-P-12) also dropped significantly at three months (-87%, Table 2-9). Monomeric pigments (-87%, Table 2-9, Figure 2-P-13) and total color at pH 4.9 (-86%, Table 2-9, Figure 2-P-14) also dropped at three months. After two years, all these differences from control had disappeared.

Volutan final treatment, Pinot Noir, post-fermentation.

The Volutan treatments in the Pinot Noir wines, the final treatments did not show significant differences from control for visible color (Figure 2-P-1), blueness (Figure 2-P-2), hue (Figure 2-P-3), visible anthocyanins (Figure 2-P-4), color density (Figure 2-P-5), color due to copigmentation (Figure 2-P-6), polymeric pigments by direct spectrophotometry (Figure 2-P-7),

total phenolics (Figure 2-P-8, 2-P-9), monomeric pigments (Figure 2-P-13), or total polymeric pigments (total color at pH 4.9; Figure 2-P-14), during two years of aging.

Total condensed tannins (e.g. iron reactive tannins, Figure 2-P-10) showed a significant decline at three months (-75%, Table 2-9), with no significant differences from control after two years. Large polymeric pigment levels (Figure 2-P-11) for the Volutan final treatment were elevated on 10/16/09, after pressing (+63%, Table 2-9), and then declined significantly (-73%, Table 2-9) at three months. SPPs were only significantly different from control at three months (-44%, Table 2-9), but not at other times. This treatment appeared to have very little overall impact on the Pinot Noir wines during aging.

SR-Terroir initial treatment, Pinot Noir, post-fermentation.

The SR-Terroir initial treatment did not show significant differences from control for Visible color (Figure 2-P-1), blueness (Figure 2-P-2), hue (Figure 2-P-3), visible anthocyanins (Figure 2-P-4), color density (Figure 2-P-5), or polymeric pigments (Figure 2-P-7).

Some treatment related differences could be seen. Color due to copigmentation (Figure 2-P-6) was elevated at three months (+312%, Table 2-10). Conversely iron reactive tannins (-98%, Table 2-9, Figure 2-P-10), Small polymeric pigments (-76%, Table 2-9, Figure 2-P-12), monomeric pigments (-76%, Table 2-9, Figure 2-P-13) and total color (-75%, Table 2-9, Figure 2-P-14) were all low relative to control at three months aging. LPPs were elevated after pressing (Boulton 2001; Harbertson and others 2012; Lambert and others 2011; Neves and others 2010; Parker and others 2007)(+76%, Table 2-9, Figure 2-P-11), then also fell below control at three months (-99%, Table 2-9), with NSD after two years. The elevation in copigmentation color coupled with the low levels of IRT, LPP, SPP, MP and total colored compounds at the three month point may indicate that anthocyanins and various cofactors were maintained in

copigmentation stacks during the early part of aging, apparently delaying or retarding the formation of polymeric pigments during the first three months. This seems consistent with the type of phenolic material presumed to be in the SR-Terroir product (i.e. seed tannins) and the findings of several authors (Boulton 2001; Harbertson and others 2012; Lambert and others 2011; Neves and others 2010; Parker and others 2007).

SR-Terroir incremental treatment, Pinot Noir, post-fermentation.

The SR-Terroir incremental treatment produced small changes affecting only the larger polymeric pigments with significantly higher losses of large polymeric pigments at the three month point. There were no significant differences from control for visible color (Figure 2-P-1), blueness (Figure 2-P-2), hue (Figure 2-P-3), visible anthocyanins (Figure 2-P-4), color density (Figure 2-P-5), color due to copigmentation (Figure 2-P-6), polymeric pigments (Figure 2-P-7), or total phenolics (Figures 2-P-8, 2-P-9).

Levels of polymeric tannins (IRT) (-98%, Table 2-9, Figure 2-P-10), monomeric pigments (-85%, Table 2-9, Figure 2-P-13) and total color (-86%, Table 2-9, Figure 2-P-14) all fell significantly below control at three months, with NSD after two years. Large polymeric pigments (Figure 2-P-11, Table 2-9) were elevated (+71%) at the end of fermentation, then fell to significantly below control at three months (-99%), and were not significantly different at two years. SPPs were lower than control after pressing (-32%. Table 2-9, Figure 2-P-12), then also fell dramatically at three months (-88%, Table 2-9), and were NSD at two years. It does not appear that this treatment produced a measureable affect on color in the Pinot Noir, although the sensory screening panel perceived this wine as being lighter than the control in the color preference test (2-tailed significance, 0.002, Table 2-15).

SR-Terroir final treatment, Pinot Noir, post-fermentation.

The SR-Terroir final treatment showed no significant differences from control for visible color (Figure 2-P-1), blueness (Figure 2-P-2), hue (Figure 2-P-3), visible anthocyanins (Figure 2-P-4), color density (Figure 2-P-5), color due to copigmentation (Figure 2-P-6), and total phenolics (Figures 2-P-8, 2-P-9).

Polymeric pigments (Figure 2-P-7), by Somers-Boulton bisulfite bleaching, was lower than control at three months aging (-23%, Table 2-10), and NSD at two years. Iron reactive tannins (-98%, Table 2-9, Figure 2-P-10), SPPs (-80%, Table 2-9, Figure 2-P-12), monomeric pigments (-88%, Table 2-9, Figure 2-P-13), and total color at pH 4.9(-87%, Table 2-9, Figure 2-P-14) were all lower than control after three months. The large polymeric pigment fraction (Figure 2-P-11) was significantly elevated after pressing (+64%, Table 2-9), then fell significantly below control at three months (-111%, Table 2-9), to the lowest level of any treatment. After two years, however, these differences had disappeared.

Pinot Noir, Summary

In general, all treatments had significantly lower levels of iron-reactive tannins, small, and large polymeric pigments after three months of bottle aging, relative to the control wines, but these differences from control disappeared after two years of aging.

Pinot Noir, which has generally lower levels of phenolics, including anthocyanins and cofactors, than other more strongly colored grape varieties(Boulton 2001; Fischer, Strasser, Gutzler 2000; Schwarz and others 2005); in this case, for this vintage and lot of Pinot Noir, the tannin additions may have provided enough additional cofactors to impact copigmentation stacking effects and altered the rate of polymerization by protecting anthocyanins in copigmentation stacks (Boulton 2001; Darias-Martín and others 2006; Schwarz and others 2005).

At the two-year point, it appears that the tannin additions did not produce any improvements in color stability for these Pinot Noir wines. One general observation which appears to run contrary to the findings of other researchers (Harbertson and others 2012; Neves and others 2010; Parker and others 2007), was the increase of the smaller polymeric pigment fraction relative to the larger one, without any significant losses in total phenolics after two years. This is not a treatment-related difference -- both fractions (SPP and LPP) were no longer significantly different from the control at that point. Levels of SPPs (for the control and all treatments) rose in the Pinot Noir, while levels of the other polymeric fractions measured by the Adams-Harbertson assay declined predictably with aging. This may indicate that the larger phenolic compounds (LPP) were not stable and were being broken apart by rearrangement reactions and hydrolysis (Boulton 2001; Harbertson and others 2012; Neves and others 2010; Obradovic 2006; Parker and others 2007; Schwarz and others 2005). The Lemberger wines contained higher levels of total, large and small polyphenolics, and did not exhibit a similar drop in LPPs.

Lemberger, post-fermentation

No significant differences from control were apparent for any of the treatments over the aging period for visible color at 520 nm (Figure 2-L-1), with one exception. At the two-year point (10/20/11), the Volutan initial treatment (+56%, Table 2-12, Figure 2-L-1) remained significantly higher than the control and all the other treatments. Measurements for hue (Figure 2-L-3),visible anthocyanins (Figure 2-L-4), color density (Figure 2-L-5), polymeric pigments (Figure 2-L-7), and total phenolics (Figures 2-L-8, 2-L-9), showed no significant differences from the control wines for any treatment.

Both initial treatments (Volutan and SR-Terroir) exhibited elevated levels of blueness (Figure 2-L-2, Table 2-12), and polymeric pigments (Figure 2-L-7) at the three month

measurement, which persisted for the Volutan initial treatment at two years aging. The Volutan final and possibly the SR-Terroir final treatments showed elevated levels of copigmentation (Figure 2-L-6) after pressing, and the Volutan incremental treatment was significantly lower than control at three months, but had no differences after two years.

For the IRT fraction, the Volutan final treatment was elevated after pressing, but not thereafter, the Volutan initial treatment was significantly elevated at both three months and two years; the SR-Terroir initial treatment was lower than control at three months, the Volutan initial and SR-Terroir incremental treatments were elevated at two years.

LPPs were elevated for several treatments during aging: the Volutan initial, SR-Terroir incremental and final treatments at three months; by two years, LPPs for Volutan initial and incremental, and SR-Terroir incremental treatments were also elevated. The only significant difference from control for SPPs was for the SR-Terroir treatment, which was elevated after pressing. For monomeric pigments (Figure 2-L-13) and total color (Figure 2-L-14) measurements, only the Volutan initial treatment, elevated at three months, showed any difference from control.

Volutan initial treatment, Lemberger, post-fermentation.

No significant differences were noted for hue (Figure 2-L-3), visible anthocyanins (Figure 2-L-4), color density (Figure 2-L-5), or color due to copigmentation (Figure 2-L-6), total phenolics (Figures 2-L-8, 2-L-9), or SPPs (Figure 2-L-12) for this treatment.

Visible color at 520 nm (Figure 2-L-1) with this treatment showed no differences until the two-year point, when it became significantly higher than the control (+56%, Table 2-12). Blueness (Figure 2-L-2), was significantly elevated relative to the control for this treatment at the three-month (+26%, Table 2-12) point. Polymeric pigments (Figure 2-L-7) for the Volutan initial

treatment were significantly higher at the three-month (+25%) and two-year measurements (+23%) (Table 2-12).

Levels of iron reactive tannins (Figure 2-L-10) were significantly elevated for this treatment at three-months (+33%) and at the two-year measurement (+104%) (Table 2-11). Likewise, large polymeric pigments (Figure 2-L-11) were significantly higher at both the three-month (+36%) and two-year (+73%) points (Table 2-11). Both monomeric (Figure 2-L-13) and total polymeric pigments (i.e. total color at pH 4.9, Figure 2-L-14) also showed significant elevations relative to control at three months (+34%, +29% respectively, Table 2-11). This treatment resulted in significantly higher formation of large, stable colored pigments, and produced the only visible color change noted, which reinforces the conclusion that this particular treatment had a positive impact on stable color formation in the Lemberger wine.

Volutan incremental treatment, Lemberger, post-fermentation.

The Volutan incremental treatment did not show any significant differences from the control for visible color (Figure 2-L-1), blueness (Figure 2-L-2), hue (Figure 2-L-3), visible anthocyanins (Figure 2-L-4), color density (Figure 2-L-5), polymeric pigments (Figure 2-L-7), total phenolics (Figures 2-L-8, 2-L-9), small polymeric pigments (Figure 2-L-12), monomeric pigments (Figure 2-L-13), or total color at pH 4.9 (Figure 2-L-14).

Color due to copigmentation effects (Figure 2-L-6), was only different from control at three months, where it was significantly lower (-73%, Table 2-12). Iron-reactive tannins (Figure 2-L-10) were significantly elevated after pressing (+230%, Table 2-11), but not thereafter. LPPs (Figure 2-L-12), on the other hand, only became significantly elevated after two years (+45%, Table 2-11). It appears that this treatment had a slight, but positive affect on large, stable polymeric pigment formation.

Volutan final treatment, Lemberger, post-fermentation.

The Volutan final treatment showed no significant difference from control for visible color (Figure 2-L-1), blueness (Figure 2-L-2), hue (Figure 2-L-3), visible anthocyanins (Figure 2-L-4), color density (Figure 2-L-5), polymeric pigments (Figure 2-L-7), total phenolics (Figures 2-L-8, 2-L-9), IRTs (Figure 2-L-10), SPPs (Figure 2-L-12), monomeric pigments (Figure 2-L-13), or total color at pH 4.9 (Figure 2-L-14).

Color due to copigmentation effects (Figure 2-L-6) was significantly elevated after pressing (+61%, Table 2-12), but not thereafter. LPPs (Figure 2-L-11) only showed a significant difference from control at three months, where they were elevated (+26%, Table 2-11).

This treatment produced only minor changes in the Lemberger wine, and only for LPPs after three months. Wine color was not affected significantly.

SR-Terroir initial treatment, Lemberger, post-fermentation.

The SR-Terroir initial treatment exhibited no significant differences from control for visible color (Figure 2-L-1), blueness (Figure 2-L-2), hue (Figure 2-L-3), visible anthocyanins (Figure 2-L-4), color density (Figure 2-L-5), color due to copigmentation (Figure 2-L-6), polymeric pigments (Figure 2-L-7), total phenolics (Figures 2-L-8, 2-L-9), LPPs (Figure 2-L-11), SPPs (Figure 2-L-12), monomeric pigments (Figure 2-L-13), or total color at pH 4.9 (Figure 2-L-14).

IRTs were lower than control at three months (-23%, Table 2-11), but not thereafter. This treatment had no significant impact on color formation or stability in the Lemberger wines.

SR-Terroir incremental treatment, Lemberger, post-fermentation.

The SR-Terroir incremental treatment showed no significant differences from control for visible color (Figure 2-L-1), hue (Figure 2-L-3), visible anthocyanins (Figure 2-L-4), color density (Figure 2-L-5), copigmentation color (Figure 2-L-6), polymeric pigments (Figure 2-L-7),

total phenolics (Figures 2-L-8, 2-L-9), monomeric pigments (Figure 2-L-13), or total color (Figure 2-L-14).

Blueness (Figure 2-L-2) was elevated at three months (+25%, Table 2-12). Iron-reactive tannins (Figure 2-L-10) were significantly elevated relative to control only at the two year point (+110%, Table 2-11), but not before. LPPs (Figure 2-L-11) were significantly lower after pressing (-162%, Table 2-11), but significantly elevated at three months (+46%) and two years (+56%). Conversely, SPPs (Figure 2-L-12) were significantly elevated after pressing (+55%, Table 2-11), but not thereafter.

This treatment produced significant results. The impact of the smaller sized seed tannins could be seen in the elevated levels of SPPs after pressing, and the elevated IRTs at two years (Neves and others 2010). Enhanced blueness was most likely due to absorbance by one or more of the non-tannin compounds in the product. The significant elevations to the large polymeric pigment fraction throughout aging indicates that this treatment was modestly successful at stabilizing wine color.

SR-Terroir final treatment, Lemberger, post-fermentation.

The SR-Terroir final treatment showed no significant differences from control for measurement of visible color (Figure 2-L-1), blueness (Figure 2-L-2), hue (Figure 2-L-3), visible anthocyanins (Figure 2-L-4), color density (Figure 2-L-5), polymeric pigments (Figure 2-L-7), total phenolics (Figures 2-L-8, 2-L-9), SPPs (Figure 2-L-12), monomeric pigments (Figure 2-L-13) or total color (Figure 2-L-14).

Color due to copigmentation effects (Figure 2-L-6) was elevated after pressing (+43%, Table 2-12), but not thereafter. Iron reactive tannins (Figure 2-L-10) were also significantly elevated after pressing (+71% on 11/05/09. Table 2-11), but the differences were not evident at

three months or two years. Large polymeric pigments (Figure 2-L-11), showed a significant increase relative to control at the three-month point (+119%, Table 2-11), but then declined, showing no significant difference at the two-year point.

Lemberger, Summary

In general, only the Volutan initial and both incremental treatments had any significant impact over the course of aging on the Lemberger wines. The Volutan initial treatment was effective at enhancing visible color, as well as the large stable colored pigments needed for aging. Increases in IRTs, which were persistent, were most likely due to the release of gallic acid from galloylated proanthocyanidins, as observed by Neves (2010). The SR-Terroir and Volutan incremental treatments both produced significant improvements in the levels of the large polymeric pigments, and may be useful treatments for improving long-term color stability.

Sensory screening results.

In order to determine if a full-scale sensory evaluation effort was warranted, a small-scale screening panel, drawn from readily available personnel, was convened on 11/02/2011. The wines were all over two years old when evaluated. Panelists were asked to rank both appearance (color) and hedonic liking of each of the six different treatments against the control wine, marking their choices for each treatment on a centered nine-point check-box scale, with the zero (or center box) indicating no difference from the control sample. This centered check-box format added the positive or negative dimension, as well as a 'degree of difference' to this sensory assessment (Lawless and Heymann 1999). Assessing color differences was the primary goal of the experiment. However, the hedonic (liking) measurement was also included to determine if any factors relating to the treatments affected overall preference. Given the small panel size, and the apparent lack of significant differences to wine professionals in informal bench-top

evaluations, this 8-person panel did produce some statistically significant results; one sample T-test method was used to analyze color preferences (Table 2-15), while a paired sample T-test was used to rate hedonic preferences (Table 2-14). For the results, using a 95% confidence interval, a two-tailed significance of ≤ 0.05 was considered statistically significant. The upper and lower ranges determined whether the preference was positive or negative. For the Pinot Noir wines, all the Volutan treatments (2-tailed significance of 0.033, 0.002, 0.001 respectively) and the SR-Terroir incremental treatment (significance of 0.002) had a negative preference with regards to color, which means the panelists found the wines lighter in color than the control. The only hedonic differences of significance were a positive liking for the Volutan incremental (significance of 0.003) and SR-Terroir initial (significance of 0.007) treatments. For the Lemberger, the only treatment that had significant preference results was the SR-Terroir incremental treatment (significance of 0.011), which exhibited a positive preference on color (i.e. the wine was perceived as being darker than control), and a very nearly positive rating for hedonic (liking) (significance of 0.064).

When these results are compared to the instrumental results, it is unclear why the sensory panel apparently saw differences which were not apparent in the analysis of either color or polyphenolic compounds. The Volutan treatments in the Pinot Noir wines, which were all given negative sensory scores for color (i.e. perceived as lighter in color than the control), showed no significant differences for visible color, visible anthocyanins, hue, color density or color due to copigmentation at two years aging. The Lemberger SR-Terroir incremental treatment, which was rated positively for both color (i.e. darker) and liking by the sensory screening panel, was significantly difference from control for both IRTs and LPPs at two years, but not for visible color. And the other Lemberger treatments, Volutan initial and incremental, which also had

significant color or phenolic differences at the two-year point, were judged by the panelists as not significantly different for either color or liking. A much larger and more comprehensive sensory panel study would be required to resolve these discrepancies.

Another complicating factor is the actual level of extractible tannin material contained in each of these commercial products. As has been discussed by several authors (Cíchová M., Petříček J., Fiala J. 2008; Harbertson and others 2012; Keulder 2006; Laghi and others 2010; Neves and others 2010; Obradovic 2006; Obreque-Slíer 2009; Parker and others 2007; Schwarz and others 2005), commercial tannin products may have widely varying levels of different types of tannins, and low extractability into wine may further reduce the amount of actual useful phenolic material. Harbertson (2012), found that for all of the enological tannin preparations tested, less than 50% of the phenolics in the product were composed of iron reactive or protein precipitable tannins, suggesting limited solubility, as well as compositional variability. Although this explicit analysis was not performed on the two enological tannin products used in this study, it is illustrative to estimate the amount of available tannin material, assuming 50% solubility in wine, as suggested by Harbertson; the Volutan product, added at a 8.9 mL/hL dose rate may have actually contributed no more than ~4.45 mL/hL (0.0445 mL/L) of polymeric phenolics and the SR-Terroir product, added at a dose rate of 3.3 g/hL, may have contributed no more than 1.65 g/hL (0.0165 g/L) of polymeric phenolics. And given the findings of Harbertson (2012) or Obreque-Slier (2009), even those levels might be over-estimations, depending on the particular product being used.

Practical Applications

The purpose of this experiment was to evaluate the impact of tannin addition timing on stable color formation. Regional winemakers are only interested in results apparent, in the bottle

or glass, to their customers. For winemakers, the only tannin addition results which really matter are those which persist after bottling, and which are discernible to untrained consumers. Understanding the changes generated by the treatments during and after fermentation are important insofar as they help us understand and better control color evolution during wine aging. Over the course of this experiment, some general trends became apparent. Different treatment lots often exhibited high degrees of variation. Samples from the same date but adjacent containers, which had undergone the same treatments and handling, could be significantly different. The control wines also exhibited this same degree of container-to-container variability. In order to account for these fermenter-to-fermenter variations, measurements from duplicate lots were averaged. Those means were plotted and used during analysis. With few exceptions, even statistically significant differences from the control were relatively small, and probably would not be apparent to consumers. Differences due to treatments were also generally short-lived phenomena, which might be evident after three months, but which disappeared by the two-year point. Varietal differences were apparent too. In the Pinot Noir wines, only the two initial treatments resulted in increases relative to control for a few fractions (small polymeric pigments, copigmentation, blueness and anthocyanins), but only at the three-month point. All other characteristics were either not significantly different from, or lower than, the controls throughout aging. In the Lemberger wines, there were clearly some statistically significant results from the two initial treatments, for appearance and phenolic content measurements. The Volutan final treatment also exhibited some appearance and phenolic compositional differences that persisted for two years. Whether any of these persistent differences meet the threshold for consumer perception of difference remains to be seen. Follow-on sensory trials would be required to

determine not only if consumers could discriminate between treatments, but also if any differences were perceived as positives or negatives with regards to wine quality or desirability.

The results of this study highlight that tannin additions are not a panacea for winemakers. This work contributes to the broader understanding tannin addition impacts on wine color by demonstrating that most of the treatment related changes to color or phenolic parameters were short-lived, relatively low in magnitude and may not be apparent to untrained observers; this supports the conclusions of several previous studies (Cíchová M., Petříček J., Fiala J. 2008; Harbertson and others 2012; Neves and others 2010; Obradovic 2006). These results reinforce the conclusions of Harbertson (Harbertson and others 2012; Neves and others 2010), that low dose rates have little or no long-term impact on red wine color stability. Boulton has argued that (Boulton 2010; Boulton 2001; Versari, A, R Boulton, and G Parpinello. 2008) red wine color is related to its composition, not just anthocyanin content, and that levels of copigmentation (and cofactors, which differ by cultivar) will affect several areas of color development: extraction from the grapes, stabilization in must and finished wine, rate of polymerization reactions, precipitation or degradation reactions, and specific polyphenolic equilibriums.

From this research, it appears that any impacts from tannin additions supplying cofactors might be fairly short-lived, and that polymeric phenolics, rather than visible wine color were primarily affected. If tannin additions are used to improve wine color, the level of cofactors in the product (and their specific composition), will have a significant impact on the treatment's overall effectiveness. In this experiment, the objective of these treatments was to determine if the timing of the addition impacted overall color extraction and stabilization. The dosage chosen for this experiment was low, less than the manufacturer's recommended range, in order to avoid negative sensory impacts reported in earlier research involving higher dosages of commercial

tannin products. The tannin additions were observed to have some short-term impacts on copigmentation, visible color, and polymeric pigment formation in the Pinot Noir wines, while the Lemberger wines exhibited more persistent treatment-related effects. These observations followed the trends observed elsewhere (Bautista-Ortín and others 2007; Boulton 2010; Ginjom and others 2010; Main and Morris 2007; Parker and others 2007), demonstrating the need for winemakers to understand the compositional nature of any tannin products they wish to add to their wines, as well as dose rate and timing requirements.

Chapter 3: Future Work

After-Action: Experiment Design Changes

Streamlined Experiment Design

In retrospect, the objectives of this experiment could have been achieved with a less comprehensive, more streamlined experiment design. A streamlined design should include fewer treatments, one type of wine per experiment, and if testing the impact of different dosages, then use only one type of tannin. Reducing the number of variables tested and the total number of unique data sets requiring analysis will improve efficiency. During the course of analyzing these results, it became apparent that the large number of unique data sets actually hindered analysis. Furthermore, several calculation steps were needed to convert the measurements from the Somers-Boulton, Adams-Harbertson and Folin-Ciocalteau methods into useable data. Based on this effort, the same relative results can be obtained by using the simpler methods, requiring fewer transformations (calculations); a small loss of precision is worthwhile if it significantly speeds up the experiment. The fewer computational steps, the better!

A considerable amount of time and effort during the measurement phase of this experiment was dedicated to strictly mechanical processes: identifying and optimizing sample handling, reagent preparation, and the workflow involved in taking measurements and recording raw data. Determining throughput bottlenecks and optimizing workflow proved to be much more important than originally anticipated, especially due to the very manual nature of the methods being used to obtain data points. Even though duplicate samples were used when making measurements, margins of error would be tighter with more replication. A major finding from this experiment is the need to increase number of replicated measurements per sample (per

analytical measurement). However, adding additional measurements tends to have a snowball effect. The workload needed to produce a single, but more precise, data-point requires more sample preparation time, reagents, disposable materials and repetition of the same handling steps (pipetting liquid into test tubes or cuvettes, mixing, adding reagents, incubation time, multiple measurements, etc.). To facilitate this, the rest of the experiment needs to be focused on fewer variables, fewer treatments and fewer wines.

Methodology Issues

One of the key challenges relating to current color or phenolic assessment methodologies is their lack of specificity. Categorization of results is neither precise, nor clearly delineated; categories overlap. Measurements made by different methodologies of the same phenolic fractions capture different components of the wine matrix in their measurement, making direct comparisons difficult or meaningless. Each method has different strengths and weaknesses, and gathers a different sub-set of phenolic data from the wine matrix being measured. However, when the methods overlap, are the differences between them due to double counting, missing, or under counting a particular fraction? Improved specificity with regards to the phenolic fraction being measured should be made a priority for future methodology improvement. Reducing the overlap between the different assays available to researchers will not only increase precision, but also help with experiment design and methodology selection.

Four different methodologies were used in this experiment; direct spectrophotometry, the Somers-Boulton method, the Adams-Harbertson method, and the Folin-Ciocalteau method. One of the challenges of employing several methods is that the different methodologies, despite using similar terms to describe their results, are actually measuring slightly different fractions of the wine's phenolic matrix. All the methodologies produce estimates based on relatively simple,

indirect assessments of different phenolic fractions, which reduces their ability to finely discriminate between different classes of phenolics. To further complicate comparisons, the direct spectrophotometric methods produce only relative estimates, which the Folin-Ciocalteau and Adams-Harbertson methods employ a standard curve to convert the measurements to mg/L equivalents in either gallic acid or catechins. For example, both the Folin-Ciocalteau and direct spectrophotometry methods produce estimates of the wine's total phenolic content. However, the direct spectrophotometric method for total phenolics ($A^{280} - 4$) uses a generic constant to represent the non-colored phenolic fraction that absorbs at 280 nm (Somers 1971), while the Folin-Ciocalteau method measures absorbance at a completely different wavelength (765 nm) and then converts the results into gallic acid equivalents via a standard curve. Although ostensibly measuring the same phenomena, and generally having good agreement, the two methods produced slightly different estimates, complicating analysis. Likewise, challenges exist for interpreting results from the Somers-Boulton assay (visible anthocyanins, color due to copigmentation effects, non-bleachable polymeric pigments) against those from the Adams-Harbertson assay (iron-reactive tannins, total colored polyphenolics, monomeric pigments, small and large polymeric pigments). The problem is that these categories are not discrete – they overlap, and include fractions of different sizes, as well as other colored compounds (Vitisin-A, for example) and non-colored phenolics too. The total color at pH 4.9 and Somers-Boulton $A^{520}{}_{SO_2}$ both purport to estimate total polymeric pigments, and do produce generally similar, but not identical results. Even the direct spectrophotometric measurements for wine color (red at A^{520}, yellow at A^{420} and blue at A^{620}) are only approximations, because the human eye does not perceive color as a single discrete wavelength (Lawless and Heymann 1999), nor do phenolics only produce color at a single wavelength. The measurements are merely a convenient point of

approximate maximum absorbance by a compound or group of compounds. A good example of this is the apparent disconnect between color due to copigmentation effects (which is described in the literature as both increasing color intensity and shifting it towards the blue end of the spectrum), and blueness (measured at 620 nm). Large spikes in copigmentation effects were not necessarily matched by corresponding spikes in blueness. This is because color changes due to copigmentation absorb across a range of wavelengths, not just at 620 nm, and that different copigmentation cofactors impact color in different ways (Bautista-Ortin 2005; Birse 2007; Boulton 2001; De Beer and others 2004; Goto and Kondo 1991; Obradovic 2006).

As a result of this overlap between what the different methodologies measure or report, vice what is actually happening to the different compounds of the wine matrix over time, making comparisons and drawing definitive conclusions about the dynamics of wine color evolution over time can be fraught with peril (Keulder 2006). Therefore, the conclusions arising from this experiment must, of necessity, be somewhat imprecise. All of the methodologies used for this experiment were developed to be relatively simple, inexpensive and quick to use, so that they might benefit industry users, as well as research labs. These methodologies should therefore be viewed more as screening assays, rather than ultra-precise assessment tools.

Recommendations

Overall, the ease of use and lack of potential errors from reagents or multiple wet chemistry steps recommends use of the direct spectrophotometry method for future wine color experiments at the Geneva Station. The Somers-Boulton method can also be easily combined with direct spectrophotometry readings. The reagents are simple and incubation times are reasonable; when combined with direct spectrophotometry, Somers-Boulton provides very useful data on polymeric pigments, copigmentation, and other visible colored fractions. Estimation of total

phenolic content by direct spectrophotometry ($A^{280} - 4$) might be better accomplished using the Folin-Ciocalteau method (which provides quantified rather than relative results), unless suitable disposable cuvettes can be found (which do not affect readings at 280 nm). If the proper cuvettes can be acquired, allowing the direct spectrophotometric readings for all wavelengths to be made with one sample in one cuvette at one time, then the simplicity of direct measurement should probably be preferred over the somewhat more involved Folin-Ciocalteau method. The results from both methods of estimating total phenolics seem to be consistently close to one another for all but the most refined experiments.

Alternatively, if the design of the experiment would benefit from phenolic quantifications (rather than a measure relative to the control), the use of the Adams-Harbertson method is called for, especially if sensory studies on physiological responses (e.g. astringency) are part of the plan.

Modifications to direct spectrophotometry

Direct spectrophotometry measurements are rapid and easy to perform, but somewhat less precise than the oxidative or protein precipitation methods. The simplicity of this method is both a strength and a weakness – a strength due to ease and speed, a weakness due to the assumptions and lack of precision inherent in the simplified approach. In particular, measuring colors at a single peak absorbance wavelength (420 nm for yellow, 520 nm for red, and 620 nm for blue) undoubtedly introduces error into the analytical results, because phenolics of different sizes or compositions do not have uniform peak absorbance points. In addition, human visual perception of color is not confined to single wavelengths. A truer representation of the colored components of wine requires increasing the number of wavelengths being measured. Expressing color measurements in terms of the *Commission internationale de l'éclairage* CIELAB color

coordinate system (HunterLab 2008) seems to be a desirable approach, and one that other laboratories investigating wine color have moved to adopt. It is a more complex methodology, however, requiring additional specialized equipment and software for analysis(Bautista-Ortín and others 2007; Birse 2007; Darias-Martín and others 2006; Lawless and Heymann 1999; Parker and others 2007; Versari, Boulton, Parpinello 2008; Zanoni and others 2010).

Readings at 280 nm

Direct spectrophotometric assessment of total phenolics required taking readings at 280 nm. This presented several challenges; interference from the cuvette body material and path length limitations, as well as absorbance due to non-phenolic material in the sample. This reduces the accuracy of the assay, since a generic correction factor is used when calculating the phenolic fraction. The actual amount of non-phenolic material present is highly variable. Disposable cuvettes from Fischer Scientific, advertised as suitable for readings at 280nm, were tried, but produced distorted readings at that wavelength (data not shown) and could not be used. The red wines being tested produced out of instrument range readings (> 4.0) with 10 mm path length cuvettes (both quartz and plastic) at 280 nm, mandating use of 1 mm path length cuvettes. Using the specialized quartz 1-mm cuvettes was very time-consuming due to size, shape and washing requirements, and should be avoided if other options are available. They proved difficult to use in the Genesys spectrophotometer, needing special spacers, and tended to become stuck in the tray. They are also fragile, required washing between samples, are very expensive and therefore available in only limited numbers, limiting the number of samples that could be processed simultaneously.

Somers-Boulton Method

The Somers-Boulton assay is a fast and simple extension of the direct spectrophotometric method (Darias-Martín and others 2006). Incubation times are reasonably short, and the method requires only simple reagent additions. The results, when combined with direct spectrophotometric measurements, are somewhat less precise than the oxidation-precipitation methods, but are also more versatile, enabling the researcher to produce estimates for a wide range of color fractions; total and visible anthocyanin content, copigmentation, polymeric pigments, visible color losses. These results are appropriate where quantitative measurements of phenolic content are not required. One important measurement was omitted from this experiment, but in retrospect, was required. Calculation of total monomeric anthocyanins required an additional assay, an acidification step (A^{520}_{HCL}), which drops the sample pH <1, in order to force all anthocyanins into their colored forms. When used with other measurements, different fractions and ratios can be calculated. Future work should include this extra acidification assay.

Folin-Ciocalteau method

The Folin-Ciocalteau assay produced good results for a single data point, namely total phenolic content. While the assay was fairly simple to perform, throughput was limited by the 1.5-hour incubation time necessary for both calibration curve and sample measurement. Reagent sensitivity was also found to be an issue with this assay. A new lot of reagent had been purchased for this experiment, but produced non-linear standard curves and therefore could not be used. Fortunately, an older batch of reagent was available which did produce consistently linear results. Because there was consistently good correlation between this assay's results and the total-phenolics estimates produced by the direct spectrophotometric method (similar, but not

identical), the latter should be used for experiments where higher volumes of sample processing are needed and where specific quantification of results are not required.

Adams-Harbertson method

The Adams-Harbertson assay provides extremely useful data, but is also very complex and was the slowest and most cumbersome of the methods to execute at the lab bench. The results, which characterize different colored and non-colored phenolic fractions, allow for direct comparisons between results within the method. One of the components of this assay, however, was not particularly useful. The "iron-reactive tannins" assay produces an estimate covering an overly broad category (all phenolics except anthocyanins and monohydroxylated phenols), and did not prove analytically useful for this experiment. The Adams-Harbertson method also carries additional potential for errors, due to the multiple handling and liquid-solid separation steps during sample processing. In particular, the removal of supernatant and re-suspension of the solids (precipitate) proved problematic. There was significant potential for losing some or all of the precipitate during pipetting. Re-dissolving the precipitate also proved very challenging. Both these issues were a potential source of non-systemic errors.

HPLC

In order for HPLC analysis to be useful for this type of experiment, the equipment and methodology must have improved sensitivity for large phenolic compounds. Although samples were assessed by HPLC, no differences attributable to tannin additives were apparent. The main challenge, most likely due to the very low addition dose rates used, seemed to be that any spike from the additions was lost in the background noise of other wine phenolics, rendering the analysis useless. One future approach would be to conduct phenolic analysis on the candidate tannin additives before conducting the winemaking trials. A separate experiment might be to

conduct HPLC analysis of multiple different commercial products to compare actual to labeled composition, as well as price vs. specific phenolic content.

Dose Rate Selection

One of the key findings from this experiment was the need for higher tannin addition dose rates. Future experiments with tannin additions should therefore start at doses no lower than minimum manufacturer recommended levels, increase to the manufacturer's maximum dose rate, and then test addition levels significantly higher than the maximum suggested dose rates. Actual doses and increments between treatment rates will depend on several factors, including type of tannin products being tested, grape type and condition at harvest, and scale of the proposed project.

Sampling Protocol

Sampling protocols can be significantly improved upon. During fermentation, rapid changes in the phenolic composition of the must suggests that samples be collected daily and that treatment addition times (with thorough mixing) be separated from sample collection to ensure proper diffusion of the treatment materials.

Even more critical for comprehensive analysis is the post-maceration period. Many more data points need to be collected after pressing. One suggested schedule would be for weekly sampling for first 6 months, followed by monthly sample collection thereafter.

All samples should be collected in duplicate and frozen promptly for later analysis. In addition, better organization of sample storage space in the freezer should be planned before sample collection, to avoid unnecessary confusion or loss of samples. Samples should be pre-sorted by wine type, then by collection date and finally be arranged by treatment. Use of disposable cardboard sample-holders, which can be clearly labeled, will make handling much

more efficient. In addition, organization of the freezer space used to store the samples would be invaluable; organizing cold storage so that samples could be sorted as they enter the freezer would save considerable time and reduce confusion.

Equipment Issues

Several equipment issues should be addressed as well. To begin with, sample collection through the cap of seeds and skins during maceration was challenging. Addition of sampling/racking wands to the temperature-controlled stainless steel fermenters would speed up the collection process, allow for more frequent sample collection, and reduce sampling errors.

As noted above, disposable cuvettes are needed for taking measurements at 280nm. A better spectrophotometer, with a larger sample tray and the ability to automate and record measurements across multiple wavelengths is needed, especially if the CIELAB color coordinate system is to be used.

Cover gas application was another area that could be improved upon. Inert gas dosing equipment should be available for use with a range of container sizes (5 gal carboys, 750 ml wine bottles, 250 mL sample jars).

Likewise, access to bottling equipment is important once the amount of wine being processed goes beyond a few carboys. Not only did the vacuum bottling system save considerable time and labor, it also ensured uniform bottle filling. Manual syphoning with lengths of tubing is messy, inefficient and may introduce microbial contaminates into the wines. Incorporating an inert gas sparging system into a racking/filling machine would eliminate an extra manual step, reduce oxidation and further speed up processing.

Sensory Trials

Sensory trials were always envisioned as a part of this tannin addition experiment; unfortunately, the scale of the project, the time it took to complete analytical measurements and the lack of strong results (due to dose rate decisions) resulted in a decision to downsize the sensory evaluation phase. Although the screening panel did demonstrate several statistically significant differences from the control wines, larger panels are clearly needed for future work. They should determine if any treatment differences are apparent to consumers, if those differences are positive or negative, and to define its temporal signature; e.g. when did differences first become apparent to the consumer and when did they cease to be apparent. Panels should be composed of consumers rather than professionals, and several discrimination trials should be run throughout the aging process, in addition to a thorough more descriptive trial.

Future Research Projects

Methodology improvement

Better discrimination between the phenolic fractions being measured by the colorimetric assays is needed. Previous research was focused on creating simple, fast and inexpensive assays suitable for an industry setting. Wet chemistry assays remain useful screening tools even when high-precision (i.e. HPLC) technology is available for use. However, the usefulness of these methods would be enhanced if the resolution between phenolic fractions could be improved without adding excessive complexity.

Commercial tannin characterization by HPLC

Another useful project would be the independent characterization of commercial tannin products, of interest to local winemakers, by HPLC. The goal would be to provide regional

winemakers with objective data on which they could plan their tannin addition strategy. This type of planning requires detailed information about the composition of these products, which is not generally available in product literature. This study should include data on the product composition (type, amount, and percent of phenolic material), solubility in wine, fillers or inert materials (type, amount, percentage), and pricing data. Results should be made public as an extension publication, and updated every several years with new products.

Copigmentation experiment

An experiment should be conducted to examine the impact of tannin additions on copigmentation. It is now recognized that early formation of copigmentation complexes has a positive impact on later polymeric pigment formation. However, the formation of copigmentation complexes depends upon the availability of both anthocyanins and colorless cofactors in the fermenting must. This experiment should consider several factors: the ratios of anthocyanin to cofactor content of the grape variety, potential and actual cofactor contributions from the additives, and if adding cofactors during fermentation affects polymeric pigment formation, timing or stability during aging.

High Dose Rate Trials

Future work should concentrate on improving our understanding of the dose rates required to impact stable color formation. Evidence from numerous experiments to date indicate that low to moderate dose rates are relatively ineffective; most earlier trials focused on the impact of tannin additions in comparison to other maceration techniques, or in combination with enzyme treatments. In order to understand the impact of dose rates on color or sensory properties, experiments should be conducted where only dose rate varies. Furthermore, trials should include manufacturer's maximum dose rate, and levels above that maximum recommended rate.

Initial vs. Final Addition Trial

The addition timing experiment should be done again, but using a high dose rate addition instead of a very low one. The experiment should be kept narrowly focused, using a single wine, a single dose rate, and only two addition timings – at the beginning of fermentation (initial), and a final addition after pressing. Measurements should be taken for color and phenolic fractions. Sensory trials should be conducted to determine the aroma or flavor impacts of the additions, if any.

Sensory Evaluation during aging

Another interesting follow-on study would be to follow sensory changes in tannin addition wines during aging. This should be a detailed descriptive analysis, including panelist training, descriptor development, color, aroma and taste evolution. Ideally, the same panelists should make repeated assessments of the same wines at multiple points during aging.

Aroma Trials

An important question regarding tannin addition treatments is their impact wine aroma. Several questions should be asked: do tannin additives affect aroma development? Do tannin additions add aroma producing compounds to the wine? Do the additions mask aromas? Are affects of treatment dose dependent? Are affects temporary or persistent? This project should examine different commercial tannin products, but focus on one type of wine, one dose rate, and one treatment timing. Measurements could be conducted by instrumental methods (gas chromatograph, olfactory) and by trained sensory panels.

Tables

Table 2-1: Enological Tannin Products. ***Institut Oenologique De Champagne (IOE), France.***

Product Name		Mfr.	Composition		Recommended Dosage	Form
Volutan		IOE*	100%White Grape tannins		15-40 mL/hL	Liquid
Tannin SR-Terroir		IOE	Catechin + grape seed tannins		5-15 g/hL	Powder
Treatments (addition amounts and rates by timing)						
Wine	Tannin	Initial	Incremental (X4)	Final	Rate	Notes
Pinot Noir	Volutan	8 mL /91L	2 mL/day	2.7mL/30.3L	8.9 mL/hL	Final in 2 carboys (19+11 L)
	SR-Terroir	3 g /91L	0.75g/day	1g/30.3L	3.3g/hL	Powder, in 100mL dH_2O Final in 2 Carboys (19+11 L)
Lemberger	Volutan	6.7mL /76.5L	1.7mL/day	2.7 mL/30.3L	8.9 mL/hL	Final in 2 carboys (19+11 L)
	SR-Terroir	2.53g /76.5L	0.63g/day	1g/30.3L	3.3 g/hL	Powder, in 100mL dH_2O Final in 2 carboys (19+11 L)

Table 2-2: Harvest Characteristics of Finger Lakes Red Wine Grapes used

Grape type:	Pinot Noir	Lemberger
Source:	Sheldrake Point	Cornell Lansing vineyard
Harvest date	10/06/2009	10/26/2009
Amount (kg)	1020	1020
Average brix	19.6	19.2
Estimated TA	11 mg/L	---

Table 2-3: Pinot Noir, treatments and additions

Pinot Noir			
Action or Activity	Reason	Date	Rate
Initial SO_2	Anti-microbial	10/07/09	50 mg/L
Fermaid®	Nutrient	10/07/09	0.25 g/L
Go-Ferm®	Nutrient	10/07/09	0.3 g/L
Add Yeast (ICV-D254®)	Start Fermentation	10/07/09	0.26 g/L
Press	End Maceration	10/14/09	---
Add *O. oeni*	Start MLF	10/14/09	0.2 g/L
Post-MLF SO_2	Stop MLF	11/03/09	60 mg/L
Cold Room (-4° C)	Cold Stabilization	01/05/10	---
Racking SO_2	Anti-oxidation	05/11/10	50 mg/L
Racking	Remove Sediment	06/07/10	---
Copper Sulfate	Reduce H_2S	06/08/10	0.5 mg/L Cu
Bottling, 750 mL Screw Cap, Green Glass	N-sparged	06/08/10	----

Table 2-4: Lemberger, treatments and additions

Lemberger			
Action or Activity	Reason	Date	Rate
Initial SO_2	Anti-microbial	10/27/09	50 mg/L
Fermaid®	Nutrient	10/28/09	0.25 g/L
Go-Ferm®	Nutrient	10/28/09	0.3 g/L
Add Yeast (ICV-D254®)	Start Fermentation	10/28/09	0.26 g/L
Press	End Maceration	11/05/09	---
Add *O. oeni*	Start MLF	11/05/09	0.2 g/L
Post-MLF SO_2	Stop MLF	12/14/09	60 mg/L
Move to Cold Room(-4° C)	Cold Stabilization	01/05/10	---
Racking	Remove Sediments	06/01/10	---
Add Copper Sulfate	Reduce H_2S	06/02/10	0.5 mg/L Cu
Bottling, 750 mL Screw Cap, green glass	N-sparged	06/03/10	---

Table 2-5: Cornell NYSAES V&B Computer Controlled Fermentation Temperature Profile, 2009.

Stage	Event	Action	Low Limit	High Limit	Time Period (hr)
Pre-inoculation	Crush / Warm up	Bring to 20 C	---	20	12 to20
---	Inoculate	At 20 C	---	---	---
1	Heat to 35 C	---	20	35	12
2	Hold	35 C for 24hr	34	36	24
3	Cool to 30 C	---	25	30	24
4	Until finish	---	20	30	24

Table 2-6: Average Finished Wine Characteristics

Wine	Pinot Noir	Lemberger	Assay Type
Sample date	02/05/2010	02/05/2010	
%ABV	11.5	11.6	Gas chromatograph
Residual Sugars g/L	0.16	0.02	Chemwell enzymatic (glu/fru)
Malic Acid, g/L	0.04	0.06	Chemwell enzymatic
SO_2 free/total mg/L	8.49/37.15	20.35/38.99	FOSS
TA mg/L tartaric acid	5.37	4.96	Auto-titration
pH	3.77	3.61	Auto-titration

Table 2-7: Folin-Ciocalteau Standard Curve

Test Tube	Gallic acid working solution	De-ionized dH2O	Folin-Ciocalteau Reagent	7% Na2CO3 solution
#1 Blank	+0	+2.8 mL	+ 200 µL	+2.0 mL
#2-6 Gallic Acid dilutions	+ 200 µL	+2.6 mL	+ 200 µL	+2.0 mL

Table 2-8: Adams-Harbertson Catechin Standard Curve

Test Tube	Catechin solution additions	Buffer C additions	Ferric Chloride Reagent
#1 Blank	+0	+875 µL	+ 125 µL
#2-8 Catechin dilutions	+ 50, 100, 150, 200, 250, 300, 350 µL	+ 825,775, 725, 675, 625, 575, 525 µL	+ 125 µL per test tube

Table 2-9: Pinot Noir, %-difference from control, Adams-Harbertson & Folin-Ciocalteau assays.

Pinot Noir %-difference from control Assay	Sample date: Volutan (Vol), SR-Terroir (SRT) Average of treatment results, N=2	10/07/09	10/08/09	10/10/09	10/12/09	10/14/09	10/16/09	02/05/10	10/20/11	ave. diff.
Iron Reactive tannins	Vol Initial	113%	63%	258%	-5%	ND	26%	-39%	30%	69%
	Vol Incremental	39%	23%	91%	-105%	ND	-5%	-98%	-15%	-9%
	Vol Final	64%	-10%	44%	-64%	ND	-19%	-75%	-41%	-10%
	SRT Initial	74%	-9%	122%	-51%	ND	-13%	-98%	-31%	4%
	SRT Incremental	50%	-3%	132%	24%	ND	-2%	-98%	-33%	17%
	SRT final	59%	4%	150%	-58%	ND	23%	-98%	24%	13%
LPP	Vol Initial	66%	30%	29%	-51%	ND	68%	-59%	-67%	14%
	Vol Incremental	24%	44%	13%	-47%	ND	64%	-100%	-333%	0%
	Vol Final	51%	2%	-6%	-57%	ND	63%	-73%	-333%	-3%
	SRT Initial	71%	-6%	94%	-72%	ND	76%	-99%	-233%	11%
	SRT Incremental	49%	5%	-26%	-38%	ND	71%	-99%	-433%	-6%
	SRT final	17%	14%	-61%	-83%	ND	64%	-111%	-67%	-27%
SPP	Vol Initial	24%	67%	48%	-5%	ND	-27%	-57%	-14%	9%
	Vol Incremental	19%	47%	44%	-14%	ND	-29%	-87%	2%	-3%
	Vol Final	14%	28%	48%	1%	ND	-31%	-44%	-10%	3%
	SRT Initial	16%	42%	25%	-5%	ND	-22%	-76%	1%	-3%
	SRT Incremental	14%	75%	41%	-10%	ND	-32%	-88%	-4%	0%
	SRT final	8%	54%	38%	-14%	ND	-29%	-80%	-7%	-4%
Monomeric Pigments	Vol Initial	32%	61%	59%	-13%	ND	5%	-13%	4%	22%
	Vol Incremental	11%	41%	45%	-19%	ND	4%	-87%	-6%	-1%
	Vol Final	20%	24%	55%	-20%	ND	10%	-19%	-9%	12%
	SRT Initial	39%	38%	17%	-23%	ND	10%	-76%	25%	1%
	SRT Incremental	73%	54%	52%	-9%	ND	8%	-85%	0%	15%
	SRT final	18%	55%	32%	-20%	ND	1%	-88%	11%	0%
Total Color @pH 4.9	Vol Initial	41%	55%	54%	-14%	ND	13%	-16%	-10%	22%
	Vol Incremental	18%	43%	42%	-20%	ND	11%	-86%	-5%	1%
	Vol Final	29%	20%	49%	-17%	ND	14%	-18%	-15%	13%
	SRT Initial	43%	29%	25%	-22%	ND	18%	-75%	4%	3%
	SRT Incremental	47%	46%	43%	-12%	ND	14%	-86%	-9%	9%
	SRT final	15%	45%	27%	-23%	ND	9%	-87%	-3%	-2%
Folin-Ciocalteau Total Phenolics	Vol Initial	6%	73%	41%	-6%	ND	6%	-20%	-3%	14%
	Vol Incremental	8%	41%	36%	-4%	ND	2%	-14%	5%	11%
	Vol Final	3%	27%	29%	0%	ND	0%	-10%	-12%	5%
	SRT Initial	-1%	35%	20%	-14%	ND	-2%	-18%	-5%	2%
	SRT Incremental	-1%	62%	36%	7%	ND	5%	-14%	-2%	13%
	SRT final	-3%	58%	46%	1%	ND	17%	-8%	10%	17%

Potentially significant differences from control are highlighted.

Table 2-10: Pinot Noir, %-difference from control, direct spectrophotometric & Somers-Boulton assays.

Pinot Noir %-difference from control Assay	Sample date: Volutan (Vol), SR-Terroir (SRT) Average of Treatment Results N=2	0/07/09	10/08/09	10/10/09	10/12/09	10/14/09	10/16/09	02/05/10	10/20/11	Average Diff.
Total Phenolics A^{280} - 4	Vol Initial	101%	100%	49%	-15%	ND	2%	-2%	-2%	33%
	Vol Incremental	96%	72%	49%	-19%	ND	3%	-2%	-3%	28%
	Vol Final	81%	37%	40%	-15%	ND	-3%	-7%	-14%	17%
	SRT Initial	120%	54%	18%	-25%	ND	0%	-2%	-8%	22%
	SRT Incremental	116%	84%	46%	-4%	ND	2%	-2%	-8%	33%
	SRT final	143%	86%	45%	-18%	ND	7%	-1%	2%	38%
Visible Color	Vol Initial	31%	63%	54%	2%	ND	1%	-6%	-11%	19%
	Vol Incremental	14%	38%	41%	-9%	ND	-8%	2%	-7%	10%
	Vol Final	22%	32%	47%	8%	ND	-3%	0%	-11%	14%
	SRT Initial	21%	39%	16%	-14%	ND	-4%	11%	0%	10%
	SRT Incremental	25%	64%	48%	14%	ND	3%	-5%	-9%	20%
	SRT final	7%	60%	42%	0%	ND	7%	-6%	-5%	15%
Color Density	Vol Initial	27%	51%	50%	-13%	ND	0%	-8%	-11%	14%
	Vol Incremental	13%	34%	39%	-21%	ND	-5%	2%	-7%	8%
	Vol Final	17%	22%	44%	-8%	ND	-2%	0%	-12%	9%
	SRT Initial	18%	30%	17%	-23%	ND	-1%	12%	0%	8%
	SRT Incremental	22%	45%	43%	-5%	ND	1%	-9%	-9%	13%
	SRT final	4%	44%	37%	-16%	ND	2%	-10%	-5%	8%
Hue	Vol Initial	-5%	-14%	-5%	-1%	ND	-1%	-4%	2%	-4%
	Vol Incremental	-1%	-8%	-2%	4%	ND	11%	0%	1%	1%
	Vol Final	-6%	-11%	-4%	-2%	ND	2%	-1%	-1%	-3%
	SRT Initial	-4%	-13%	5%	8%	ND	12%	2%	0%	1%
	SRT Incremental	-4%	-22%	-8%	-6%	ND	-3%	-7%	2%	-7%
	SRT final	-4%	-18%	-9%	-6%	ND	-10%	-7%	1%	-8%
Blueness	Vol Initial	30%	38%	44%	-17%	ND	-5%	-11%	-14%	9%
	Vol Incremental	12%	31%	32%	-24%	ND	-9%	5%	-9%	5%
	Vol Final	23%	14%	34%	-11%	ND	-6%	-1%	-14%	6%
	SRT Initial	22%	19%	16%	-21%	ND	-2%	18%	-1%	7%
	SRT Incremental	26%	23%	33%	-13%	ND	-5%	-14%	-11%	5%
	SRT final	1%	30%	29%	-23%	ND	-4%	-14%	-6%	2%
Visible Anthocyanins	Vol Initial	35%	72%	55%	-11%	ND	2%	-1%	-8%	21%
	Vol Incremental	19%	40%	43%	-20%	ND	-8%	5%	-18%	9%
	Vol Final	29%	40%	49%	-4%	ND	-1%	5%	-2%	17%
	SRT Initial	29%	53%	3%	-26%	ND	-5%	10%	4%	10%
	SRT Incremental	27%	79%	47%	-5%	ND	5%	5%	-4%	22%
	SRT final	29%	71%	50%	-9%	ND	11%	5%	7%	23%
Color due to Copigmentation	Vol Initial	-44%	136%	102%	-9%	ND	-1%	75%	2%	37%
	Vol Incremental	281%	73%	75%	-34%	ND	-20%	232%	-8%	86%
	Vol Final	0%	100%	79%	-5%	ND	-9%	225%	-18%	53%
	SRT Initial	-59%	93%	-43%	-33%	ND	-23%	312%	84%	47%
	SRT Incremental	-163%	152%	101%	25%	ND	4%	198%	-27%	41%
	SRT final	-206%	134%	68%	2%	ND	5%	252%	-39%	31%
Polymeric pigments	Vol Initial	28%	40%	48%	-17%	ND	-5%	-15%	-13%	10%
	Vol Incremental	10%	35%	33%	-28%	ND	-9%	-5%	-3%	5%
	Vol Final	17%	11%	38%	-17%	ND	-9%	-7%	-14%	3%
	SRT Initial	17%	3%	15%	-25%	ND	-3%	12%	-1%	2%
	SRT Incremental	24%	24%	50%	9%	ND	-5%	-18%	-11%	10%
	SRT final	-6%	29%	16%	-30%	ND	-7%	-23%	-9%	-4%

Potentially significant differences from control are highlighted.

Table 2-11: Lemberger, %-difference from control, Adams-Harbertson & Folin-Ciocalteau assays.

Lemberger %-difference from control Assay	Sample date: Volutan (Vol), SR-Terroir (SRT) Average of Treatments, N=2	10/27/09	10/29/09	10/31/09	11/02/09	11/05/09	02/05/10	10/20/11	Ave. diff.
Iron Reactive tannins	Vol Initial	-24%	18%	79%	114%	47%	33%	104%	53%
	Vol Incremental	13%	46%	48%	74%	230%	-1%	67%	68%
	Vol Final	2%	45%	8%	0%	7%	-5%	28%	12%
	SRT Initial	-3%	65%	50%	25%	19%	-23%	13%	21%
	SRT Incremental	-2%	7%	24%	80%	18%	-2%	110%	34%
	SRT final	22%	-12%	-43%	19%	71%	7%	8%	10%
LPP	Vol Initial	0%	-1%	61%	87%	38%	36%	73%	42%
	Vol Incremental	-219%	17%	43%	42%	14%	14%	45%	-6%
	Vol Final	73%	44%	1%	-38%	-20%	26%	-5%	12%
	SRT Initial	5%	33%	4%	-4%	29%	4%	-11%	9%
	SRT Incremental	-197%	-13%	33%	62%	-162%	46%	56%	-25%
	SRT final	127%	5%	-15%	128%	9%	89%	-19%	46%
SPP	Vol Initial	7%	23%	-6%	18%	3%	13%	9%	9%
	Vol Incremental	216%	20%	-4%	7%	-8%	-1%	2%	33%
	Vol Final	45%	28%	-13%	7%	-19%	-20%	-10%	3%
	SRT Initial	23%	55%	-15%	7%	-5%	-8%	-5%	7%
	SRT Incremental	216%	9%	3%	16%	55%	4%	2%	44%
	SRT final	59%	-4%	-7%	25%	-13%	-12%	-9%	6%
Monomeric Pigments	Vol Initial	6%	17%	-4%	31%	7%	34%	29%	17%
	Vol Incremental	22%	42%	3%	13%	1%	12%	9%	15%
	Vol Final	-6%	25%	-15%	-8%	-18%	-20%	-10%	-8%
	SRT Initial	14%	41%	-17%	1%	2%	-2%	-5%	5%
	SRT Incremental	33%	4%	-5%	23%	13%	5%	18%	13%
	SRT final	-84%	-11%	-19%	0%	-13%	-12%	-3%	-20%
Total Color at pH 4.9	Vol Initial	5%	16%	-1%	32%	8%	29%	27%	17%
	Vol Incremental	19%	34%	4%	13%	0%	9%	11%	13%
	Vol Final	34%	28%	-14%	-7%	-19%	-15%	-9%	0%
	SRT Initial	15%	44%	-16%	2%	2%	-3%	-6%	5%
	SRT Incremental	29%	3%	-1%	24%	12%	10%	17%	13%
	SRT final	25%	-7%	-16%	16%	-12%	-1%	-8%	-1%
Folin-Ciocalteau Total Phenolics	Vol Initial	9%	17%	10%	22%	20%	14%	18%	16%
	Vol Incremental	8%	45%	0%	4%	14%	5%	2%	11%
	Vol Final	11%	44%	6%	11%	21%	1%	13%	15%
	SRT Initial	11%	-3%	-3%	24%	12%	10%	16%	10%
	SRT Incremental	2%	34%	-2%	-2%	-5%	-4%	4%	4%
	SRT final	-1%	12%	-11%	3%	6%	-4%	-1%	1%

Potentially significant differences from control are highlighted.

Table 2-12: Lemberger, %-difference from control, Somers-Boulton & direct spectrophotometric assays

Lemberger %-difference from control Assay	Sample date: Volutan (Vol) SR-Terroir (SRT) Average ofTreatments, N=2	10/27/09	10/29/09	10/31/09	11/02/09	11/05/09	02/05/10	10/20/11	Average Diff.
Total Phenolics A^{280}-4	Vol Initial	22%	20%	15%	30%	13%	24%	23%	21%
	Vol Incremental	32%	49%	7%	6%	1%	7%	3%	15%
	Vol Final	14%	49%	6%	20%	10%	11%	10%	17%
	SRT Initial	37%	-9%	-7%	19%	10%	14%	18%	12%
	SRT Incremental	20%	29%	-3%	-4%	-11%	-6%	2%	4%
	SRT final	-5%	3%	-3%	3%	-4%	1%	-2%	-1%
Visible Color A^{520}	Vol Initial	6%	18%	-11%	1%	0%	10%	56%	12%
	Vol Incremental	8%	38%	1%	1%	2%	4%	7%	8%
	Vol Final	24%	37%	-1%	-2%	-5%	-9%	-9%	5%
	SRT Initial	3%	122%	-1%	-6%	-3%	-1%	-15%	14%
	SRT Incremental	21%	0%	0%	1%	1%	7%	14%	6%
	SRT final	17%	-3%	-2%	-1%	-5%	-8%	-9%	-2%
Color Density	Initial	3%	15%	4%	16%	5%	16%	26%	12%
	Incremental	4%	32%	2%	8%	9%	5%	9%	10%
	Vol Final	20%	36%	-9%	-3%	-10%	-9%	-8%	3%
	SRT Initial	11%	57%	-3%	1%	-2%	0%	-6%	8%
	SRT Incremental	15%	2%	0%	14%	4%	12%	15%	9%
	SRT final	15%	-2%	-7%	0%	-8%	-2%	-7%	-1%
Hue	Vol Initial	-4%	-5%	6%	32%	10%	11%	2%	8%
	Vol Incremental	-5%	-7%	4%	17%	14%	3%	4%	4%
	Vol Final	-8%	-3%	-16%	0%	-13%	-1%	3%	-5%
	SRT Initial	-5%	-2%	-7%	6%	-3%	1%	4%	-1%
	SRT Incremental	-8%	3%	1%	29%	7%	8%	2%	6%
	SRT final	-6%	3%	-11%	3%	-8%	11%	3%	-1%
Blueness A^{620}	Vol Initial	2%	12%	11%	42%	15%	26%	34%	20%
	Vol Incremental	2%	28%	5%	19%	26%	9%	14%	15%
	Vol Final	47%	52%	-22%	-5%	-19%	-5%	-7%	6%
	SRT Initial	15%	87%	-10%	5%	-6%	2%	-5%	13%
	SRT Incremental	19%	10%	4%	36%	11%	25%	21%	18%
	SRT final	45%	1%	-13%	4%	-11%	16%	-7%	5%
Visible Anthocyanins	Vol Initial	21%	19%	0%	-9%	-2%	4%	24%	8%
	Vol Incremental	47%	44%	-1%	-3%	-4%	1%	2%	12%
	Vol Final	42%	33%	4%	-4%	-3%	-10%	-10%	8%
	SRT Initial	36%	45%	2%	-4%	0%	-1%	-7%	10%
	SRT Incremental	62%	-3%	-3%	-8%	-2%	1%	12%	8%
	SRT final	30%	-4%	-1%	-4%	-4%	-16%	-5%	-1%
Copigmentation color	Vol Initial	-135%	46%	-17%	-72%	11%	-40%	45%	-23%
	Vol Incremental	-143%	80%	-22%	-33%	-6%	-73%	-38%	-34%
	Vol Final	-155%	47%	42%	-4%	61%	32%	-27%	0%
	SRT Initial	-133%	64%	11%	-16%	3%	-25%	-5%	-15%
	SRT Incremental	-159%	4%	-17%	-60%	-26%	-19%	-24%	-43%
	SRT final	-184%	7%	27%	-9%	43%	6%	41%	-10%
Polymeric pigments	Vol Initial	-1%	14%	4%	46%	12%	25%	23%	17%
	Vol Incremental	-11%	14%	7%	17%	27%	10%	12%	11%
	Vol Final	16%	53%	-21%	6%	-16%	-7%	-8%	3%
	SRT Initial	5%	90%	-9%	10%	-6%	0%	-9%	12%
	SRT Incremental	1%	12%	9%	41%	10%	23%	16%	16%
	SRT final	10%	2%	-6%	12%	-9%	15%	-13%	2%

Table 2-13: Calculations, direct spectrophotometry, Somers-Boulton & Adams-Harbertson methods

Spectrometric Color and Phenolic Content Calculations (Sources: Jacobson, 2006; Ribereau-Gayon, 2006; Somers, 1977, Harbertson 2003)				
Color Fraction	P.L. mm	Abs. λ nm	Calculations	Comments
Total Phenolics (corrected A^{280}-4)	1	280	$10(A^{280})$-4	AU 280 Corrected for 10 mm path length (P.L.);'4'=Absorbance (Abs.) from non-phenolic material
Visible Red Color	10	520	A^{520}	Red component visible color
Yellow-Brown color	10	420	A^{420}	Yellow-brown component of color
Blueness	10	620	A^{620}	Blue component of visible color
Color Density	10	420 520 620	$A^{420} + A^{520} + A^{620}$	Intensity and total amount of color
Hue	10	420 520	A^{420} / A^{520}	Purity of color; development towards orange
SO_2 Resistant Polymeric Color	10	520	$A^{520}{}_{SO_2}$	Visible color due to large and small polymeric pigments
Anthocyanins without Copigmentation (corrected $A^{520}{}_{buffer}$)	10	520	$A^{520}{}_{buffer}$ x 20	Corrected for dilution, disrupts copigmentation stacks, anthocyanin color only
Anthocyanins (SO_2 bleachable)	10	520	$A^{520}{}_{Acet.}$	Color due to all monomeric anthocyanins (free + SO2 bound)
Color due to Copigmentation Effect	10	520	A^{520}- $20(A^{520}{}_{buffer})$	Color enhancement due to copigmentation effects; Visible color - corrected $A^{520}{}_{buffer}$
Visible Anthocyanins	10	520	$A^{520} - A^{520}{}_{SO_2}$	Ionized (colored) Anthocyanin fraction, including copigmentation
Iron reactive tannins	10	510	$A^{510}{}_{Final}$ - ($A^{510}{}_{Background}$ x 0.875)	$A^{510}{}_{Final}$ = post-Ferric Chloride addition
LPPs	10	520	$A^{520}{}_{B} - A^{520}{}_{C}$	post-bleaching samples
SPPs	10	520	$A^{520}{}_{C}$	Supernatant post-bleaching
Monomeric pigments	10	520	$A^{520}{}_{A} - A^{520}{}_{B}$	Buffered minus bleached sample
Total Color @ pH 4.9	10	520	$A^{520}{}_{A}$	Total polymeric pigments

Table 14: Sensory Panel Results, Hedonic Preference (liking) vs. Control

Paired Samples Test											
		Pairs tested		Paired Differences			95% Confidence Interval of the Difference				
Wine	Treatment	Control	Treatment	Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	Sig. 2-tailed
Pinot	Volutan Initial	9-70	9-73	.375	2.066	.730	-1.352	2.102	.513	7	.623
	Volutan incremental		9-77	2.125	1.356	.479	.991	3.259	4.432	7	.003
	Volutan Final		9-72	.125	1.553	.549	-1.173	1.423	.228	7	.826
	SRT Initial		9-75	3.000	2.268	.802	1.104	4.896	3.742	7	.007
	SRT incremental		9-79	.250	1.581	.559	-1.072	1.572	.447	7	.668
	SRT Final		9-82	1.375	2.200	.778	-.464	3.214	1.768	7	.120
Lemberger	Volutan initial	9-213	9-215	-.125	1.553	.549	-1.423	1.173	-.228	7	.826
	Volutan incremental		9-219	.875	2.100	.743	-.881	2.631	1.178	7	.277
	Volutan final		9-223	1.000	1.512	.535	-.264	2.264	1.871	7	.104
	SRT initial		9-217	.750	1.581	.559	-.572	2.072	1.342	7	.222
	SRT incremental		9-221	1.375	1.768	.625	-.103	2.853	2.200	7	.064
	SRT final		9-225	1.000	1.512	.535	-.264	2.264	1.871	7	.104

Table 2-15: Sensory Panel Results, Color Preference vs. Control

One-Sample Test									
Color Preference Control vs. Sample				Test Value = 0					
								95% Confidence Interval of the Difference	
Wine	Treatment	Control	Sample ID	t	df	Sig. 2-tailed	Mean Difference	Lower	Upper
Pinot	Volutan Initial	9-70	9-73	-2.646	7	.033	-.500	-.95	-.05
	Volutan incremental		9-77	-4.965	7	.002	-1.125	-1.66	-.59
	Volutan Final		9-72	-5.292	7	.001	-1.000	-1.45	-.55
	SRT Initial		9-75	-.683	7	.516	-.250	-1.12	.62
	SRT incremental		9-79	-5.000	7	.002	-1.250	-1.84	-.66
	SRT Final		9-82	-1.821	7	.111	-.750	-1.72	.22
Lemberger	Volutan initial	9-213	9-215	.893	7	.402	.375	-.62	1.37
	Volutan incremental		9-219	.000	7	1.000	.000	-.77	.77
	Volutan final		9-223	-.357	7	.732	-.125	-.95	.70
	SRT initial		9-217	-1.488	7	.180	-.625	-1.62	.37
	SRT incremental		9-221	3.416	7	.011	1.250	.38	2.12
	SRT final		9-225	-1.667	7	.140	-.625	-1.51	.26

Figures

General

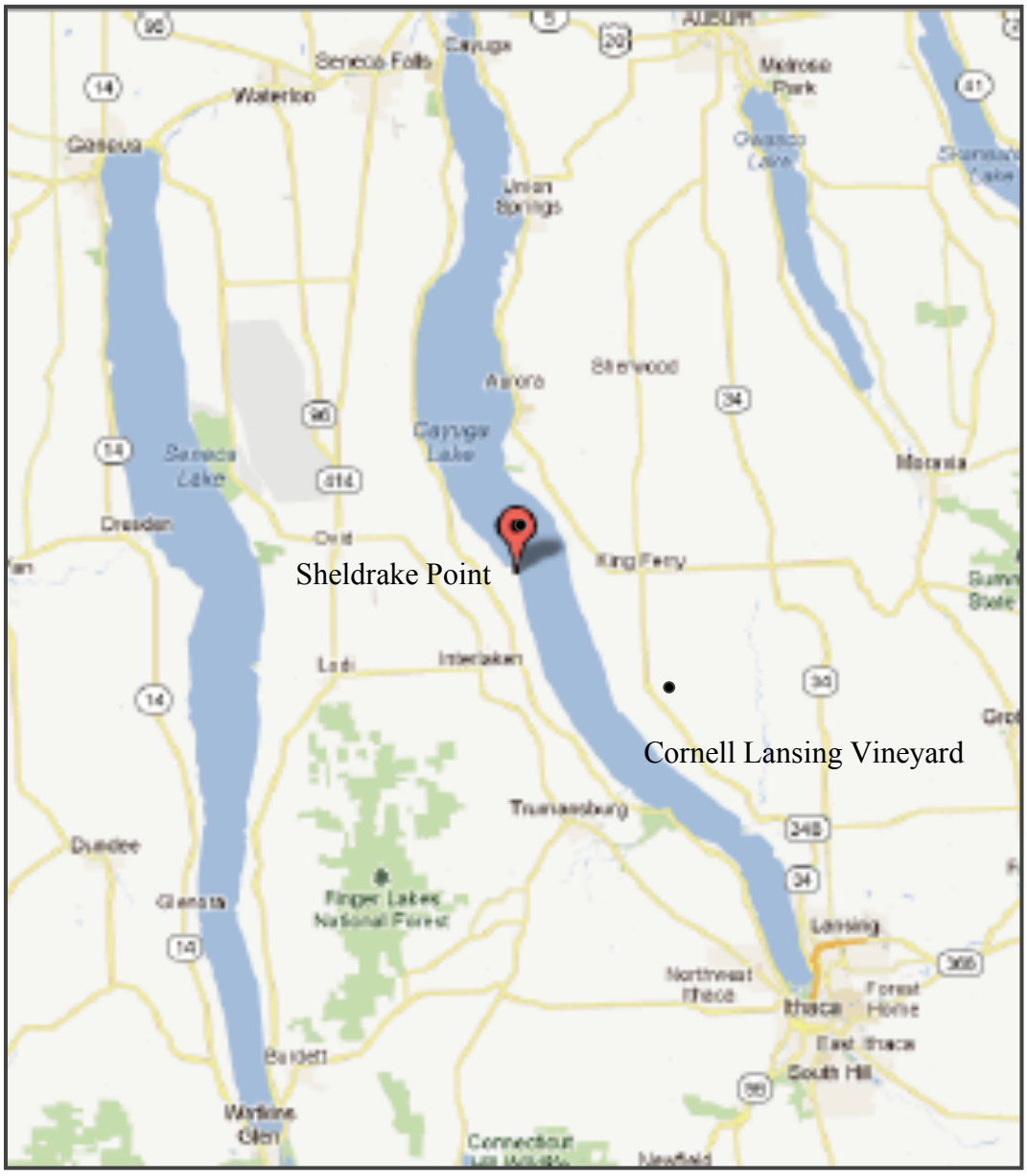

Figure 2–1

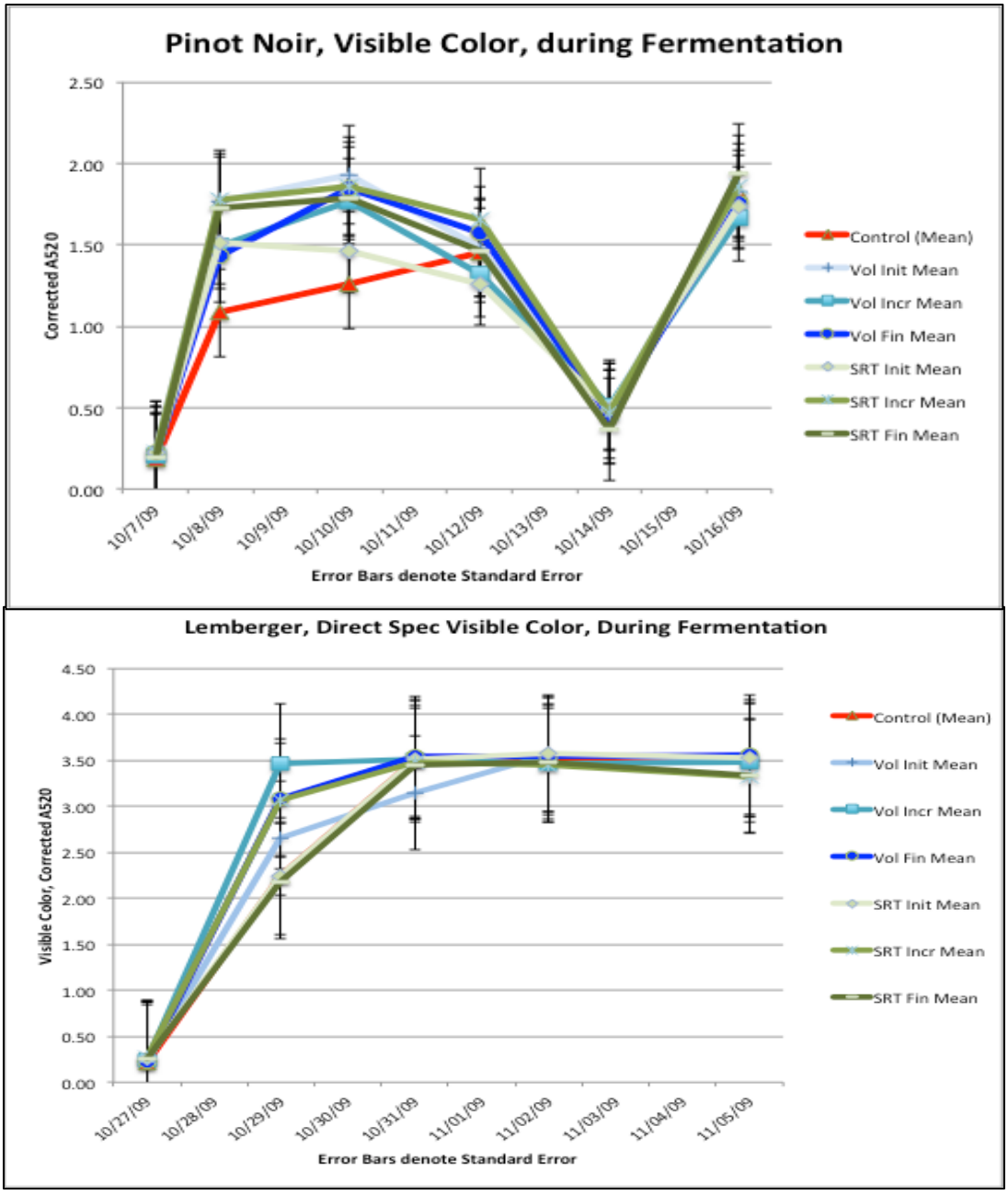

Figure 2–2: Differences between Pinot Noir and Lemberger fermentations

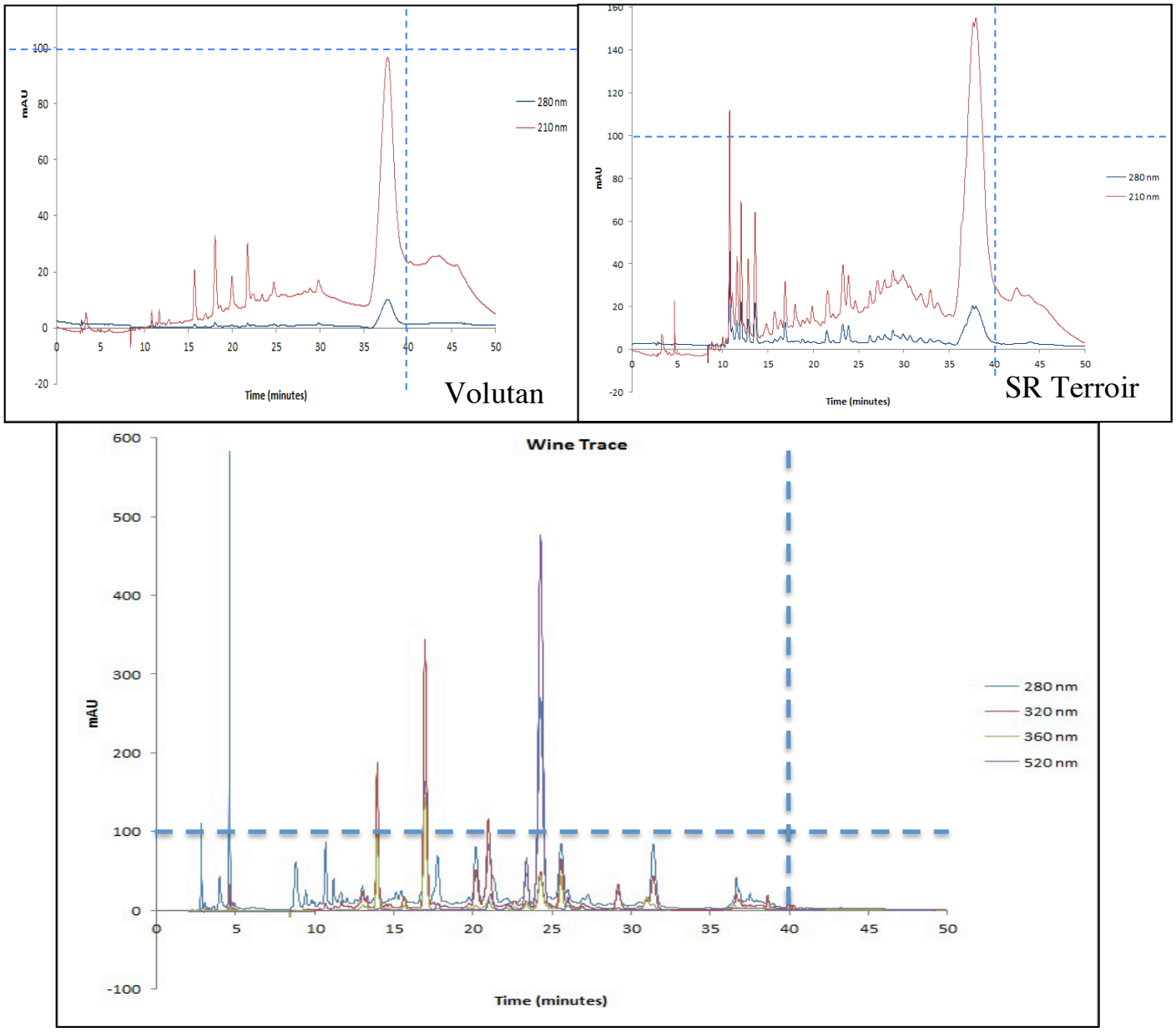

Figure 2–3: HPLC Trace Comparison

Pinot Noir

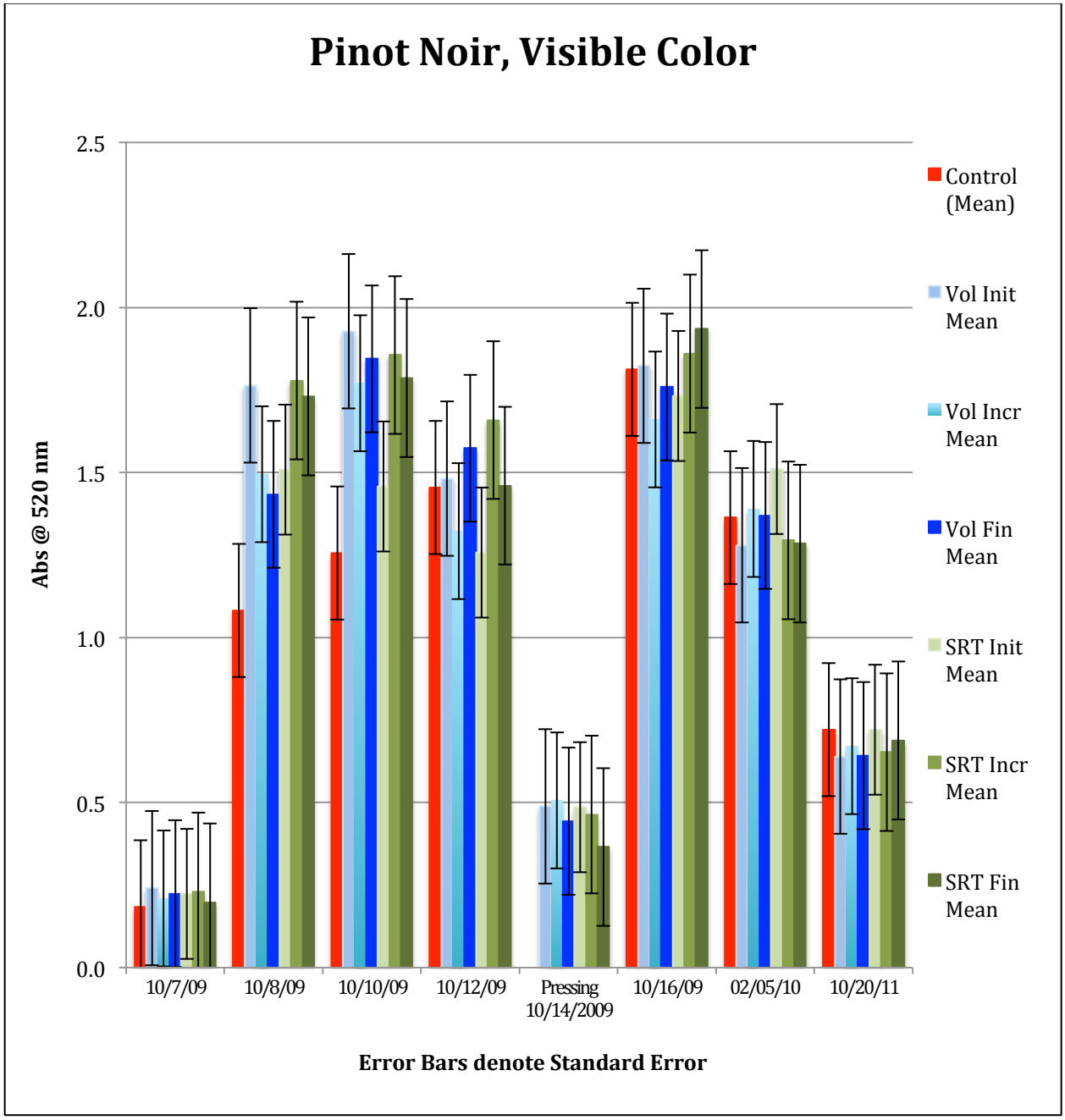

Figure 2-P–1: Pinot Noir, visible color

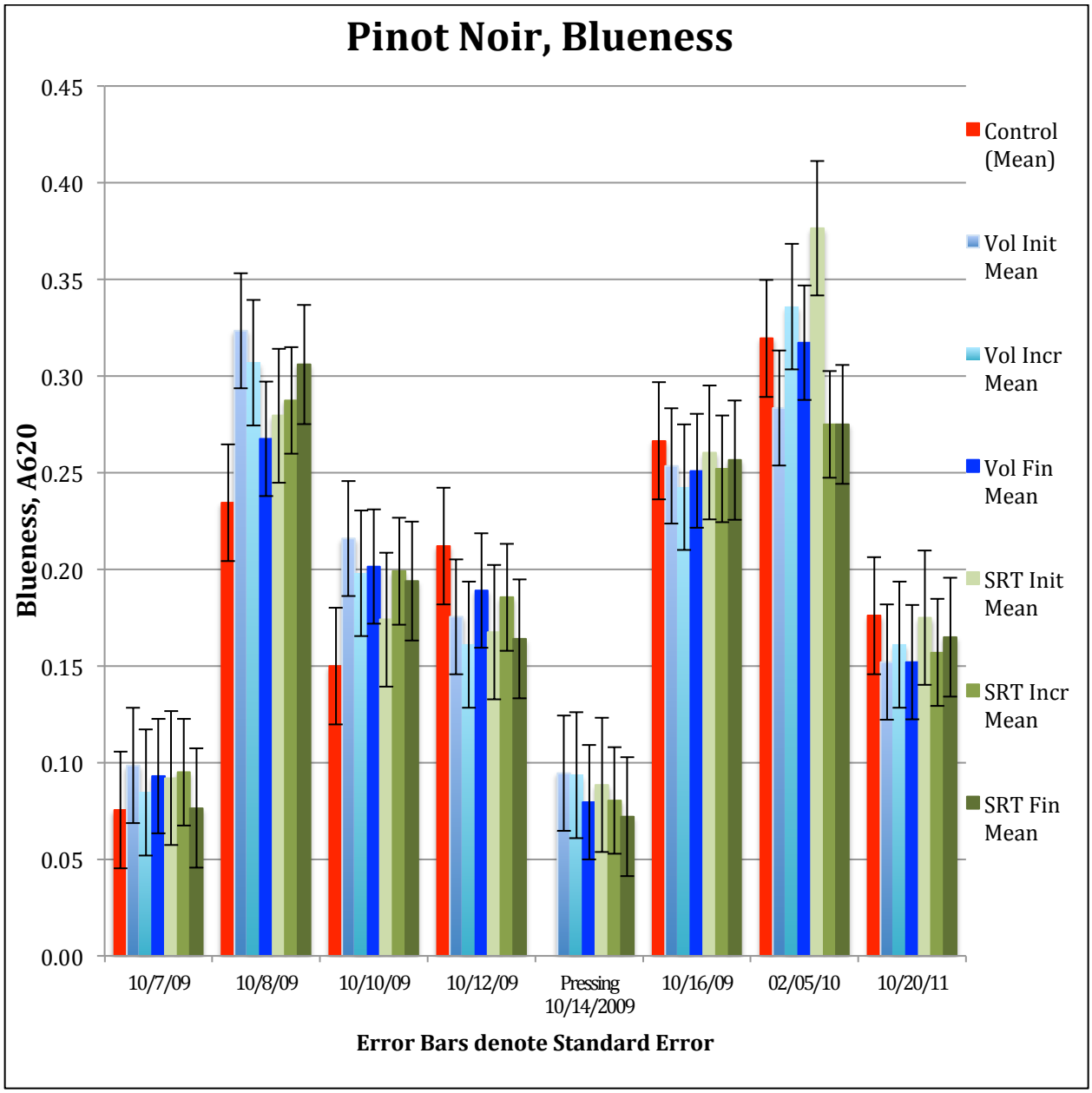

Figure 2-P–2: Pinot Noir, blueness

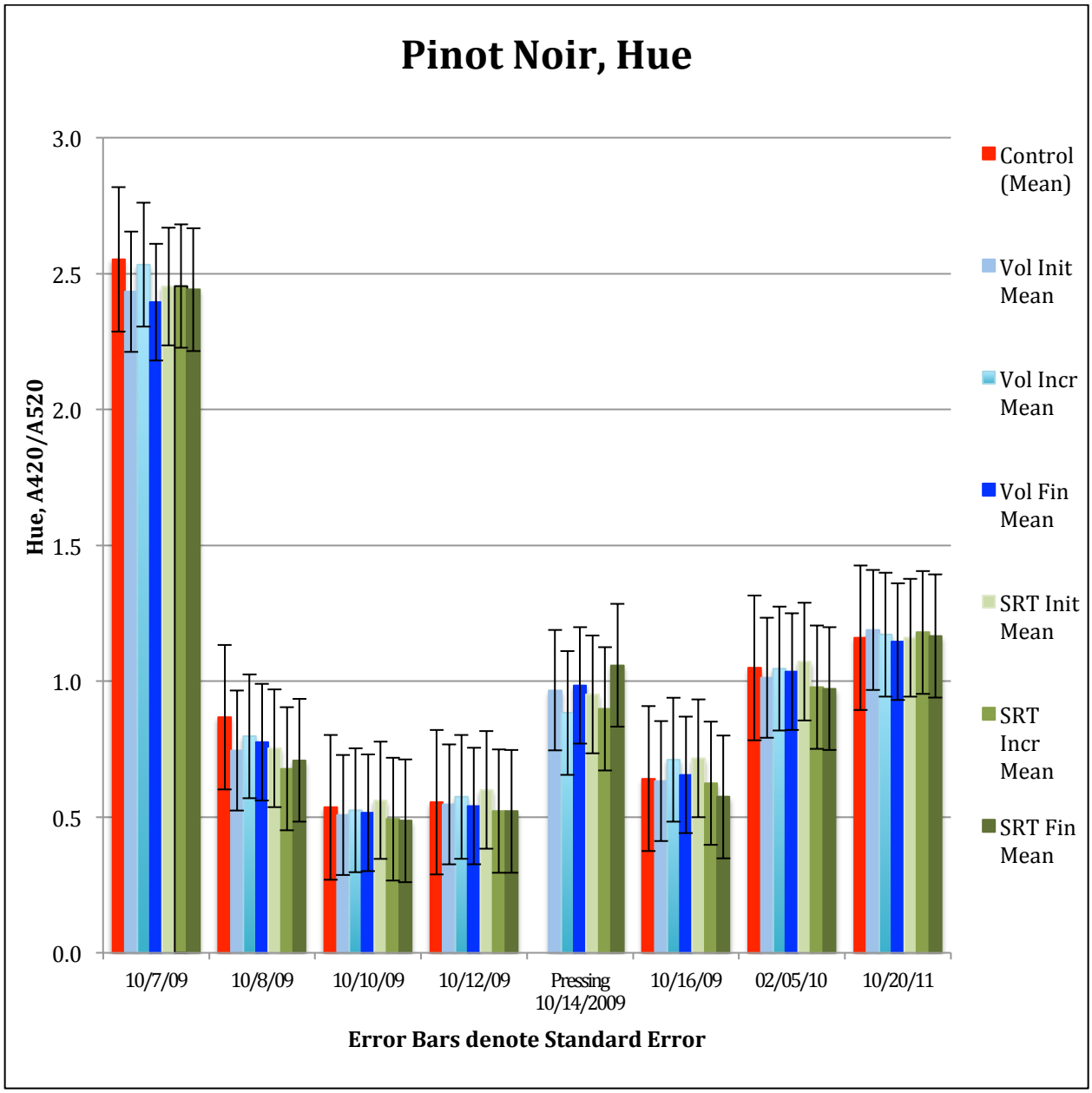

Figure 2-P–3: Pinot Noir, hue

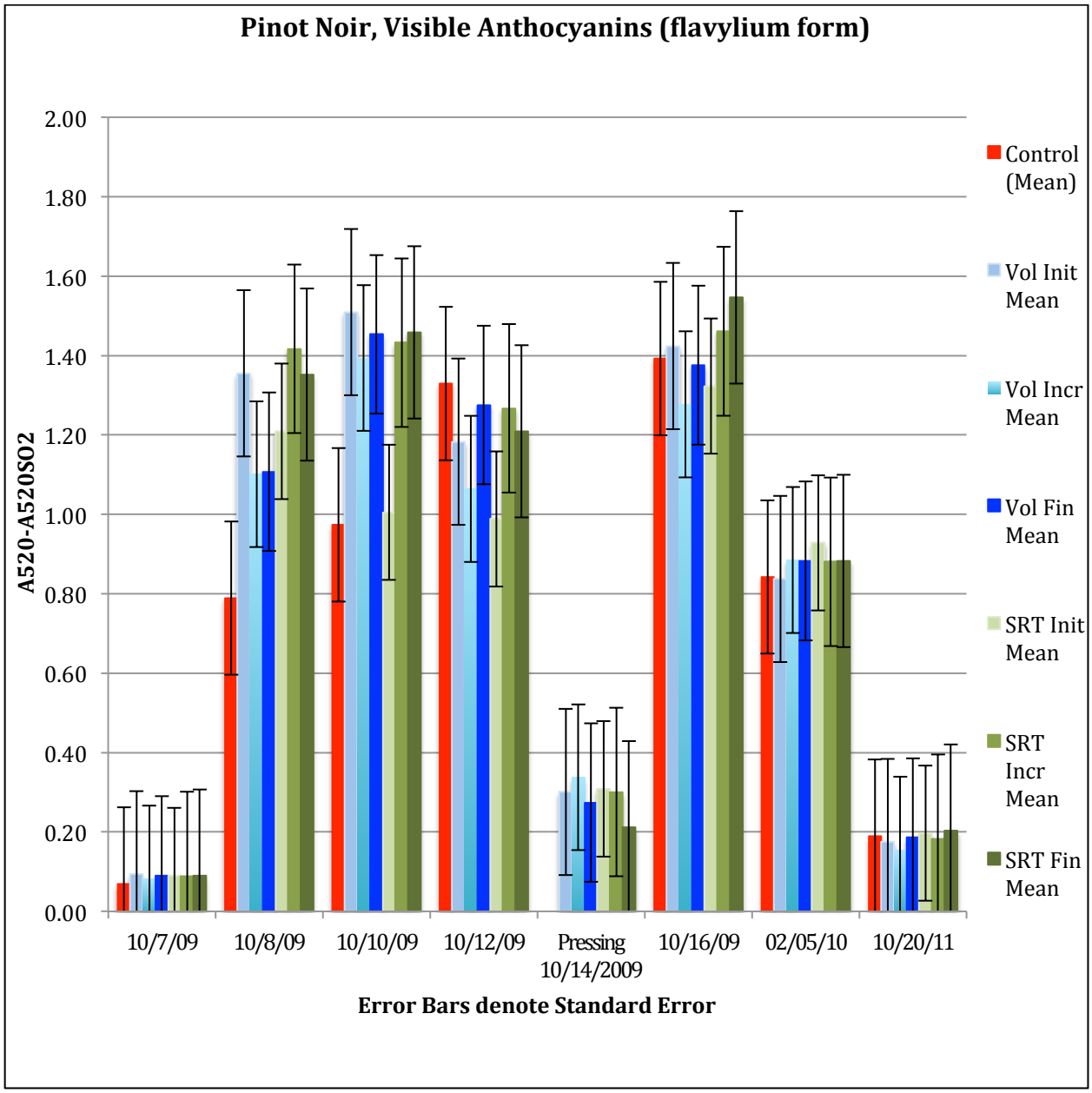

Figure 2-P–4: Pinot Noir, Visible Anthocyanins

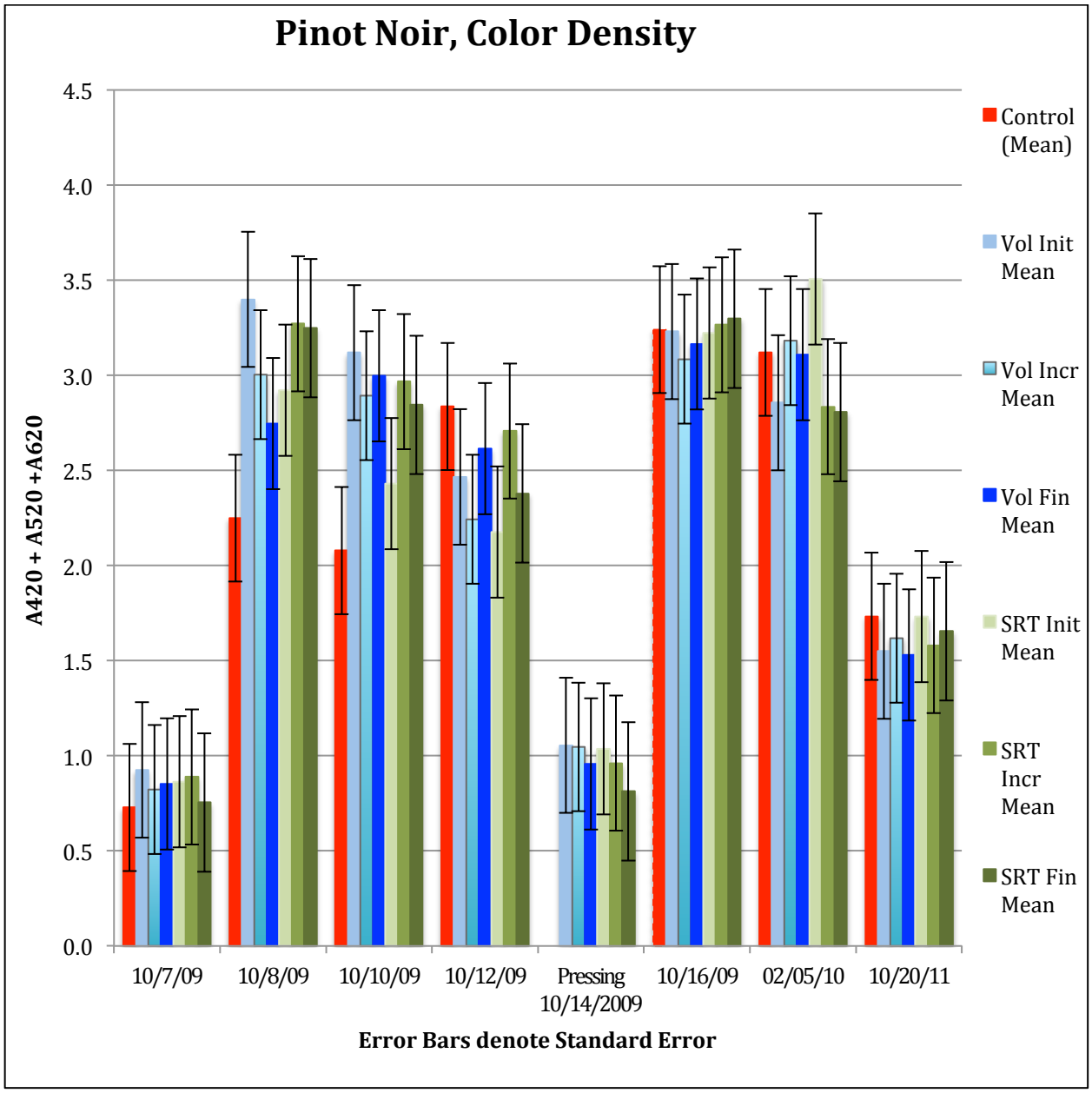

Figure 2-P-5: Pinot Noir, color density

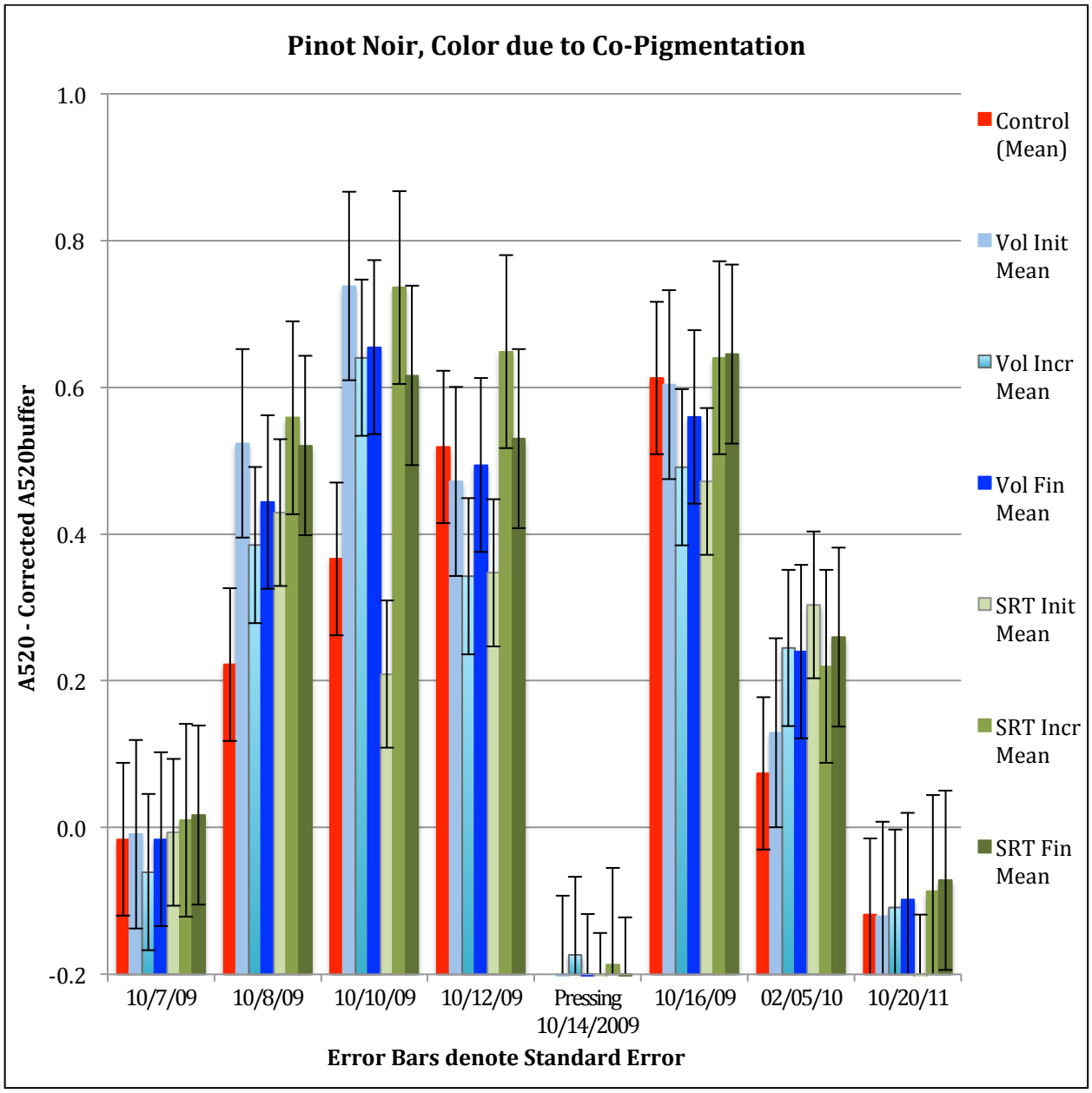

Figure 2-P–6: Pinot Noir, color due to copigmentation

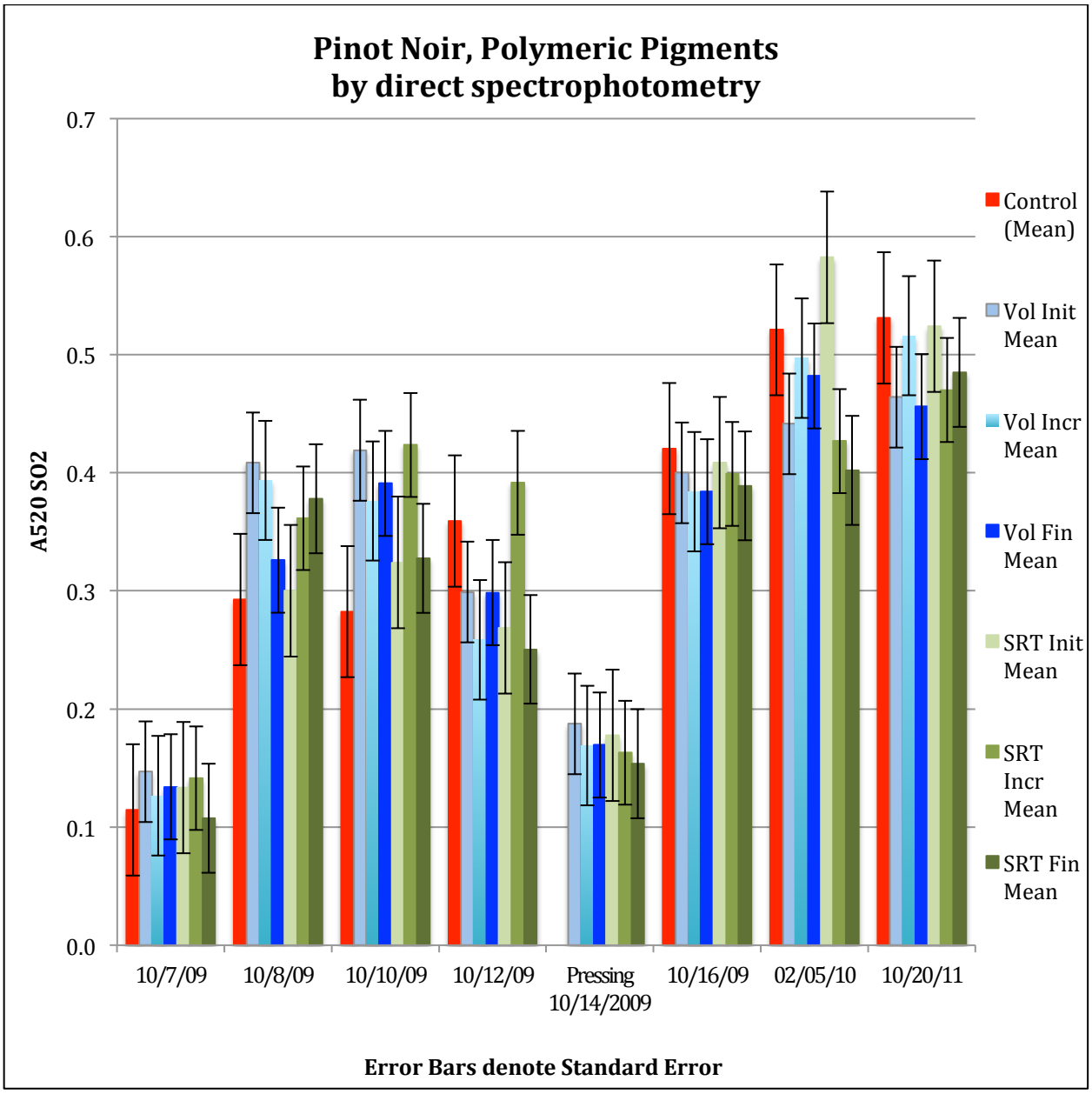

Figure 2-P–7: Pinot Noir, polymeric pigments

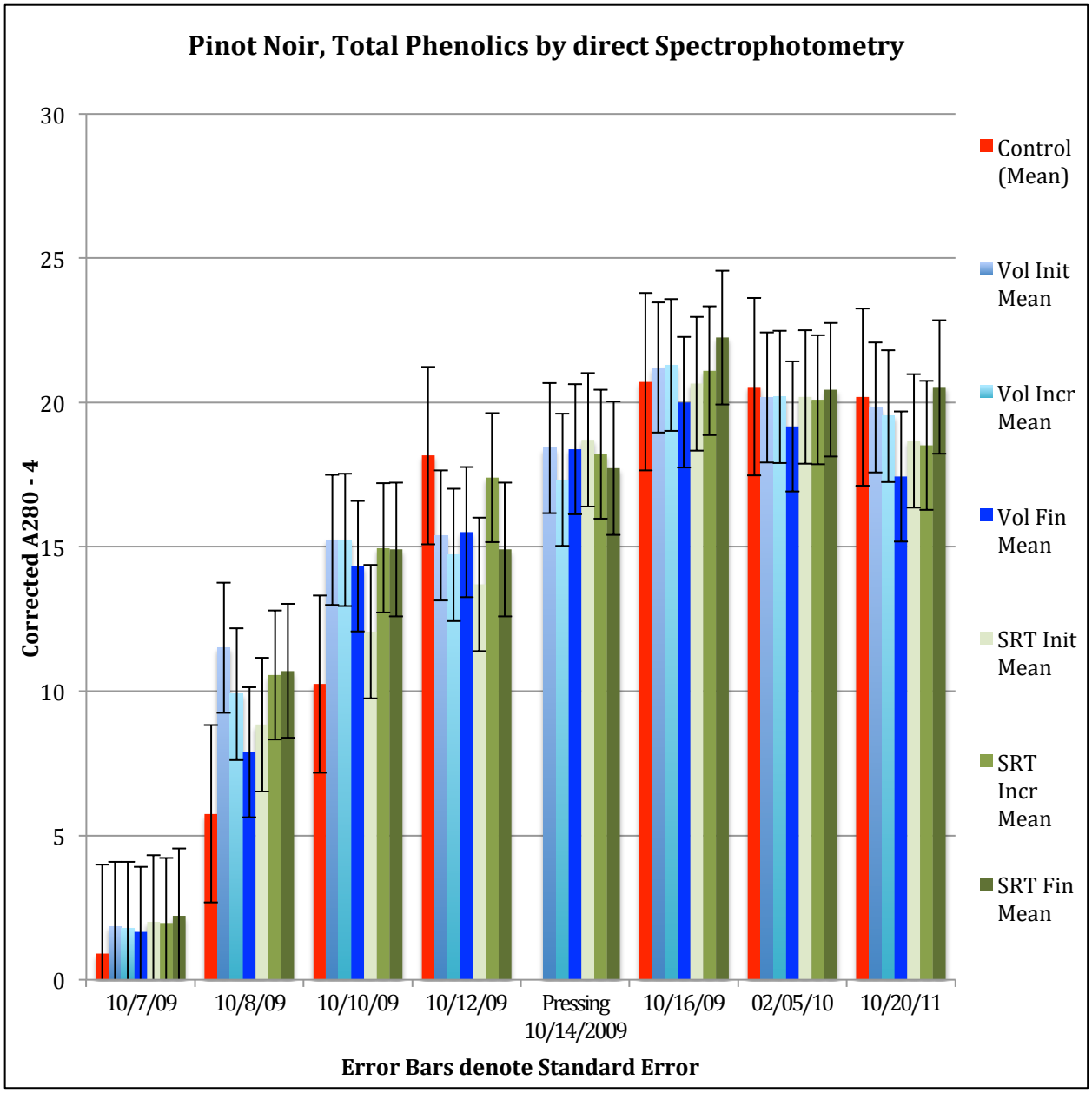

Figure 2-P-8: Pinot Noir, total phenolics by direct spectrophotometry

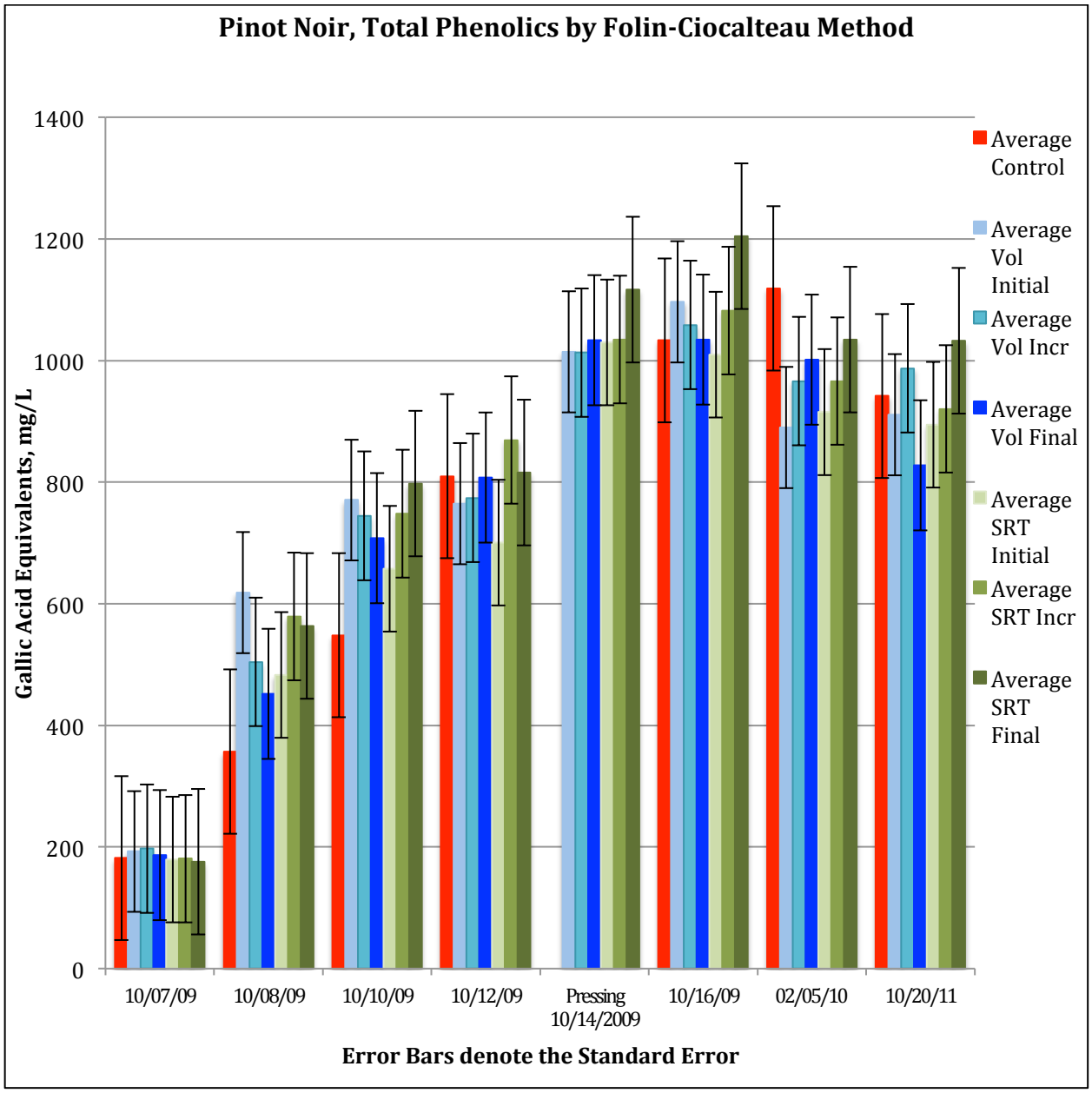

Figure 2-P–9:Pinot Noir, Total Phenolics by Folin-Ciocalteau

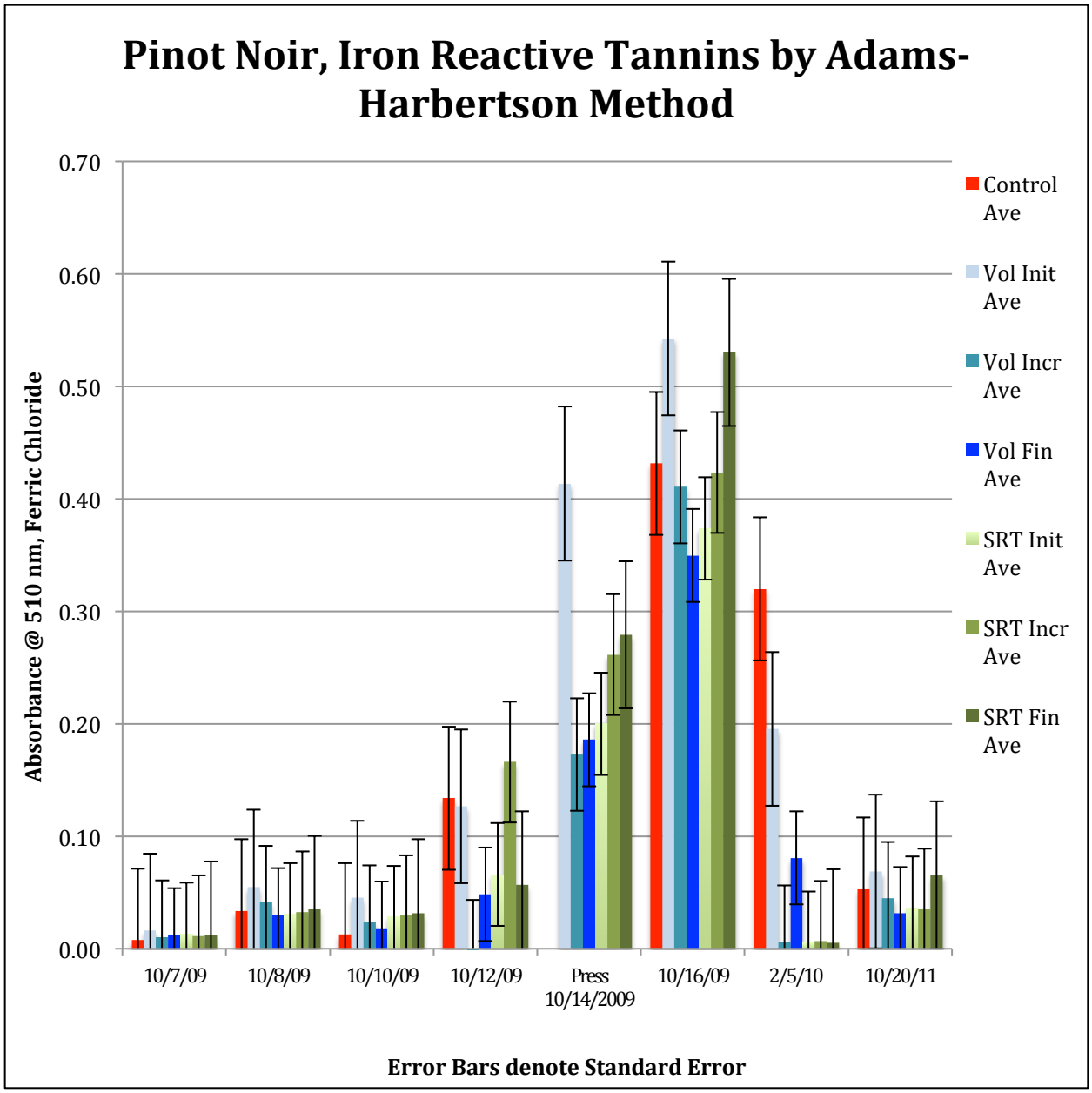

Figure 2-P–10: Pinot Noir, Iron-reactive tannins

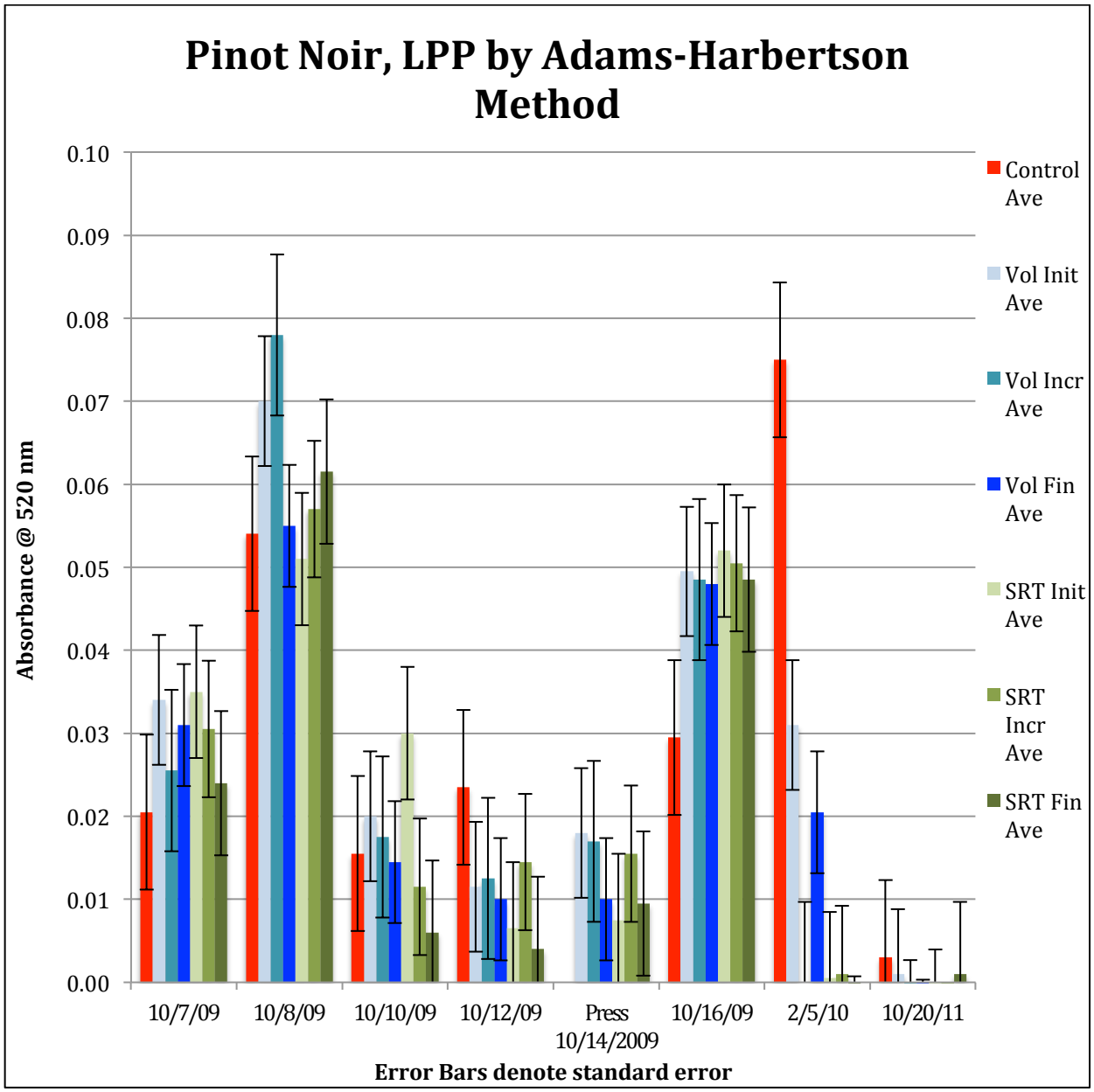

Figure 2-P–11: Pinot Noir, large polymeric pigments

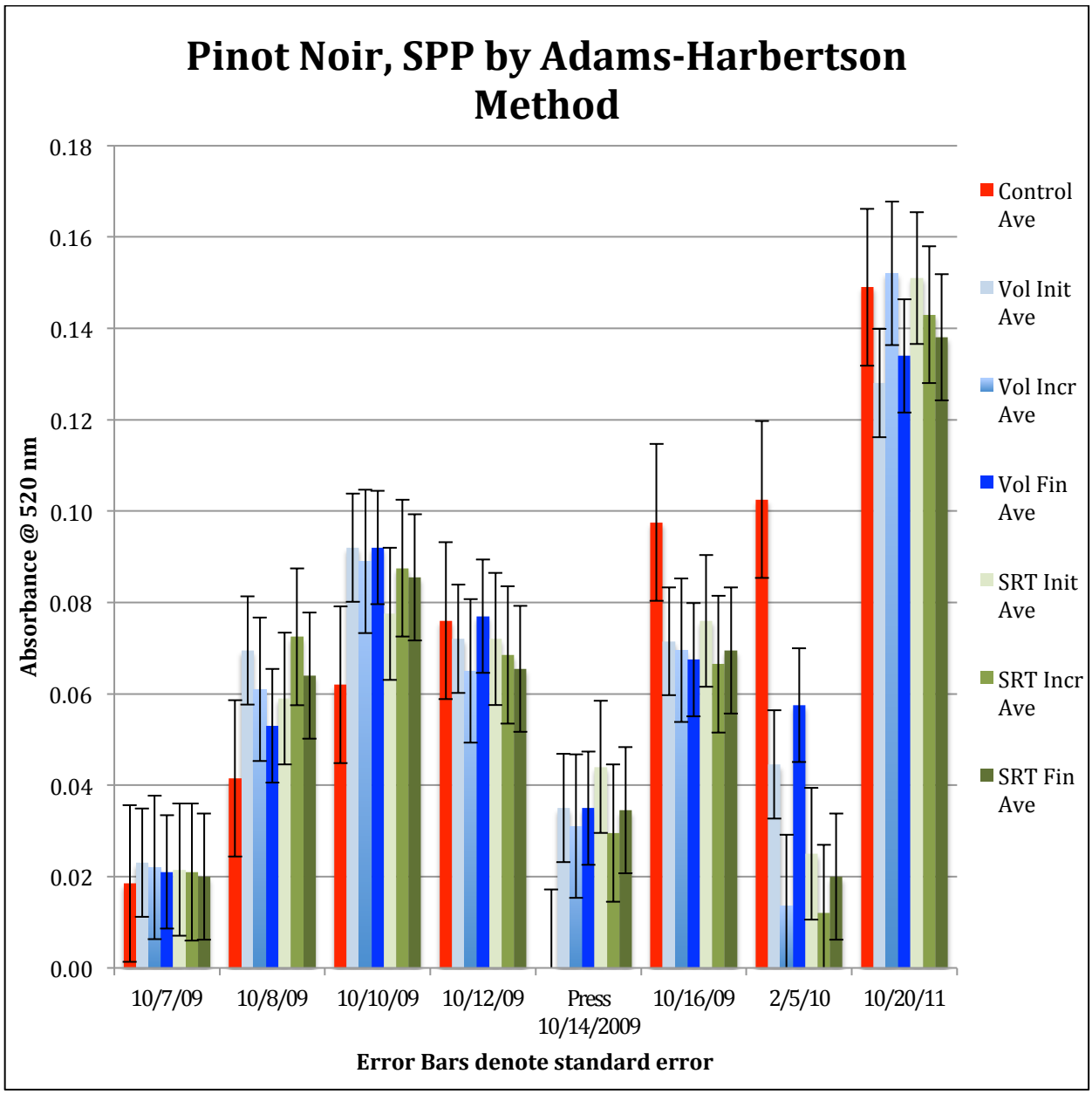

Figure 2-P–12: Pinot Noir, small polymeric pigments

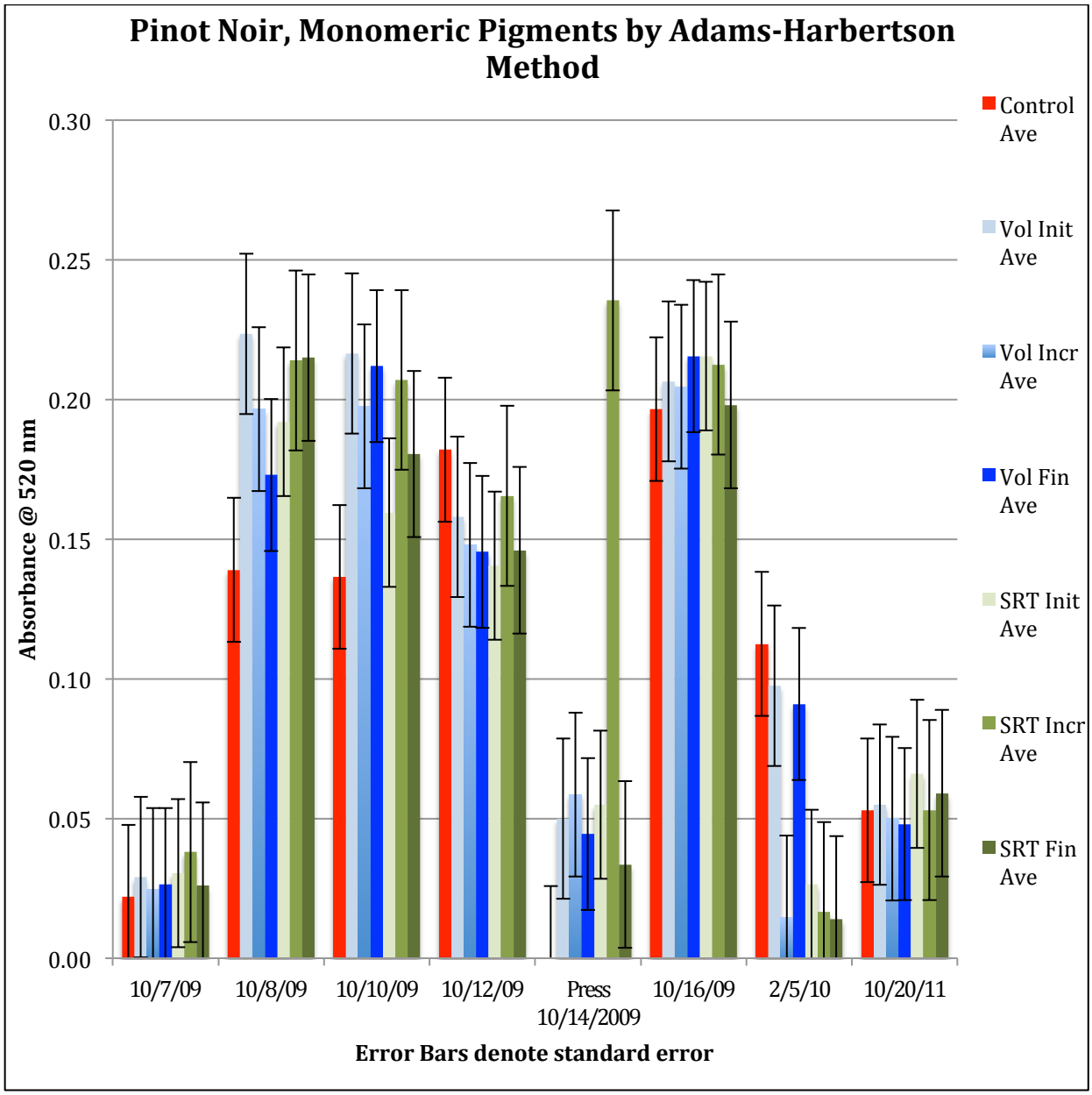

Figure 2-P–13: Pinot Noir, monomeric pigments

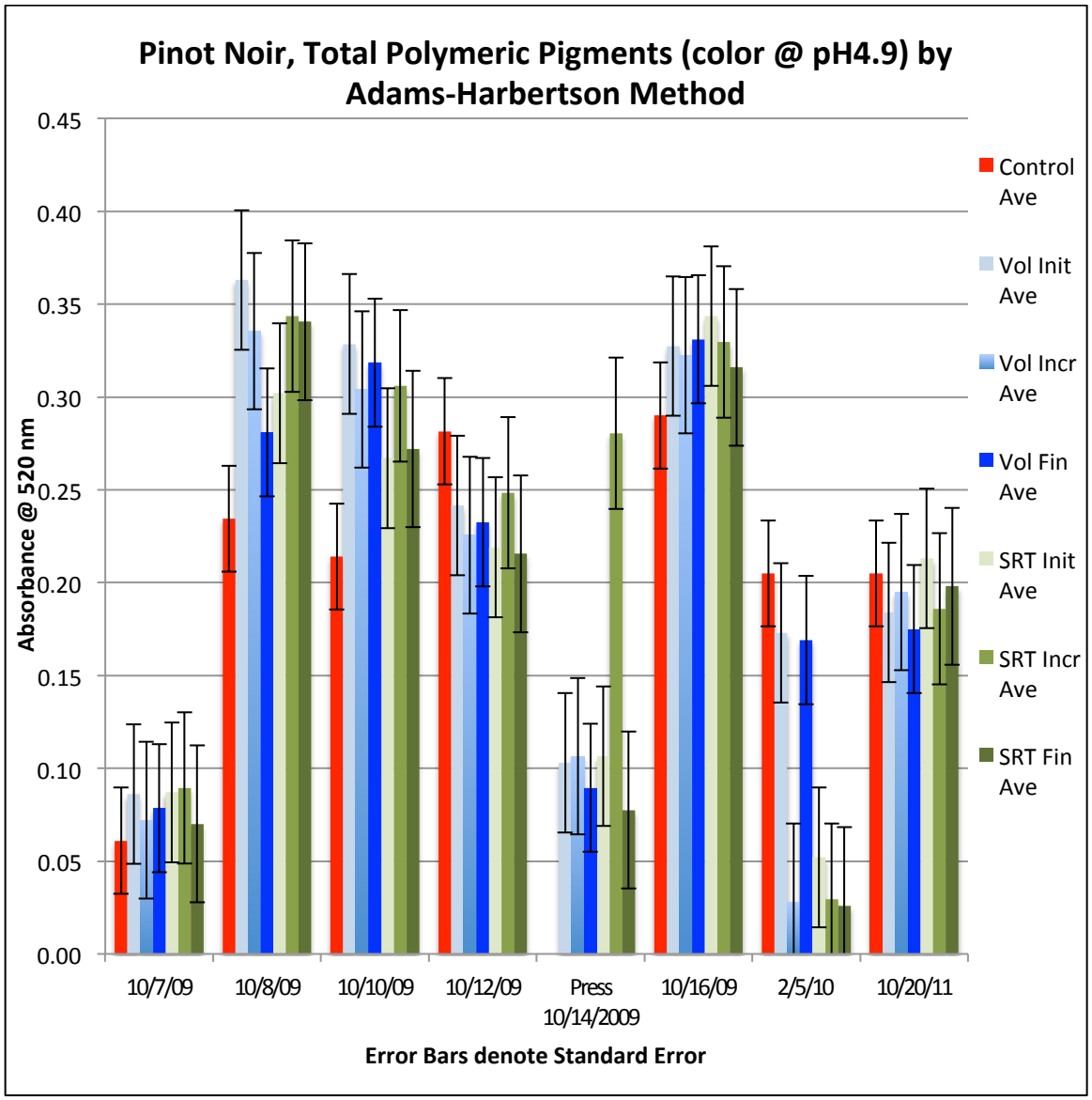

Figure 2-P–14: Pinot Noir, total polymeric pigments (color at pH 4.9)

Lemberger

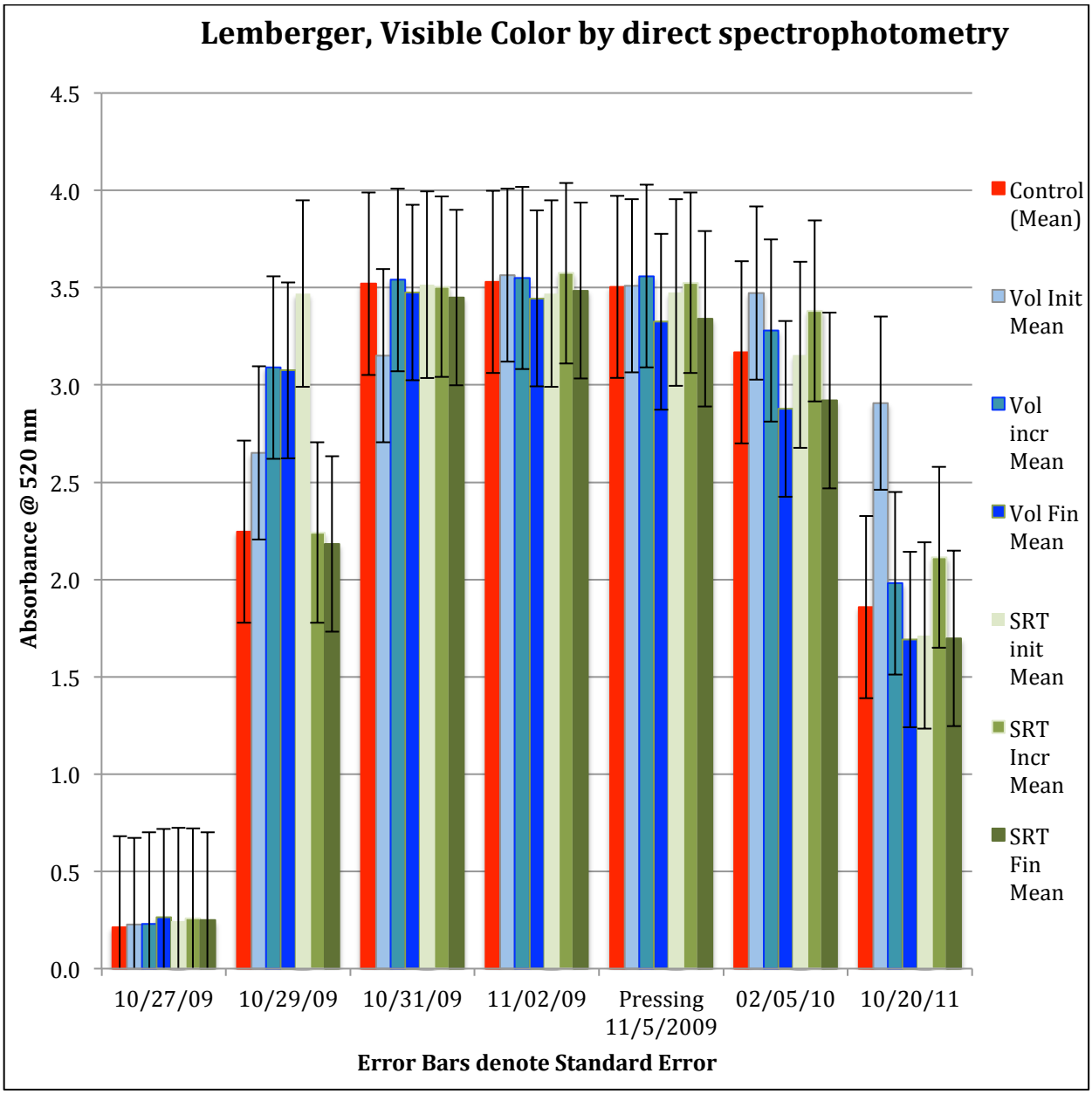

Figure 2-L-1: Lemberger, visible color

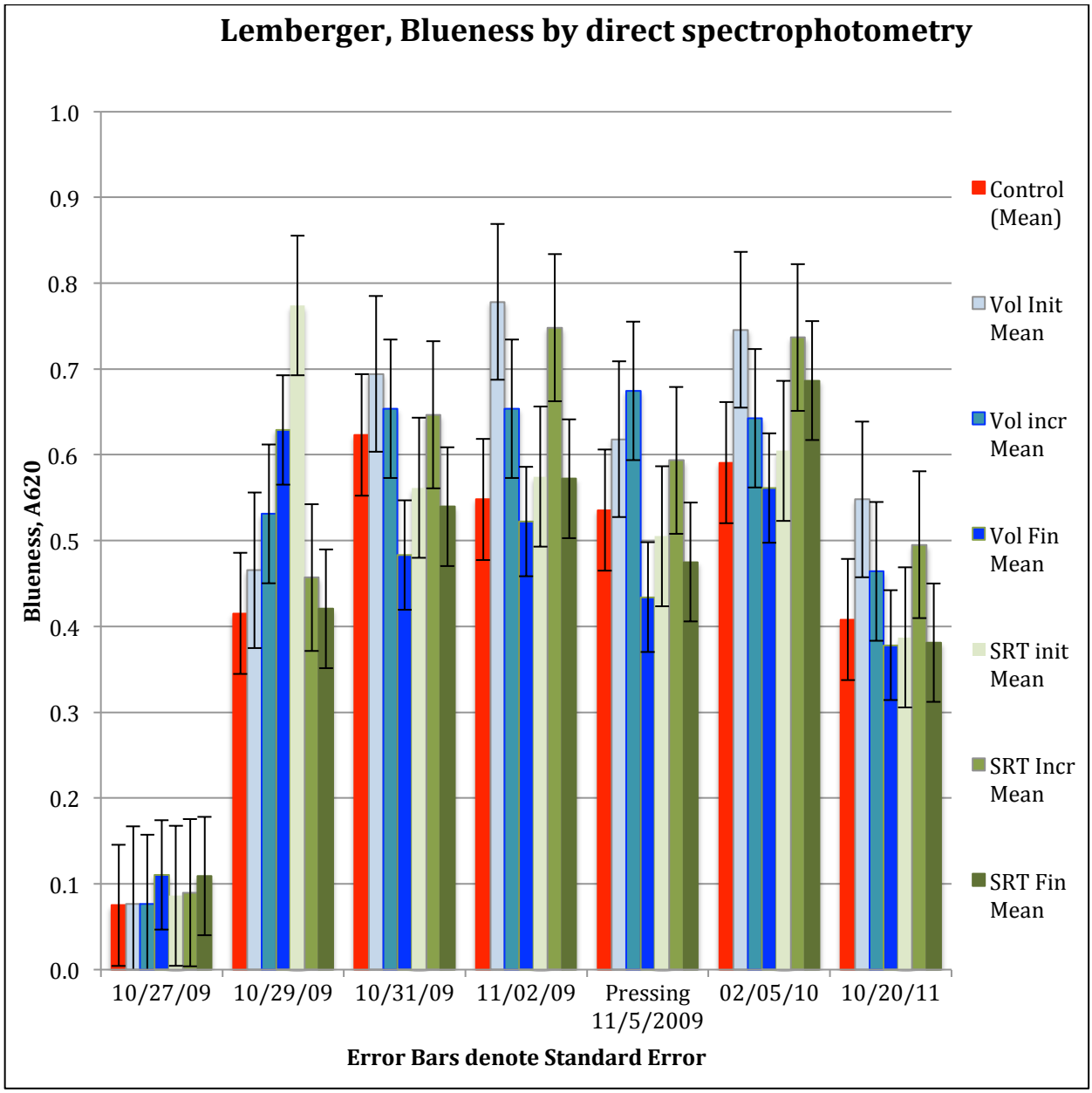

Figure 2–L–2: Lemberger, blueness

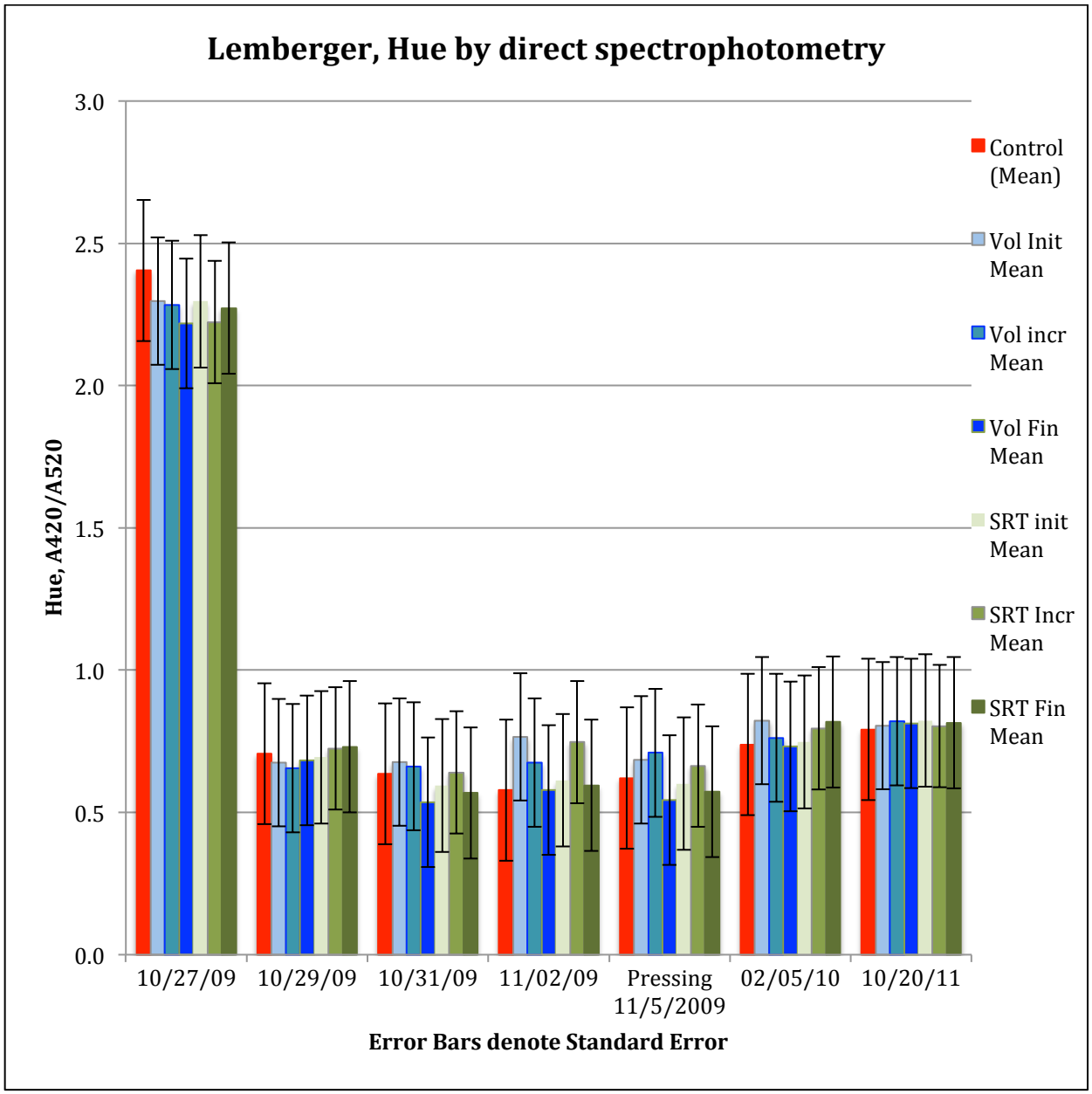

Figure 2–L–3: Lemberger, Hue

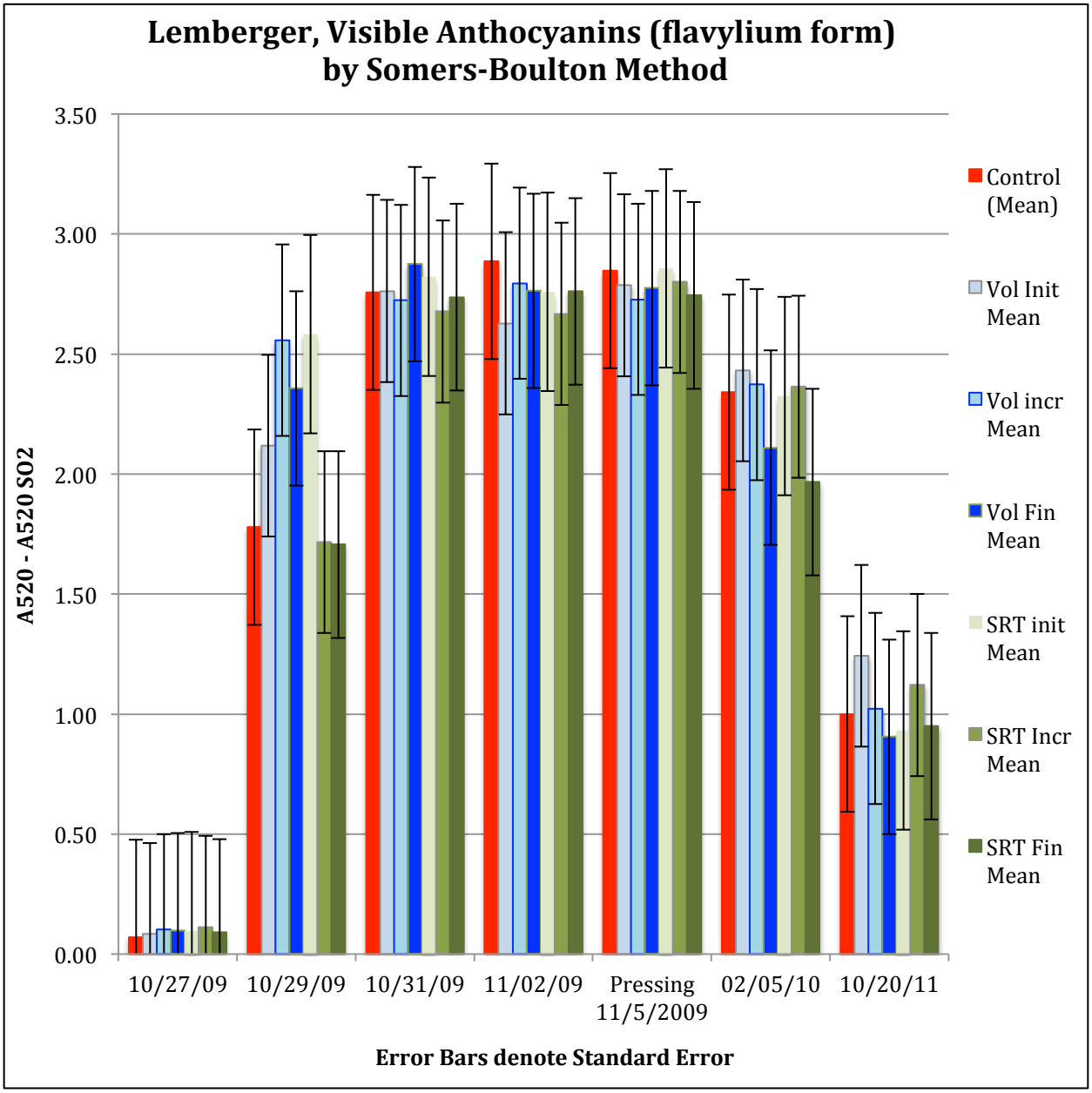

Figure 2–L–4: Lemberger, visible anthocyanins

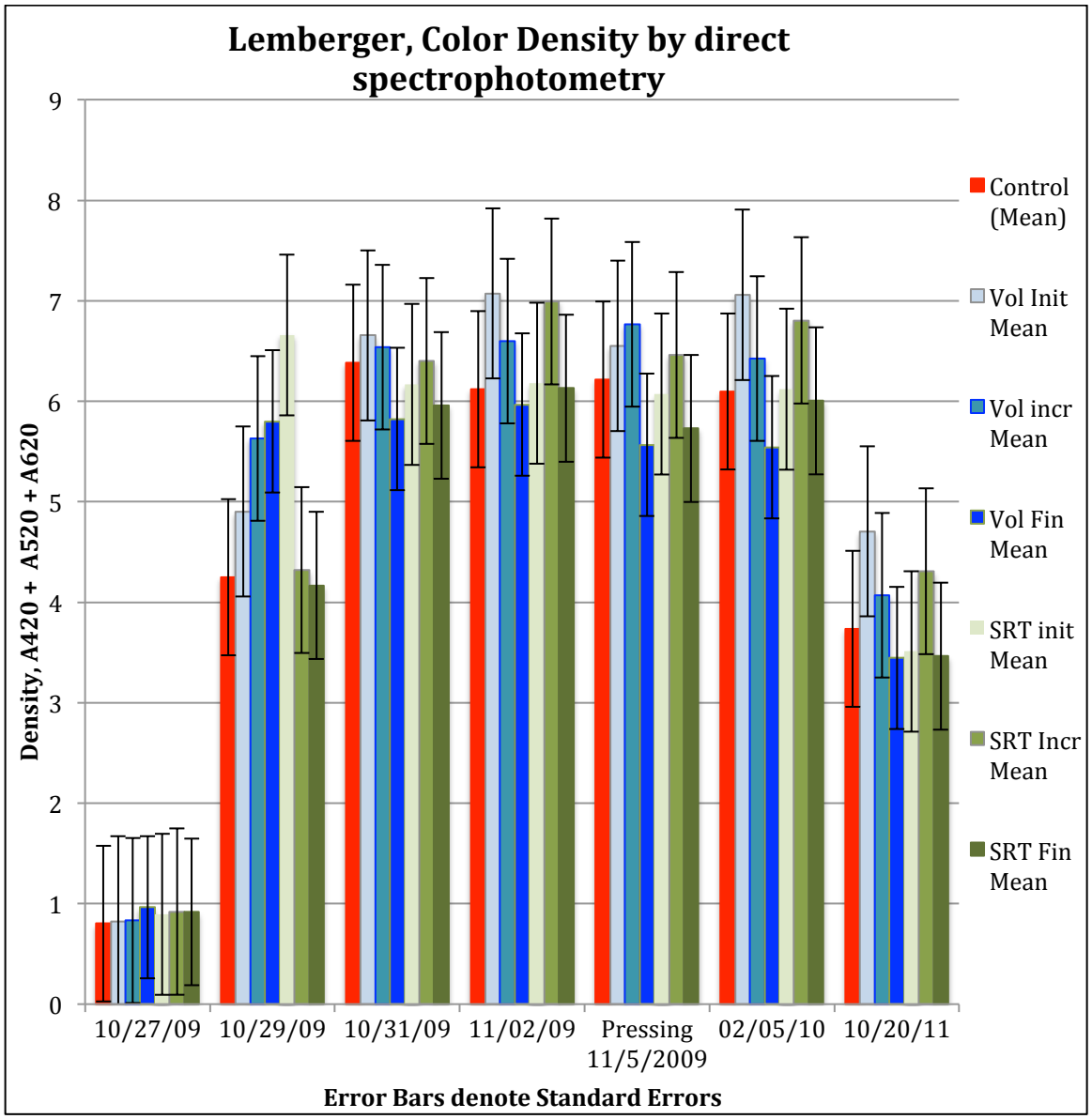

Figure 2–L–5: Lemberger, color density

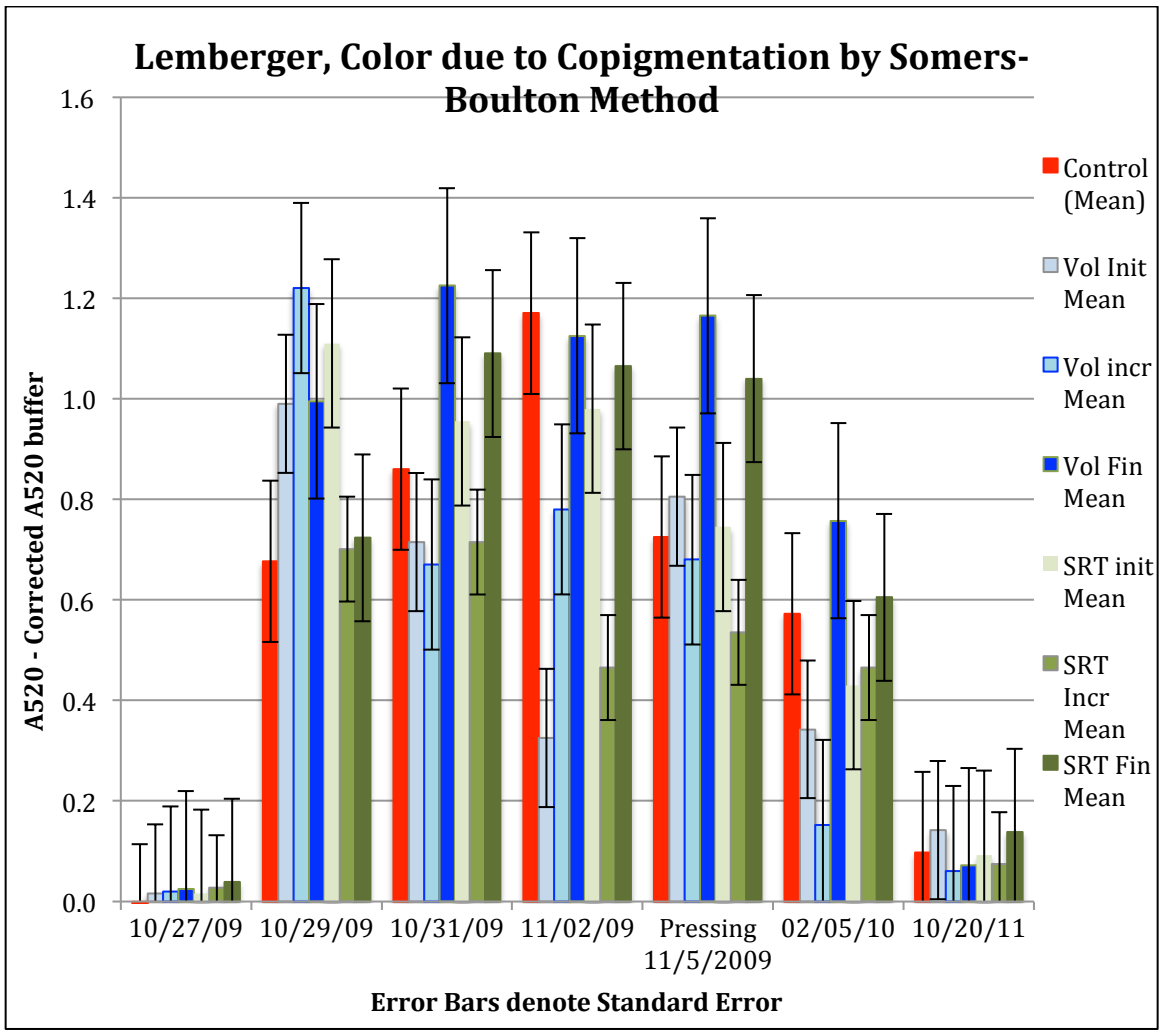

Figure 2–L–6: Lemberger, color due to copigmentation

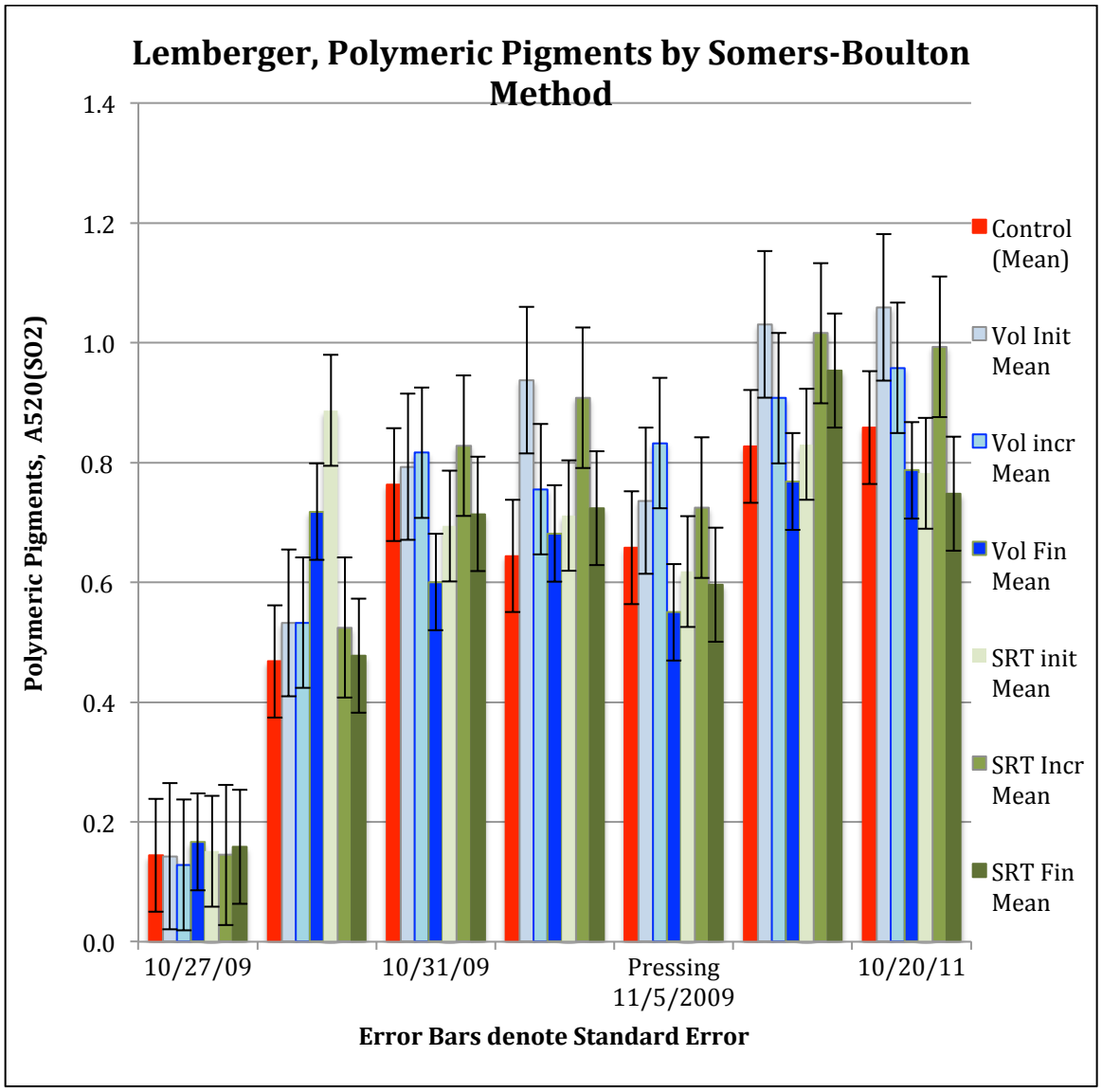

Figure 2–L–7: Lemberger, polymeric pigments

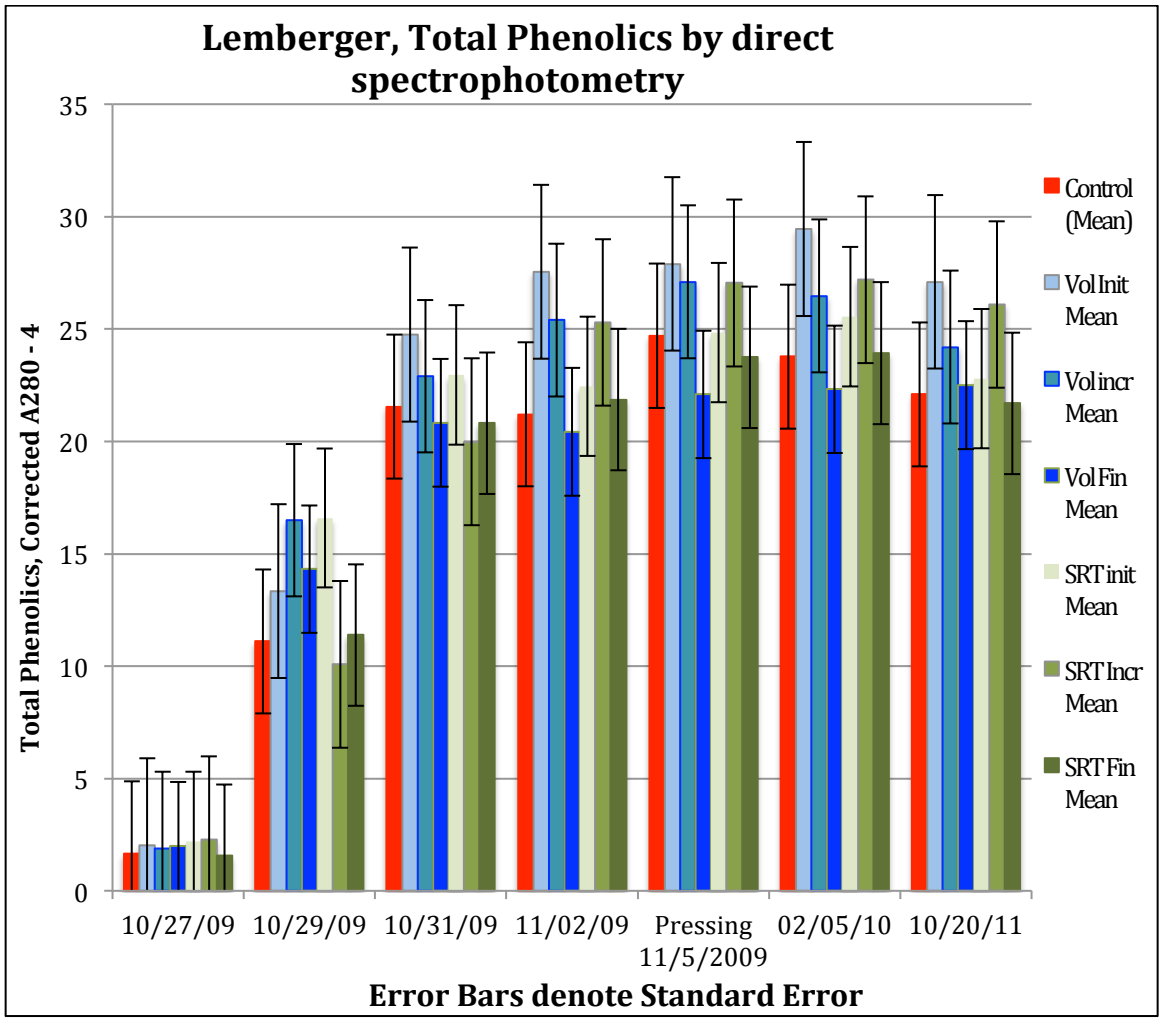

Figure 2–L–8: Lemberger, total phenolics by direct spectrophotometry

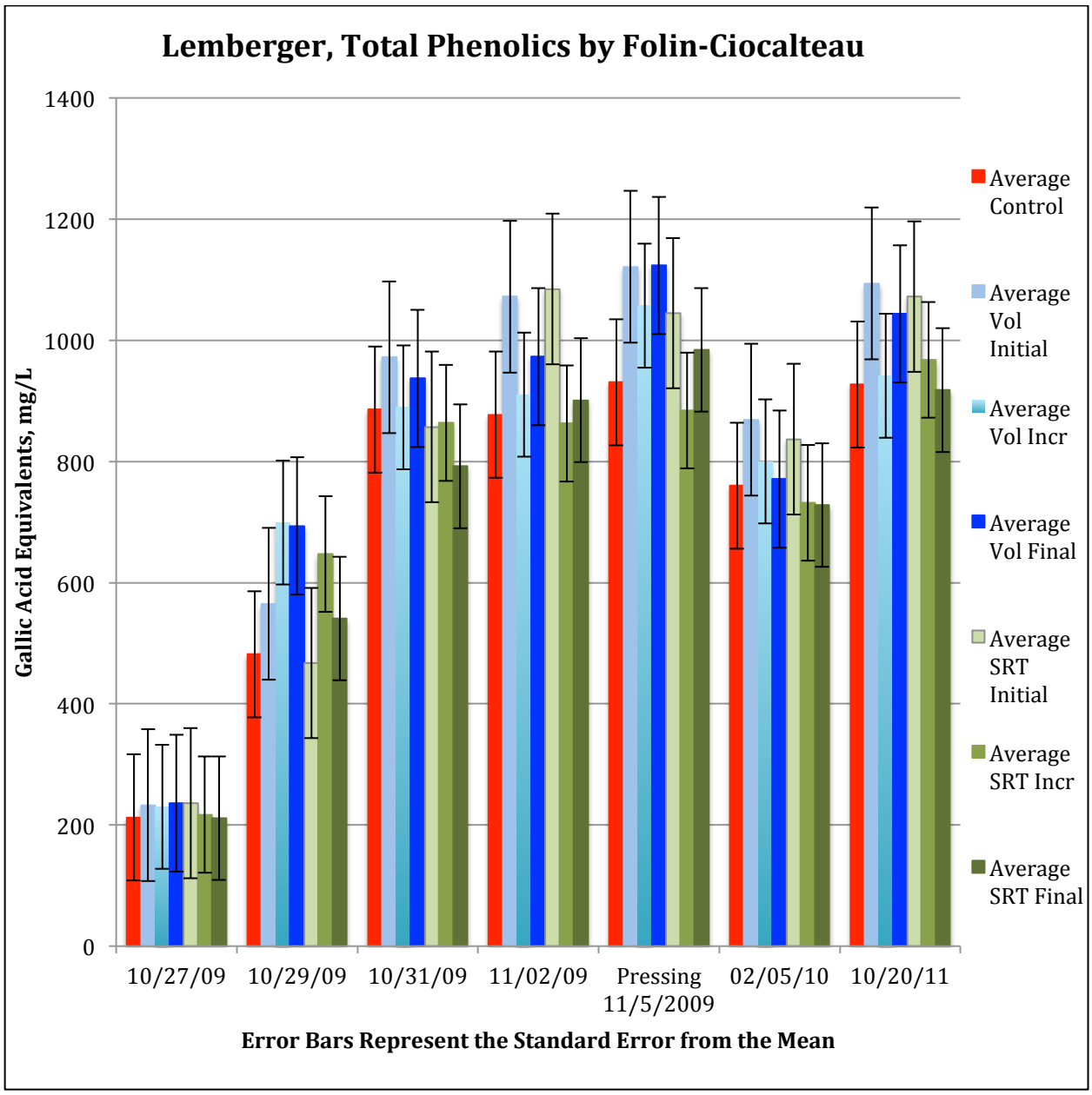

Figure 2–L–9: Lemberger, total phenolics by Folin-Ciocalteau

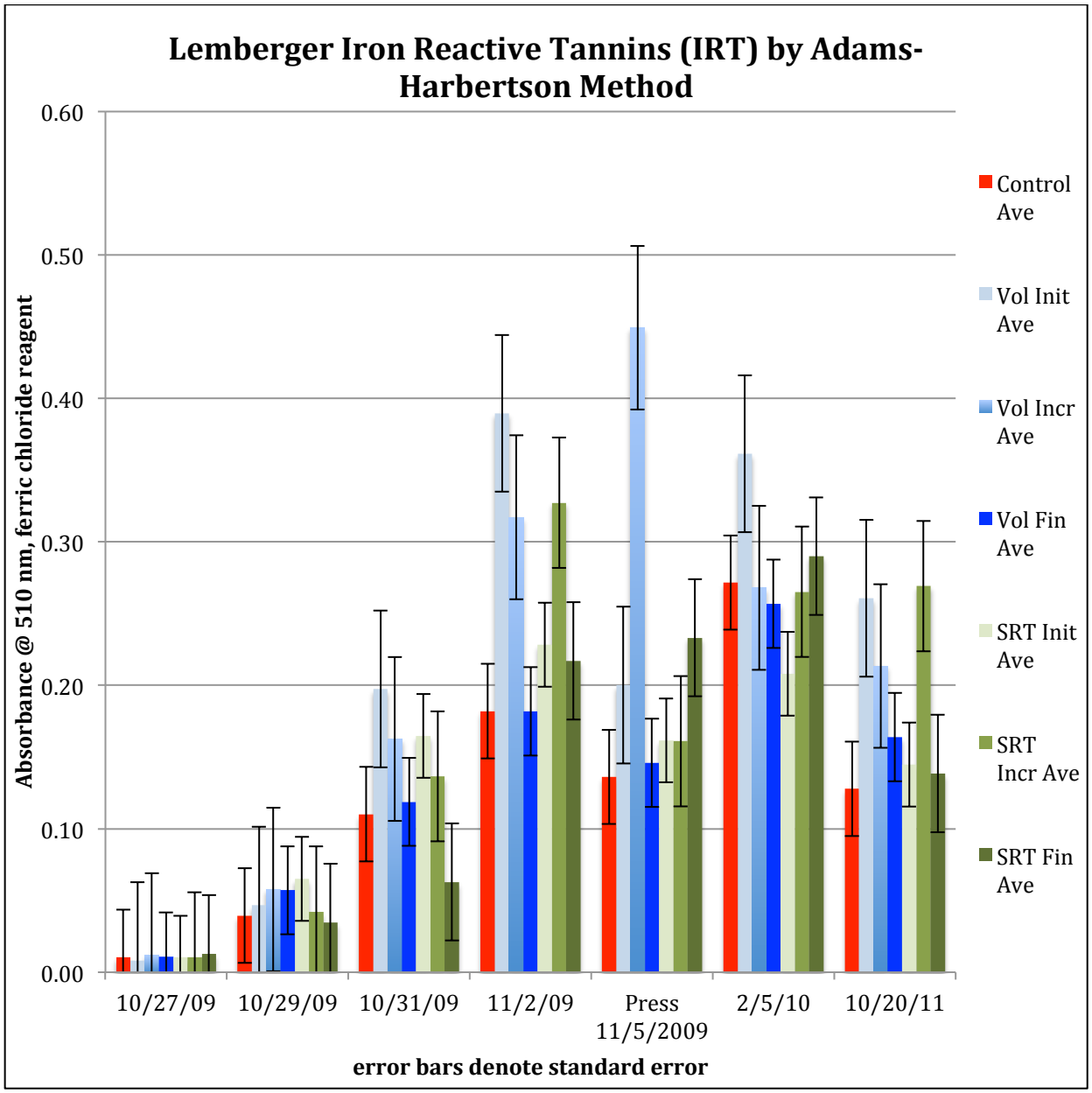

Figure 2–L–10: Lemberger, Iron Reactive Tannins

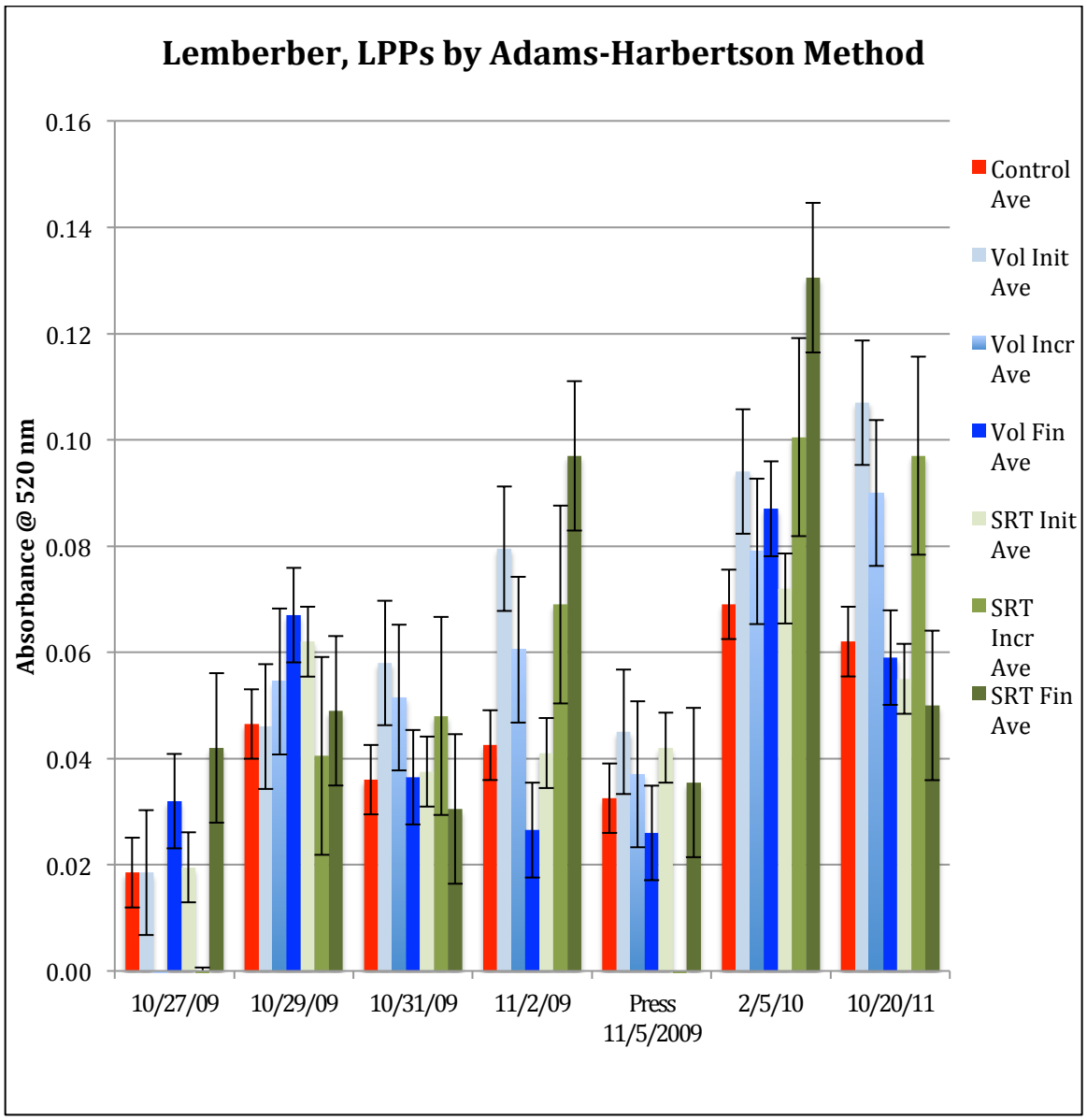

Figure 2–L–11: Lemberger, large polymeric pigments

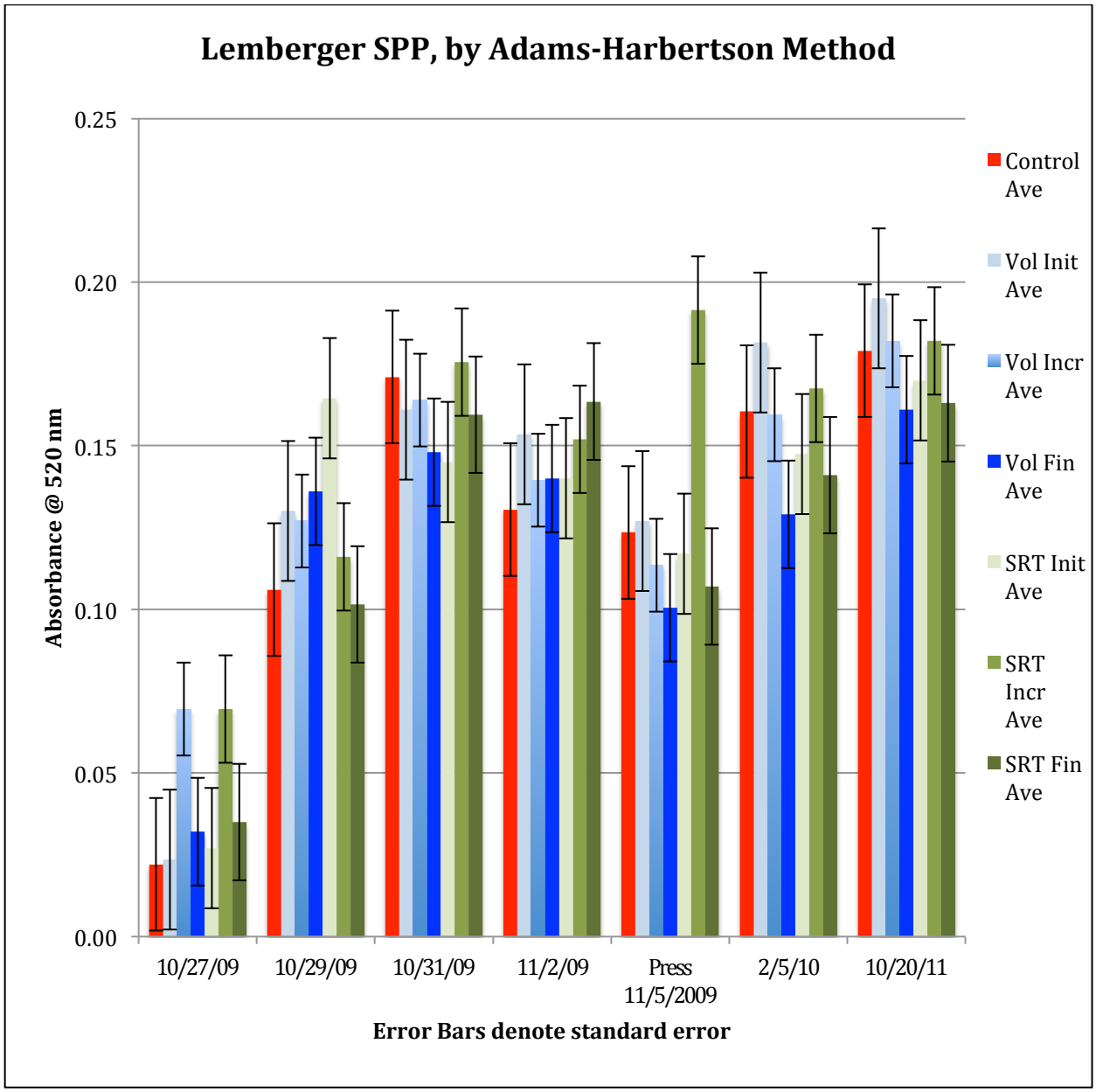

Figure 2–L–12: Lemberger, small polymeric pigments

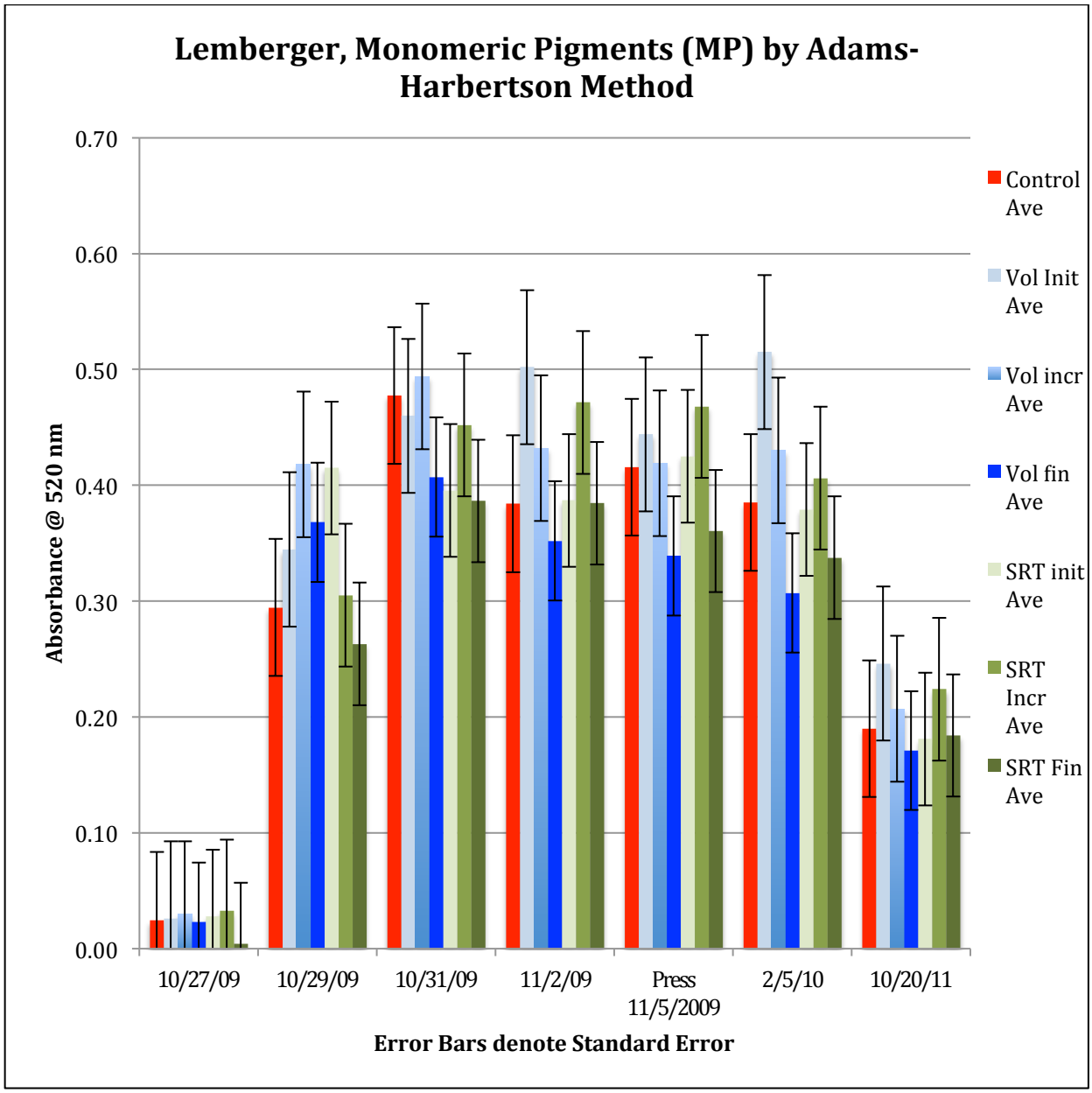

Figure 2–L–13: Lemberger, monomeric pigments

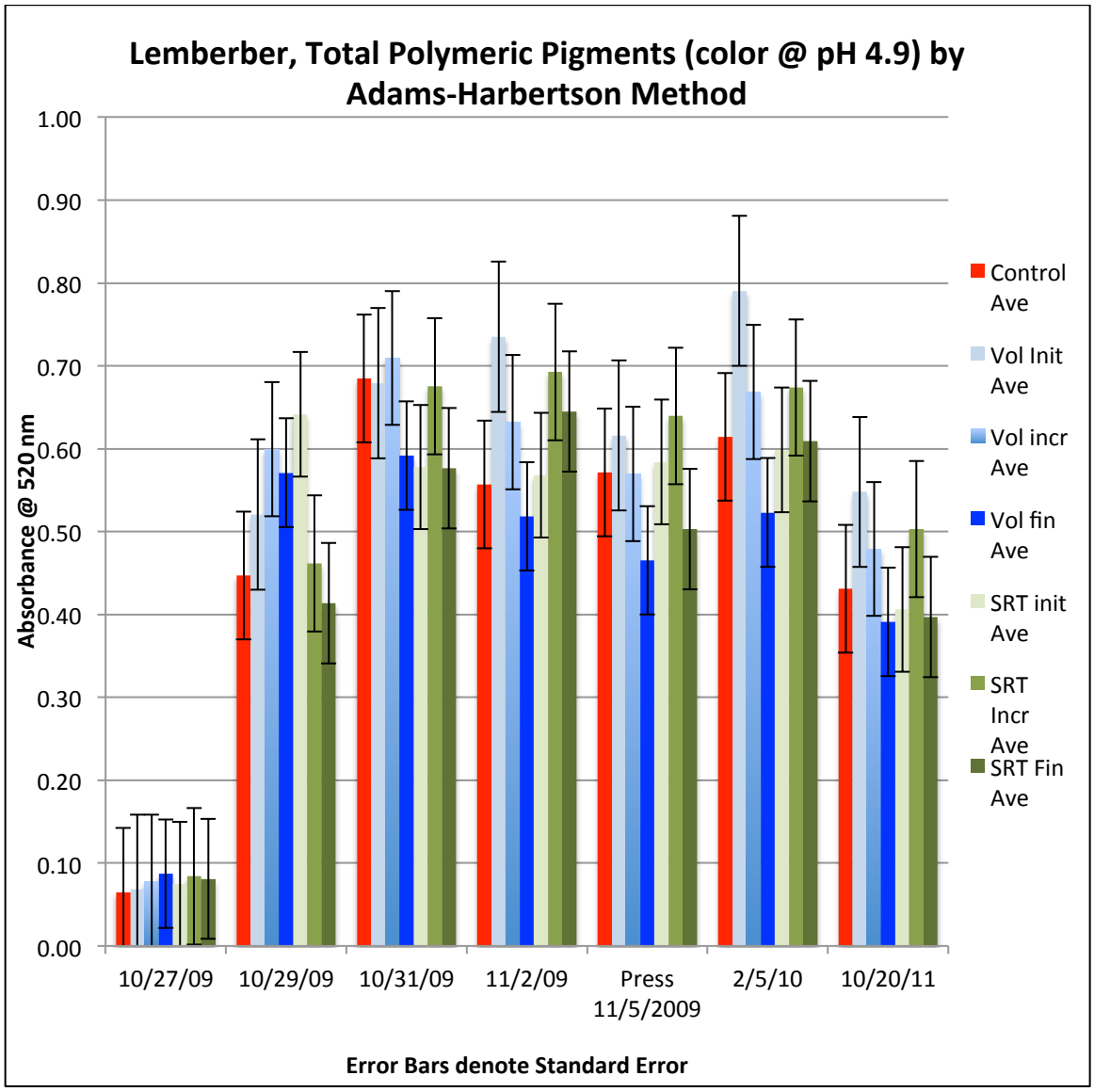

Figure 2–L–14: Lemberger, total color at pH 4.9

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