## Title

Polyphenolic Changes Throughout Maturation in Vitis Vinifera: Understanding Non-Oxidative Interactions Between Procyanidins and Anthocyanins



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# Polyphenolic Changes Throughout Maturation in *Vitis Vinifera*:

## Understanding Non-Oxidative Interactions Between Procyanidins and Anthocyanins

By

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DISSERTATION

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Committee in Charge

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Throughout my adult life, I have received a great deal of support and assistance, whether in academics or the military.

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# Abstract

Interactions between proanthocyanidin material and anthocyanins can lead to the formation of pigmented oligomers and polymers. Although it is visually important that pigmented polymer forms, for the stability and ability to age a red wine, it also can have impacts on the taste and smell of the wine. Given that the synthesis of both proanthocyanidins and anthocyanins occur during fruit development, understanding the factors to improve them in the vineyard seems logical. In 2015 the 'tannin activity' method was used to understand how proanthocyanidin material extracted from grape skins and seeds changed over the ripening period. In that work, which ran from veraison until commercial harvest, there were clear changes in the proanthocyanidin molecular mass, activity, and the formation of pigmented polymer. Based on the sample size (N=372) across the growing season, it was expected that a controlled experiment would increase the correlations seen in the 2015 work. Due to the inverse relationship between activity and pigmented polymer formation in 2015, an experiment was designed in 2016 to look at shade cloth application to decrease the concentration of anthocyanins in the skin. Results from this work showed that changes in anthocyanin concentrations in the berry can impact the activity and molecular mass of proanthocyanidin material. Both molecular mass and activity have been shown to be correlated with wine astringency. The 2018 study built upon both earlier experiments by using white grapes and adding purified anthocyanins into the extracts to look at the ramifications of proanthocyanidin and anthocyanin interactions. The results from this experiment clearly showed the decrease in proanthocyanidin material size as the addition rate of anthocyanins was increased. Thus, increased anthocyanin levels in grapes will change the tannin composition during the winemaking process, and that consequently changes tannin activity. The combined results from these experiments suggest that viticulture techniques can have broad ranging implications for wine quality when

looking at flavonoids and mouthfeel. They also suggest that the formation of pigmented polymer through the acid-catalysis $S_N1$ reaction is happening much faster than previously suggested and warrants further investigation.

# Chapter 1: A Review of Polyphenolics in *Vitis Vinifera*

## 1.1: Introduction

Flavonoids are a large class of secondary metabolites produced in higher plants. Flavonoids fall into the larger class of polyphenolics; this group of molecules is of relative importance due to the high variation in uses by the plant. Recent review papers have discussed these classes produced in *Vitis Vinifera* and higher plants in depth.[1] The focus of this dissertation is primarily about two subclasses of flavonoids called anthocyanins and flavan-3-ols. These two subclasses represent arguably the most important polyphenolics produced by the plant for red wine quality. Both molecules, anthocyanins and flavan-3-ols, are products of a metabolic pathway referred to as the phenylpropanoid pathway.

Anthocyanins are a highly conjugated class of polyphenolics which give many fruits, flowers and vegetables their distinct colors. Anthocyanins are a variable class of polyphenols in that they have considerable variation in substitution patterns on the B-ring. Moreover, they can also have a multitude of glycosylation, coumarylations and acylation patterns in variable positions. These attached glucose molecules can have further substitutions such as acylation or coumarylations. In grapes they are produced in the second half of the growing season – generally considered the "ripening" phase. It has been postulated that the evolutionary reason to produce anthocyanins is likely for the attraction of seed dispersers such as birds.

In red wine production, anthocyanins combine with other molecules in solution to form stable pigment. Stable pigment is part of the reason in which red wine can age for a long period of time. These stable pigments can be a combination of anthocyanins and proanthocyanidins (PAs), but also include reactions with other molecules in a wine system, such as acetaldehyde, pyruvic acid, and coumaric acid to form Vitisins. Vitisins are formed through a fourth ring formation between the 4 and 5 carbon. Due to this positioning, it makes the molecule more stable, for instance by

preventing the anthocyanin from further nucleophilic attacks at the 4-position, such as sulfur dioxide bleaching. Moreover, monomeric anthocyanins in the presence of acetaldehyde can combine through ethylidene bridges. These anthocyanin dimers can, through a condensation reaction, lead to the visually yellow xanthylium form.

Although anthocyanins appear highly colored, they are in constant equilibrium with uncolored forms. The observed pKa for anthocyanins between the colored and colorless forms (flavylium

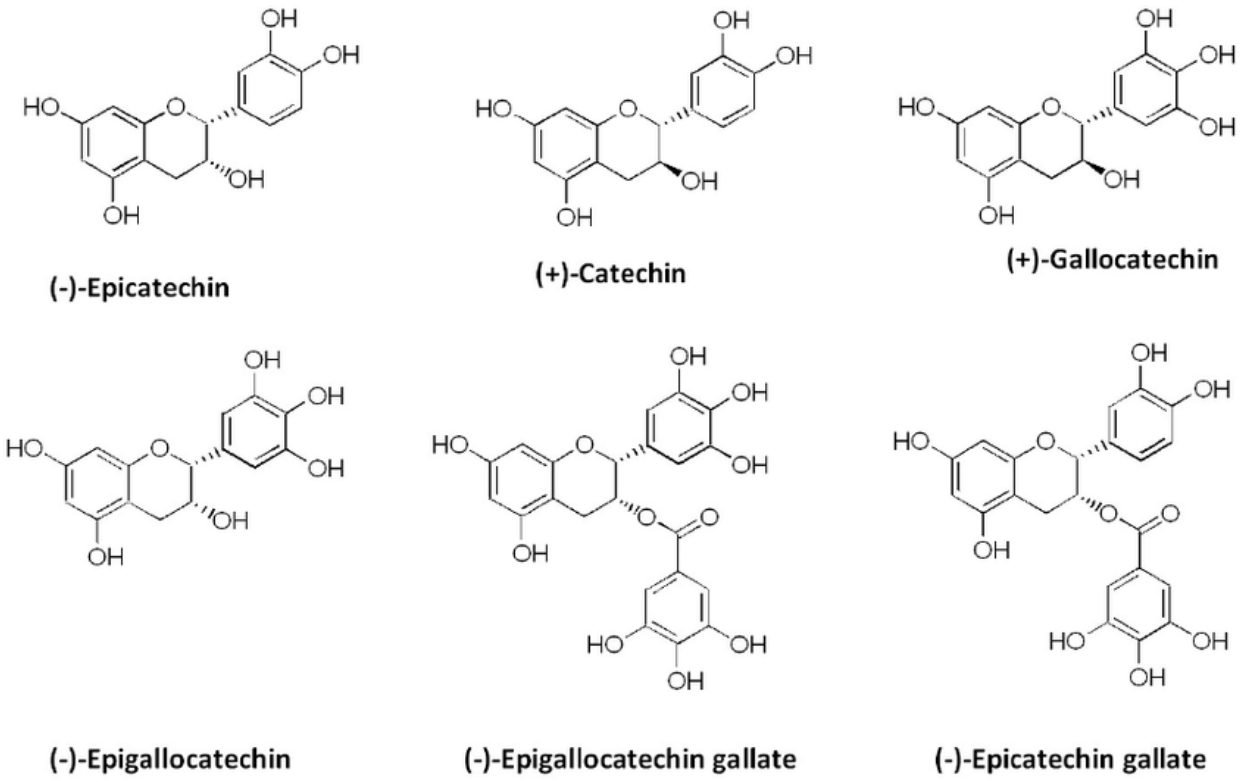


Figure 1.1: Flavan-3-ol monomers

and hemiketal respectively) is 2.66. Given this pKa, at wine pH most anthocyanin molecules are in the colorless form. However, because of the positive charge on the flavylium this form is unlikely to participate in the formation of stable pigment. It has been discussed that while the flavylium provides color, the hemiketal form likely provides the stability through nucleophilic

attack of other components. Given this, it is likely that the hemiketal form likely acts as a nucleophile attacking an electrophilic substrate, such as acetaldehyde, and then the product shifts to the flavylium form, providing stable color.

As described above, one route to the formation of stable color is an interaction between PAs and anthocyanins. Proanthocyanidins are can generally be described as a low to medium molecular weight polymer consisting of flavan-3-ols (**Figure 1.1**). The proportion of the specific flavan-3-ols subunits within these polymers is dependent on where the PA material is derived from. Proanthocyanidin material derived from the skin of grapes is generally higher proportionally in (-)-epicatechin and (-)-epigallocatechin, the former being called a procyanidin while the latter being described as a prodelphinidin. Grape seeds generally contain higher proportions of galloylated flavan-3-ols. Regardless of location wherein they were derived from, the basic structure of PAs taken from plant material is flavan-3-ols subunits attached in a series through 4β→8 or 4β→6 bonds.

The historical importance (>10K years) of tannins is based on their use in tanning game hides. Because of this importance, the chemistry of these polymers has been studied by chemists for a long time. The ability for these polymers to tan hides is likely due to their ability to precipitate proteins. This high affinity for protein material is important in the context of food and beverage in that it gives an *astringent* feeling on the palate as proline-rich proteins (PRPs) in the saliva are precipitated during gustation. The sensation of astringency has been evaluated in wine through sensory studies, the results of which show that there is indeed a structure-activity relationship. The larger the molecular weight of the material, the more PRPs tend to aggregate and precipitate, leading to an increased feeling of astringency. However, substitutions such as galloylation of lower molecular weight material also seem to impart increased feelings of astringency. Lastly, PA that

has become pigmented seemingly decreases the overall astringency. Not only has this relationship been shown in sensory studies, but also in industrial uses for tannin wherein lower molecular weight material is not nearly as effective as higher molecular weight material in, for instance tanning hides. While research has shown polymers with molecular weights reaching into the range of 15,000 Daltons, this outcome may be an artifact of methodology.[2] The way in which PAs size may change after it has been extracted is dependent on polymer size and conditions in solvent. Two of the major changes that can happen to PAs is through acid-catalyzed reactions and

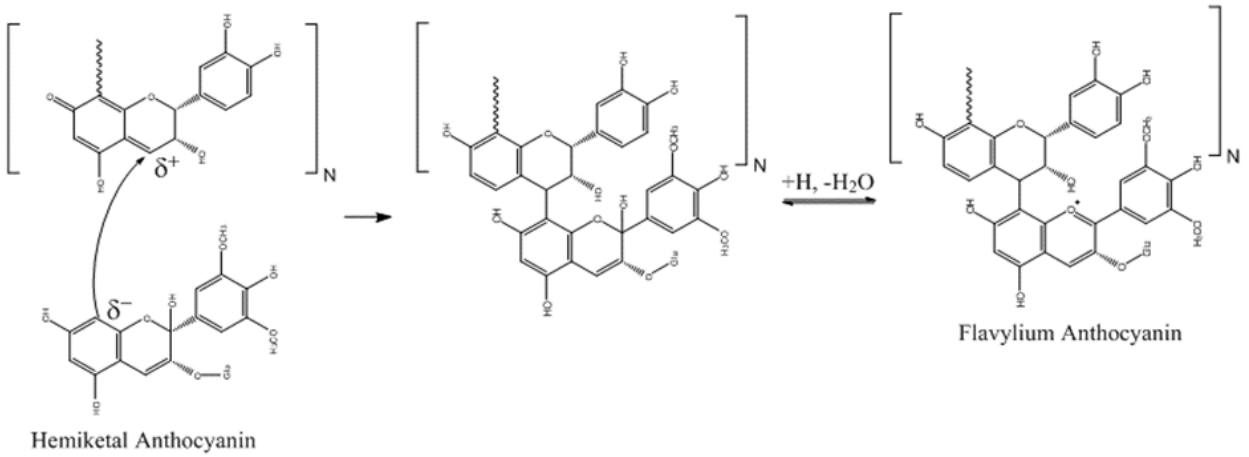


Figure 1.2: Nucleophilic attack of a condensed tannin cleavage intermediate by an anthocyanin in the hemiketal form to form a pigmented tannin product. A partial positive exists at the 4-poistion of the extension subunit following acid-catalyzed cleavage

acetaldehyde-mediated reactions. In acid-catalyzed reactions, the interflavan linkage (IFL) is cleaved with the lower subunit acting as a leaving group and the upper subunit being left with a carbocation. Due to resonance from the 7-position of the "A-ring", it is much more likely that this is a quinone-methide intermediate rather than a true secondary carbocation (**Figure 1.2**). In the second type of reaction ethanol is oxidized to acetaldehyde through iron-mediated catalysis. Acetaldehyde has an electrophilic resonance structure that can be attacked by the 6 and 8-position

carbons on a flavan-3-ol or anthocyanin. This reaction is one of the ways in which stable polymeric pigment can form in a wine solution (**Figure 1.3**).[3]

The *p*-quinone methide intermediate allows for the formation of a new oligomer or polymer, as any other nucleophile in solution may now attack the C-4 position of former upper subunit.

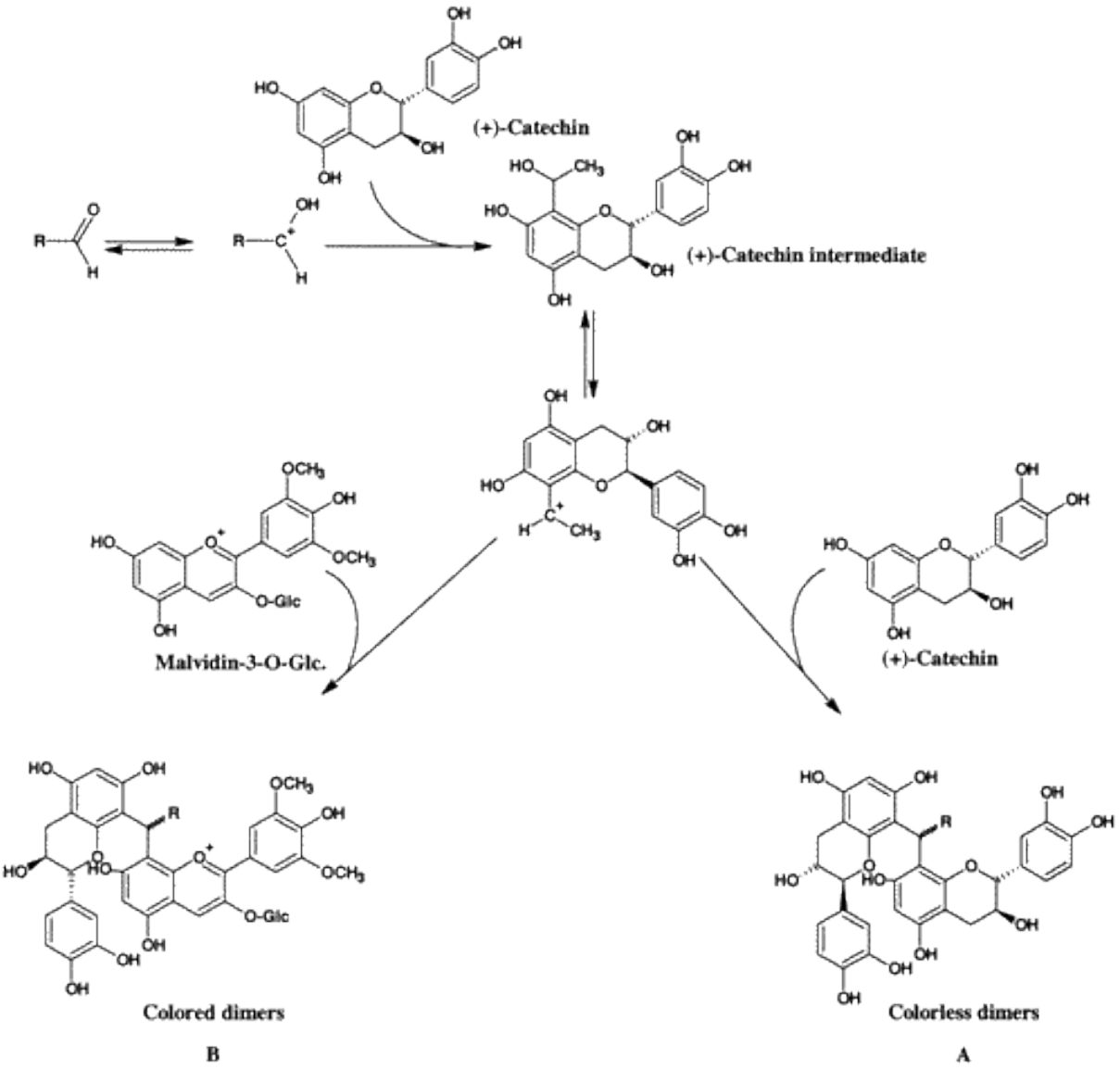


Figure 1.3: Acetaldehyde mediated formation of colored and colorless dimers (Es-Safi et al. 2002)

Although research has shown that anthocyanins can partake in this reaction, forming pigmented PA, other nucleophiles in the solution can also interact with this subunit. Recent work has been conducted showing that sulfur dioxide, a commonly used preservative in wine, can act as a nucleophile at the 4 position, possibly altering further reactions as well as sensory perception.[4] Historically, the acid labile IFL has been used for characterization, quantification and kinetics experiments.[5-10] The basic procedure for this is to isolate the PAs and put them into solution of methanol with 0.1M HCl in the presence of a nucleophile, which will act as a marker for cleavage. Following reaction, excess acid is quenched through sodium acetate and the sample is then checked through various chromatography techniques to quantify subunit proportions and tannin concentrations. Kinetics for the both the 4β→6 and 4β→8 IFL were conducted in 1983 by Hemingway.[6] The results from this experiment suggested a relative slow rate of cleavage for both bonds under wine conditions, with the former having a higher bond enthalpy and therefore more stable.

## 1.2: The Phenylpropanoid Pathway

The phenylpropanoid pathway is ubiquitous in the plant kingdom. This pathway is a rich source of secondary metabolites in plants, producing compounds responsible for the attraction of animals for reproduction, the defense of the plant from pathogens, and the production of structural

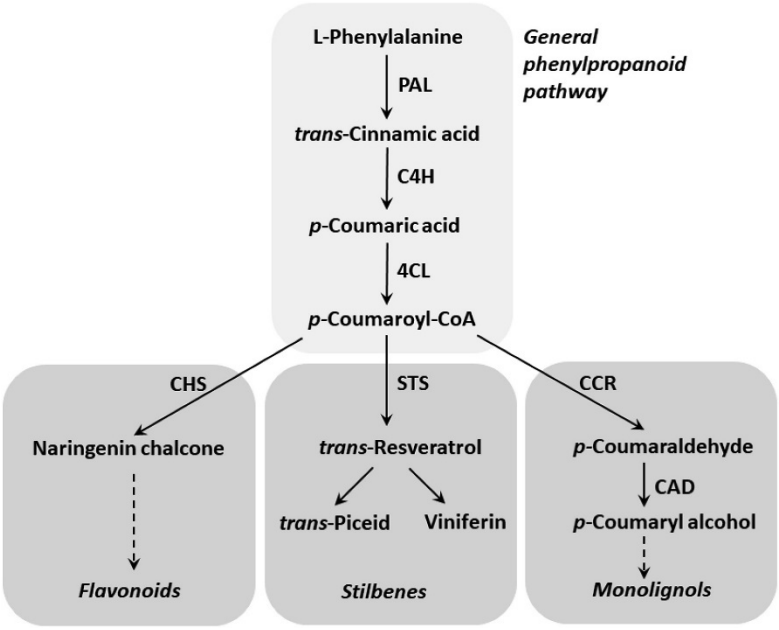


Figure 1.4: The Phenylpropanoid Pathway

components such as lignin. The first committed step in the phenylpropanoid pathway is the conversion of phenylalanine to cinnamic acid following catalysis by phenylalanine ammonia lyase (PAL), which can be seen in **Figure 1.4**. From the formation of cinnamic acid, there are a multitude of compounds – both flavonoid and non-flavonoid – that can be created off the same base structure. The production of flavonoids from the phenylpropanoid pathway of grapes is considered bimodal; the accumulation of condensed tannins in the skin and seeds occurs as early as flowering while the accumulation of anthocyanins occurs following lag phase. In this way, the mechanics of the phenylpropanoid pathway change at the onset of the ripening phase to synthesize anthocyanins rather than condensed tannin. This allows for the plant to use a singular pathway to upregulate different secondary metabolites based on the environmental conditions and time of year. Below are subsections discussing some of the synthesis along with the subclasses of flavonoids that are produced from the phenylpropanoid pathway.

### 1.2.1: Overview of Flavonoid Synthesis

Flavonoids are a class of plant secondary metabolites with a C6-C3-C6 structure as seen in **Figure 1.5**. This class of metabolites is of importance in wine production due to being responsible for the ability to age red wine for long periods of time. The synthesis of these compounds begins following the catalysis of a chalcone to a flavanone (naringenin) by chalcone isomerase (CHI). Following the formation of dihydroflavonols, the pathway branches allowing for the formation of various classes of flavonoids as well as variations in hydroxylation and glycosylation moieties within those classes. Research has shown that there is subcellular distribution of enzymes in the phenylpropanoid pathway and that the enzymes responsible for

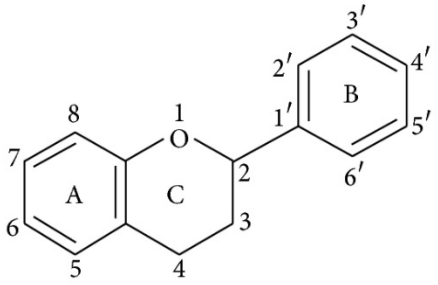

Figure 1.5: Basic C6-C3-C6 structure of flavonoids

flavonoids such as chalcone synthase (CHS) mainly occurs in the chloroplast, tonoplast, and the face of the endoplasmic reticulum of developing grape skins.[11]

### 1.2.1.1: Classes of Major Flavonoids Present in Grapes and Wine

Although there are more than 6 subclasses of flavonoids that are synthesized in the phenylpropanoid pathway, the major classes of importance in grapes and wine are anthocyanins, flavonols, and flavanols, including condensed tannin (**Figure 1.6**). Condensed tannin (CT) is synonymous with PAs, a term derived them tannins ability to turn into anthocyanidin monomers when heated in acidic ethanol. The subsections below will provide more information about each of these subclasses of flavonoids.

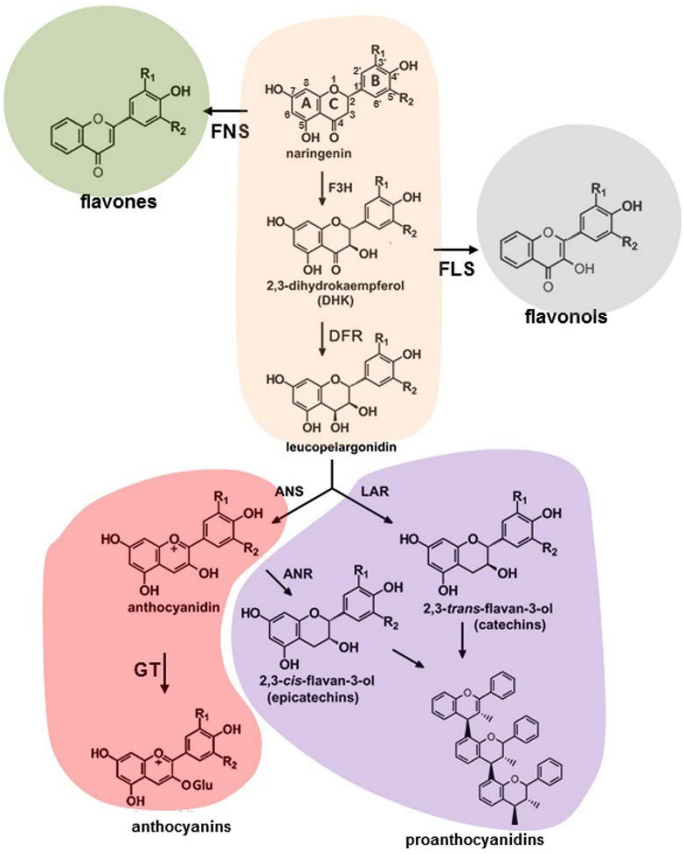


Figure 1.6: Different Products of the Phenylpropanoid pathway

#### 1.2.1.1.1: Anthocyanins

Anthocyanins are a class of flavonoids responsible for the color in many different species throughout the plant kingdom. Anthocyanins are in a pH driven equilibrium in solution which impacts the concentration in the colored form. They have the typical C6-C3-C6 carbon structure seen in all flavonoids, but with a higher level of conjugation on the heterocyclic C-ring leading to absorption at a higher wavelength. The pKa of the equilibrium is approximately 2.66 between the colored flavylium form and the colorless hemiketal form, which can be seen in **Figure 1.7**.[12]

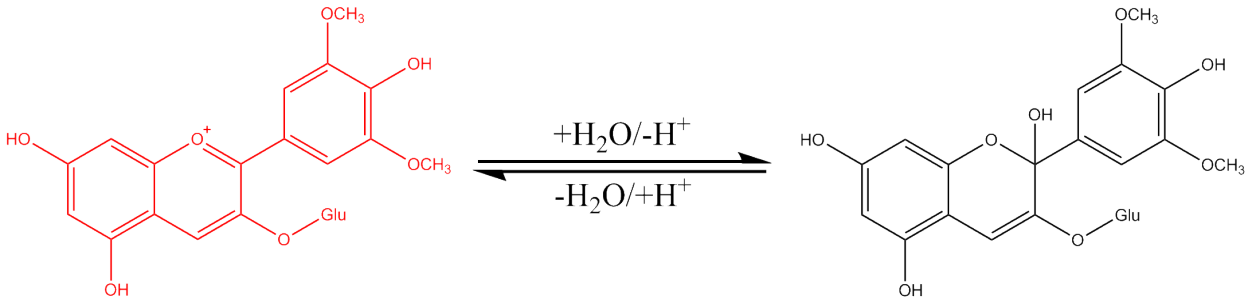


Figure 1.7: Anthocyanin pigment equilibrium

In red winemaking, this equilibrium is of importance because of the nucleophilic and electrophilic reactions that take place during wine aging. The hemiketal form has nucleophilic centers at the 6 and 8-positions, while the flavylium form has an electrophilic center at the 4-position. This equilibrium-dependent nucleophilicity and electrophilicity can lead to a multitude of different reactions within a wine system. Overall, reactions between anthocyanins and other wine constituents lead to the color stability required for long term aging of red wine. However, these reactions can also lead to the yellowing or "bricking" of a red wine over time as further reactions take place – such as the formation of pyranoanthocyanins and xanthylium's. The formation of these anthocyanin derivatives is outside of the context of this introduction, but they have been discussed in other reviews.[13]

**1.2.1.1.2: Flavonols**

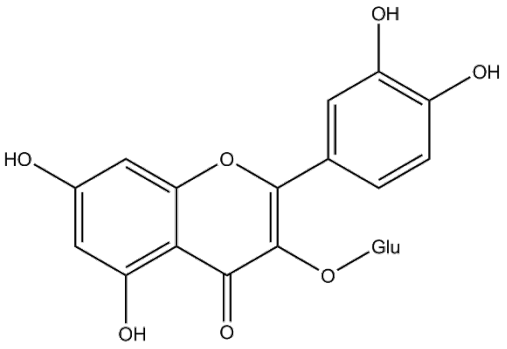


Figure 1.8: The flavonol glucoside of quercetin

Flavonols are a class of flavonoids that unique for having a ketone functional group at the 4-position and a double bond between the 2 and 3-carbons, as seen in **Figure 1.8**. The synthesis of flavonols is generally regarded as a function of cluster sun exposure in grapevines. Flavonols play a key role as an electron acceptor to scavenge radicals formed through UV light excessive exposure on grape clusters. This is likely due to the wavelength at which

flavonols absorb light – 365nm. This absorption in the UV-A area helps prevent photooxidation from occurring and photosystem degradation of important cellular components such as DNA. During wine aging, particularly in young wines, flavonols can also have implications in a wine system because they can have a direct impact on color. The ketone and double bond described earlier create a planar structure which allows for aromatic interactions (π-stacking). In this case flavonols such as quercetin can act as an intermolecular copigment leading to increases in anthocyanins in the flavylium form above those expected based solely on the equilibrium constant. An example of intermolecular copigmentation can be seen in **Figure 1.9**, wherein the planar flavonol shares weak bonding with the anthocyanin leading to increases in electron delocalization.[14] Flavonols have also been reported to affect the astringency of wine like solutions.[15]

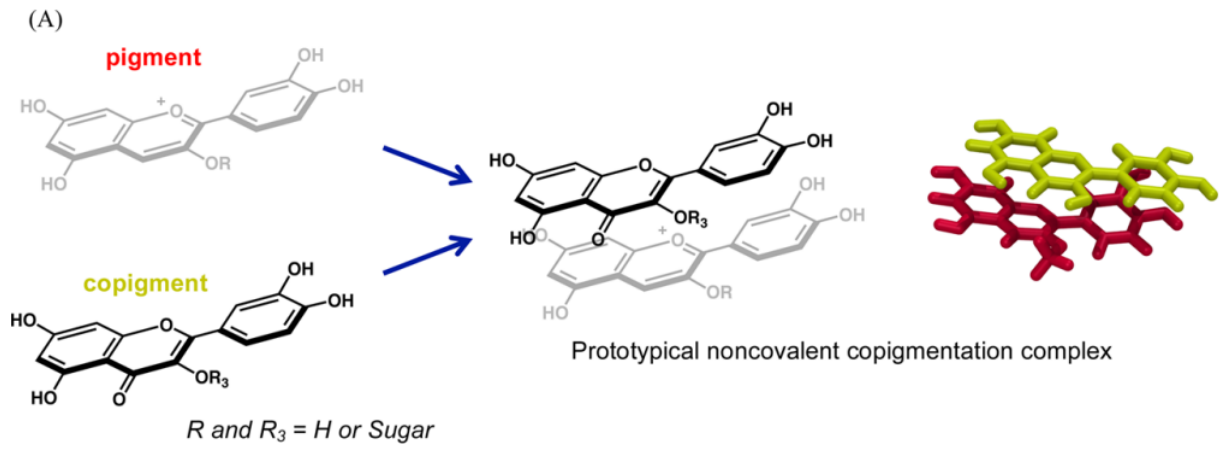


Figure 1.9: An example of non-covalent copigmentation occurring due to interactions between an anthocyanin and a flavonol. (Trouillas et al. 2016)

### 1.2.1.1.3: Flavanols

Flavanols, sometimes called flavan-3-ols due to the hydroxyl functional group coming off the 3-carbon. Although afzelechin, a flavan-3-ol with a single hydroxyl substitution on the B-ring, is not

present in any significant level in grapes, there are four flavan-3-ols that are considered important: (-)-epicatechin, (+)-catechin, (-)-epigallocatechin and (-)-epicatechin gallate (**Figure 1.1)**. The major flavanols in grapes and wine are generally considered (-)-epicatechin and (+)-catechin, which differ only in the stereochemistry at the 2 and 3-carbons, the former being more abundant in skin material, while the latter is more abundant in seed material. Like the other subclasses of flavonoids, there are various types of B-ring hydroxyl substitution leading to multiple molecules within this subclass. (-)-Epigallocatechin has a B-ring which is trihydroxylated and makes up more than 40% of the subunits in PAs derived from grape skins, while (-)-epicatechin gallate is an (-)-epicatechin, but rather than having a hydroxyl functional group at the 3-carbon, there is a gallate ester. (-)-Epicatechin gallate is generally considered a flavanol that is derived from stem and seed material rather than skin material in grapes and is in higher proportion as a terminal subunit in comparison to an extension subunit, which will be described in the next section. Although these flavanols can occur as monomers in grapes, they are largely present as extension or terminal subunits in the polymerized PAs form. These polymers that are built of flavonoid subunits are common in the plant kingdom, and our diet, and will be discussed in the next section.

**1.2.1.1.4: Proanthocyanidins**

Proanthocyanidins are not actually a subclass of monomeric flavonoids, but rather they are a polymer of flavanols, as seen in **Figure 1.10**. Procyanidins and prodelphinidins are differentiated by the hydroxylation patten on the B-ring. Polymeric material with a tri-hydroxylated B-ring are considered prodelphinidins, while those with a catechol on the B-ring are referred to as procyanidins. Depending on the plant material PAs can be a single type of subunit, for instance in

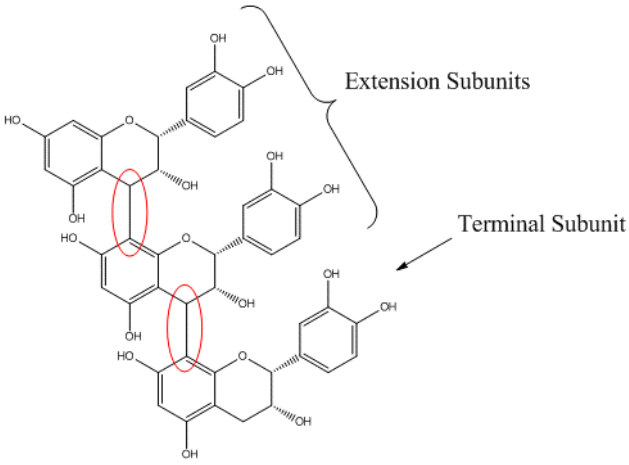


Figure 1.10: A Flavanol Oligomer

cacao, (-)-epicatechin is the primary subunit with much lower proportions of (-)-catechin. In grapes however, there are multiple subunits making up these polymers, and the proportions of the subunits change depending on the part of the plant the PAs were derived from. The seeds and skins of wine grapes have differing compositions and concentrations of PAs. In skins there is a higher proportion of prodelphinidins while the seed and stem material have higher proportions of procyanidins. Wine grape skins tend to have a high proportion of (-)-epicatechin and (-)-epigallocatechin, with lower proportions of (+)-catechin and (-)-epicatechin gallate, which are generally found in the stem and seed material.

Along with variations in the composition, PAs can range in size. Proanthocyanidin material derived from the stems and seeds tend to have a lower degree of polymerization when compared to those derived from grape skins. This is of importance because polymer size has been shown to impact the inherent astringency of red wines during gustation, with larger polymers having a higher affinity and proclivity to precipitate salivary proteins.[16] Seed and stem tannin also have higher levels of galloylation which also increases wine astringency.

The differences in size and composition have implications for the formation of stable pigments in red wine for long term aging, as smaller tannins with a higher proportion of galloylation tend to precipitate out of solution following interactions with acetaldehyde. Furthermore, due to the increased degree of polymerization, grape skin PAs have an increased abundance of extension

subunits, which can act as a nucleophile in the presence of anthocyanins, possibly leading to increased stable pigment formation.

### 1.2.1.2: Timing and Location of Proanthocyanidin Synthesis in *Vitis Vinifera*

Earlier it was briefly stated that flavonoid biosynthesis was bimodal in terms of how the pathway is used to synthesize secondary metabolites. As shown in **Figure 1.11,** PA synthesis occurs from before berry set to approximately veraison.[17] After the onset of color development, PA synthesis does not continue at a significant rate. Instead, after veraison, there is a switch to the development of anthocyanins, but continued flavonol production.[18] The importance of this will be discussed in

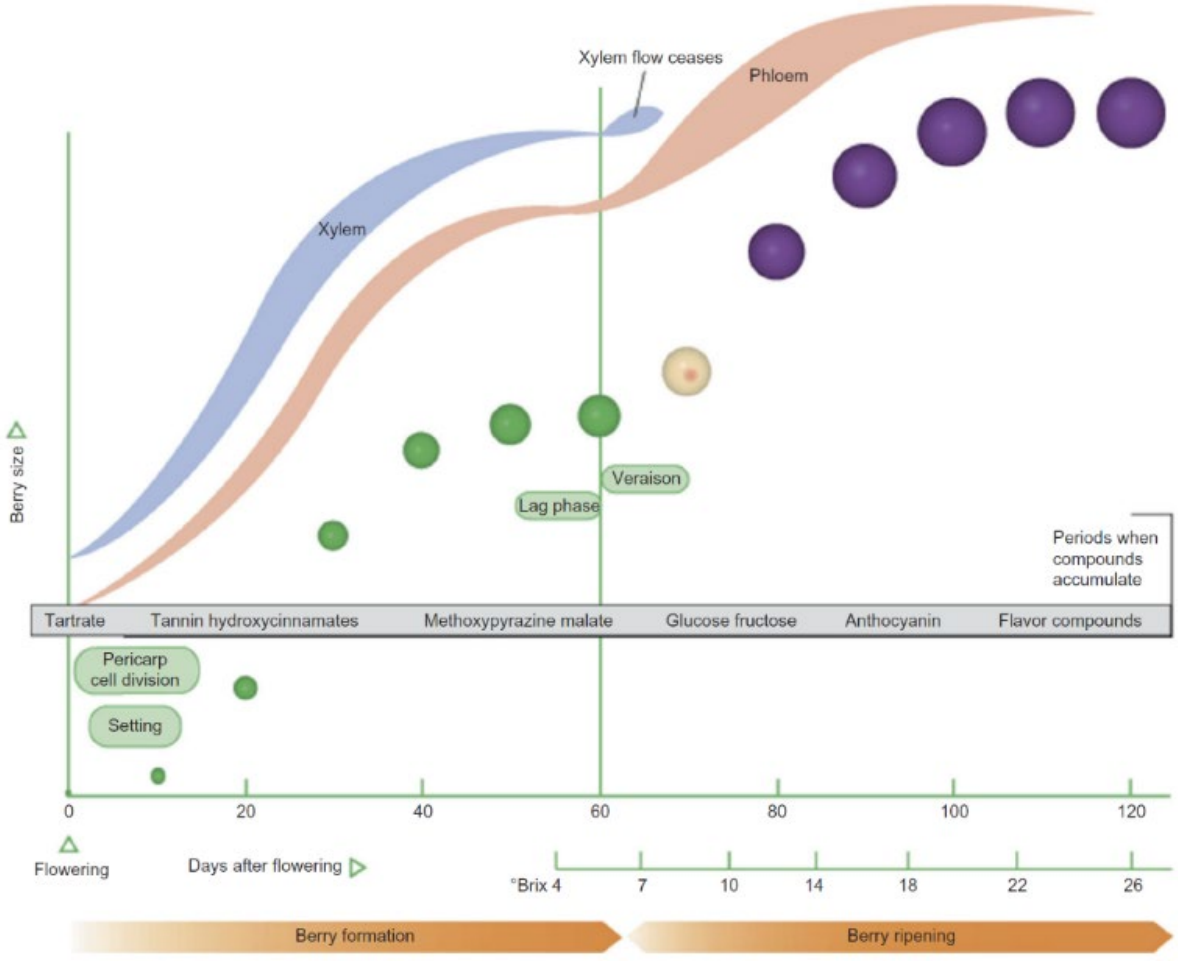


Figure 1.11: Illustration of grape berry development taken from "Understanding Berry Development" Kennedy, 2002.

section 1.4, but it is of importance for viticulturists to understand that tannin synthesis is completed in grape skins at the point when they see color development. The only way to alter the qualities of fruit from a PA perspective in the vineyard is through early season management practices between fruit set and veraison. Although the extractability of PAs may change during the ripening phase, due to changes in the cell wall material, its synthesis can only be altered based on management decisions early in the season. This is important to the wine industry, as research has shown that there is a correlation between phenolic content and bottle price.[19]

Although the specific mechanism for the linkage of its constituent monomers into the PA oligomers and polymer is still unknown, recent work has shown possible insights into *where* in the cell it is occurring and *how* it is occurring. It is likely that the synthesis and storage of these compounds is regulated due to their nonspecific binding of proteins, making them relatively dangerous for an organism to have floating around the cell unrestricted. Research conducted by Brillouet has shown that there are changes associated with the chloroplast wherein new plastids of thylakoidal origin appear to be shuttled to the vacuole. This is of particular interest because it suggests the synthesis of PAs in the chloroplast organelles, with storage in a tannin-specific vacuole.[20-23]

This idea of plastid senescence and redifferentiation was shown as early as 1978 by Whatley, wherein a diagram clearly depicts the swelling of the plastid and the unstacking of the grana thylakoids. Although this work was not focused on PAs nearly as much as that of Brillouet's, it shows that these changes have been observed for decades.[24]

The synthesis of PAs in the chloroplast may explain why enhanced light exposure can increase concentrations in the grape skin, but still begs the question of origin in the seeds, as they are insulated from sun exposure by the mesocarp and exocarp. It is already known that the chloroplast

in grape skins is different than those in the leaf material, suggesting that skin and seed chloroplasts are optimized for low-light or shaded environments. Although seeds have been shown to conduct photosynthesis, and that higher levels of chloroplast in the seeds suggests a higher level of germination, they have photosystems that favor far-red light incidence due to penetration through the pericarp layers. If chloroplasts are the site of PA synthesis, it may be that plants are producing these plastids in a non-photosynthetic manner in order to produce defense compounds from the phenylpropanoid pathway.

*How* flavanols are polymerizing into PAs has long been a question for researchers. Early work showed that leucocyanidins were a possible substrate for auto-polymerization of flavanols in the absence of an enzymatic catalysis, through the loss of a water at the 4-carbon leading to a p-quinone methide, which would then be attacked by a "starter unit" or terminal subunit flavanol.[25] However, recent work has shown that there may be an intermediate molecule that does interact with an enzyme.

Research by Liu et al. in 2016 showed that a 4β→(S-cysteinyl)-epicatechin can be converted to (+)-epicatechin by leucoanthocyanidin reductase (LAR). This allows for the creation of a terminal subunit through an enzyme in the phenylpropanoid pathway. The work also showed, through $^{13}C$ labeling, that the cysteine can also act as a leaving group allowing for the formation *in vitro* of PAs through auto-polymerization. This work suggests that there is indeed enzymatic control of the production of PAs in the plant, however it is also possible for auto-polymerization when a terminal subunit and a 4β→(S-cysteinyl)-epicatechin are in solution allowing for the formation of a Procyanidin B2 (an (+)-epicatechin dimer).[26]

## 1.3: The Impact of Biotic and Abiotic Stress on Polyphenolics

Biotic and abiotic stress in plants can lead to increased signaling to deal with change. Plants are immobile, which presents them with a unique set of challenges to survive in a local environment. Rather than moving to a more desirable location, plants must adapt to their surroundings through the upregulation of metabolites in response to the type of stimuli. Biotic stress generally comes from opportunistic viruses, invertebrates, and fungi, while abiotic stress comes from the lack of water, extreme temperatures, over exposure of light, and nutritional deficiencies. Generally, how a grapevine responds to these stresses can depend on a multitude of factors ranging from susceptibility to disease to inherent ability to deal with local environments due to evolutionary or inherited traits. In some ways modern viticulture practices have altered this paradigm due to the availability of water through irrigation as well as fungicides and insecticides. However, the performance of a plant/rootstock combination in the wrong site (dare I say 'terroir') can have long-term negative implications on overall wine quality.

### 1.3.1: Biotic Stress

Biotic stress occurs from the outward attack on the plant itself. This can come in many forms, but the major biotic attacks in a vineyard are viral and fungal. The sessile nature of plants requires that they respond through the upregulation of defense compounds for survival and preservation. There are *many* virus and fungal infections, with more than 65 known viruses that can affect grapevines.[27] Here the focus will be on those known to impact the phenylpropanoid pathway and therefore impact wine quality.

#### 1.3.1.1: Virus

It is not uncommon to see viruses inhabiting vineyards. Globally this can lead to huge reductions in the return on investment of planting and farming wine grapes. The economic losses have been

estimated at approximately $91,661 per acre over the life of a vineyard through reductions in yield and quality parameters.[28] Common viruses, such as Eutypa and Botryosphaeria dieback, prevent sap flow from the trunk to shoots leading to the loss of growing positions and eventual loss of the plant. More recently another virus, Grapevine Rupestris Stem-Pitting Virus (GRSPaV), has garnered some attention as possibly leading to vein necrosis in the 110 Richter rootstock, as well as stem pitting in *V. Rupestris*. GRSPaV is a candidate for explaining "Syrah decline", wherein young, healthy, plants suddenly crash, requiring entire vineyard replanting.

Outside of the viruses discussed above, which can lead to substantial losses through the complete shutdown of vascular tissue, there are other viruses which can lead to significant yield loss as well as impact the phenylpropanoid pathway. Given the context of this review, the following viruses will be discussed in greater depth due to how they can impact polyphenol content in the berry.

**1.3.1.1.1: Grapevine Fanleaf Virus (GFLV)**

Grapevine Fanleaf Virus is spread through the feeding of the ectoparasitic nematode *Xiphinema index.* This allows for vine-to-vine transfers of the virus; however, it will likely spread slower than other viruses due to nematodes slow movement in the soil. Grapevine Fanleaf Virus can have significant economic impacts on wine grapes. Specifically, there can be a dramatic reduction in yield between healthy and infected vines. Research conducted in 2018 looking at Schioppettino and Refošk showed a 32.2% and 28.7% reduction in yield averaged over two years, respectively, when compared with healthy vines. This dramatic reduction in yield from GFLV cannot solely be explained by the impact of GFLV on vine water status as the research showed that Refošk had no significant differences between healthy and infected vines in stem water potential. Furthermore, this work showed that GFLV infected vines had increased total anthocyanin concentrations in both Schioppettino and Refošk. This was due to an upregulation of F3'5'H, leading to an increase in

malvidin-3-glucoside and an overall shift in the anthocyanin proportions extracted.[29] A picture of leaf chlorosis occurring due to symptomatic infection of GFLV can be seen in **Figure 1.12.**

The upregulation of the phenylpropanoid pathway has been studied in other research regarding GFLV infection. Research exploring the impact of GFLV affected vines on the incidence and severity of biotic attacks from other pathogens showed that vines infected with GFLV was less likely to be susceptible to powdery mildew. An examination of the transcriptional changes between the healthy and infected vines showed an upregulation of the phenylpropanoid pathway, specifically stilbene synthase. Interestingly, there was not an upregulation of CHS, which is the first irreversible step into the phenylpropanoid pathway.[30]

Figure 1.12: Example of Grapevine Fanleaf Virus causing chlorosis in the leaf tissue of Cabernet Sauvignon

Taking together, research looking into the impacts of GFLV on red varieties shows a significant decrease in yield due to cluster weights, which cannot be explained simply through a decrease in vine water status, but possibly by an increase in the upregulation of secondary metabolites in the phenylpropanoid pathway that are known as plant defense compounds.

#### 1.3.1.1.2: Grapevine Red Blotch Virus (GRBV)

Grapevine Red Blotch Virus (GRBV) was first discovered in California in 2008 at the Oakville Research Station in the Napa Valley. Genome sequencing showing that GRBV was related to viruses in the family *Geminiviridae* in 2012 – in New York.[31] This was published while another research paper was in preparation from the University of California, Davis, also looking at the genome of a red leaf disease at the Oakville Research Station which had not been identified as Grapevine Leafroll-associated Virus (GLRaV). This second publication had a nearly identical genome sequencing to that of the first publication. The newly sequenced virus was to be called "Grapevine Red Blotch Virus".[32]

When compared to GFLV, the spread of GRBV can be significantly faster, and due to this, the economic damage can be severe. A recent publication on the economics of dealing with GRBV suggested that the infection of a vineyard can cost as much as $27,740 per acre in the Napa Valley.[33] Rather than having a soil-borne vector such as a nematode, work conducted in greenhouses has shown that GRBV is transmitted through *Spissistilus festinus* (Threecornered Alfalfa Hopper).[34] Furthermore, a recent study has shown that *Stictocephala bisonia* and *Stictocephala basalis* (Buffalo Treehopper) can harbor GRBV, although transmission to clean plant material has not been confirmed.[35] When compared with GFLV, this can present a significant problem, as hoppers can move through vineyards and feed on plants at a pace that is significantly faster than that of nematodes.

In the first paragraph of this section GFLV was discussed as causing a significant reduction in yield, but with an upregulation of secondary metabolites on the phenylpropanoid pathway. Although GRBV does not appear to impact yield in a dramatic way like GFLV or GLRaV, there can be reductions in grape and wine quality. Rather than seeing an upregulation occur in the

phenylpropanoid pathway to enhance defense compounds, the opposite occurs in GRBV-(+) vines. Research has shown a down regulation of shikimate metabolism, which generates phenylalanine.[36] Phenylalanine is the substrate from which all compounds on the phenylpropanoid pathway are generated. Therefore, it can be assumed that secondary metabolites produced from the phenylpropanoid pathway would likely be decreased. Furthermore, there appears to be an impact on the net photosynthesis of GRBV infected plant material. This is likely the cause for uneven ripening that is a key attribute to GRBV, leading to musts with higher acidity and lower sugar levels which has been observed in multiple publications.[27, 37-39]

Strictly from a polyphenol standpoint, the synthesis of PA material does not appear to be impacted, while anthocyanin synthesis significantly declines. Although there is a delay in the maturation of GRBV-(+) fruit, this does not account for the impact that the virus has on the phenylpropanoid pathway, as diseased fruit harvested at a later date but at the same sugar ripeness as healthy fruit had significantly lower anthocyanins levels.[37]

**1.3.1.1.3: Grapevine Leafroll-associated Virus**

Grapevine Leafroll-associated viruses (GLRaV) are widespread and can have significant impacts on infected grapevine materials. Eleven of these viruses have been described in literature, belonging to the family *Closteroviridae*, most of which belong to the genus *Amplerovirus*, with the exception of GLRaV-2, which belongs to the genus *Closterovirus*.[40] Transmission of these viruses is through a mealybug vector (*Pseudococcus longispinus, Pseudococcus Calceolariae, and Pseudococcus Viburni*) after feeding on the phloem of grapevine root systems. Symptoms of GLRaV are visually clear from inspection, with red leaves that have down turned margins in red varieties while white grape varieties show chlorotic symptoms. An example of the symptoms in

Pinot noir can be seen in **Figure 1.13**, where there is obvious upregulation of anthocyanins occurring in the leaves, with the margins of the leaf curling.

Grapevine Leafroll-associated virus impacts the vine by a downregulation of genes associated with photosynthesis. Research looking into the impact of GLRaV on Touriga Nacional have shown an overall decrease in photosynthetic pigments resulting in lower quantum yield, electron transport rate and photochemical quenching.[41] This decrease in photosynthetic metabolic processes is also associated with a repression of the biosynthesis of primary and secondary metabolites.[42] Work has shown that physical parameters of infected vines is also impacted, leading to significant decreases in yield.

Figure 1.13: Example of Grapevine Leafroll-associated Virus in Pinot Noir. Red coloration along with the curling of the marginal tissue on the leaf is a callsign for this virus.

Rootstocks can have significant impacts on the concentration of signaling hormones present, this can make it difficult to compare research papers across the globe where different rootstock/scion combinations are commonplace. Work has shown that the effects of GLRaV is dependent on the specific virus, along with scion and rootstock material. Cabernet Franc that was grafted to two different rootstocks (MGT 101-14 and Kober 5BB) showed significant differences in response to infection. While both rootstocks showed a decrease in overall canopy and pruning weights, 5BB clearly was impacted more severely than 101-14.[43]

There is discrepancy in the results of papers when looking at the overall impact of GLRaV on the phenylpropanoid pathway in grape berries. Vega et al. suggested an overall repression of the pathway leading to lower levels of anthocyanins in berries. This decrease in anthocyanin content, due to GLRaV has been annotated in multiple studies.[42, 44, 45] However, other studies show a lack of impact on anthocyanin content of fruit.[46, 47] Although there is a general lack of impact on anthocyanins, increases in flavonol concentrations are common throughout these studies. This is likely due to decreases in overall leaf matter leading to increases in flavonol concentration due to exposure, which is also likely the cause of increases in heat shock proteins in these studies as well.[48]

The impact of GLRaV on PAs in fruit is relatively limited. Lee and Martin quantified the total tannin of Pinot noir and saw no significant differences between healthy and infected vines.[45] However, given the clear variation of impacts on varieties and rootstocks, a single study may not be enough to definitively say whether this group of viruses can impact PAs.

In summary, the impact of GLRaV on grapevines is relatively fluid. Several studies show dramatic decreases in yield, leaf matter, sugar accumulation, and anthocyanin production while in other studies show contradictory results. In terms of the phenylpropanoid pathway it appears that

GLRaV can have a significant impact on the concentrations of anthocyanins and flavonols. There is limited literature on how these viruses may impact PA concentration and degree of polymerization.

Due to the size of this family of viruses, there are reviews specifically on the different GLRaV's present in grapevines and how they impact gene expressions. Readers who would like to look at specific variants and how they may impact grapes differently should read a recent review by Song et al. focusing on this topic.[49]

#### 1.3.1.2: Fungal Infection

Fungal infections in grapevines are a common occurrence. In general, these are by various species of opportunistic fungi growing in ideal conditions during specific times of year. Cluster and vine infections can have implications across all parameters of primary and secondary metabolite chemistry such as titratable acidity, sugar levels, and total polyphenolics levels**.** Steel et al. reviewed various species of bunch rot and their specific impact on fruit and wine quality in depth.[50] Although these fungi can impact more than just the phenylpropanoid pathway, as well as enzymatically hydrolyze different types of 'tannin' such as gallotannin and ellagitannin (found in oak barrels), here only implications having to do with anthocyanins and PAs will be discussed.

Flavonoids are not only a way to attract mammals for seed dispersal, such as anthocyanins, but they are also a defense mechanism for plants. Due to this, when plants become infected, or recognize a foreign pathogen, the phenylpropanoid pathway becomes upregulated to enhance the defenses of the host. This is true for infections of the wood, stem, leaf, or fruit material. Depending on the area in which infection is located, variable genes will become upregulated to enhance plant immune response. Generally, these upregulations having to do with infection are related to

phytohormone regulation as well as secondary metabolite pathways, such as the production of stilbenes, lignins, and flavonoids

**1.3.1.2.1: Botrytis Cinerea**

*B. Cinerea* is one of the major fungal species leading to grape infection in wine grape production, leading to significant yield and quality losses globally due to gray mold. Early infection, occurring during flowering and berry development, leads to the upregulation of the phenylpropanoid pathway.[51] This upregulation leads to increases in PA material as the plant attempts to combat localized infections. In the early stages of development, the upregulation of PAs and stilbenes can effectively fight off the infection.[52] However, in the later stages of ripening, the plant is relatively ineffective at fighting infection from gray mold pathogens. An oversimplified view of the situation would be that the inherent upregulation of pathways known to be correlated with high quality wine production would be a positive. This view doesn't consider the fact that the laccase enzyme produced by *B. Cinerea* can have huge impacts on wine quality, specifically on flavonoids in red wine production. Laccase can oxidize catechols, like that of polyphenol oxidase (PPO), to an

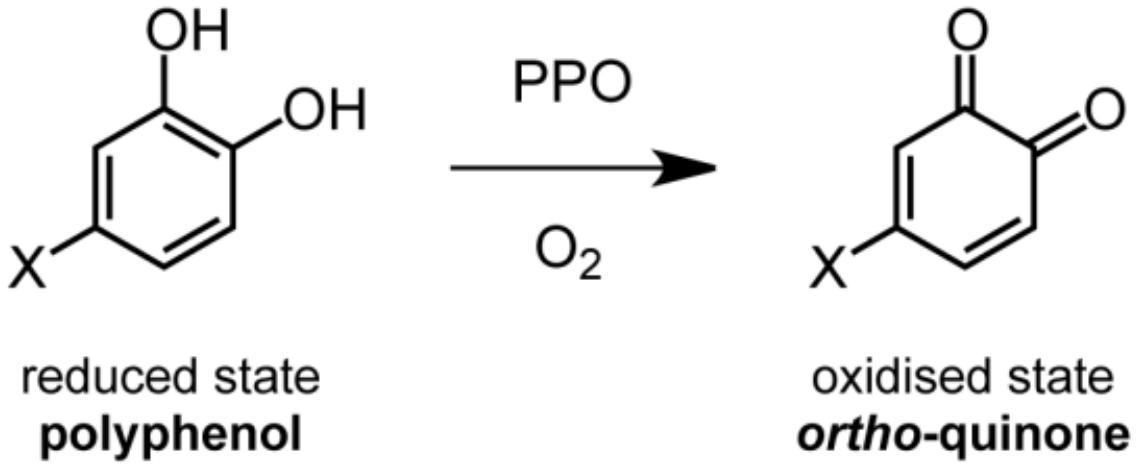


Figure 1.14: Catechol oxidation by Polyphenol Oxidase (PPO)

orthoquinone (**Figure 1.14**).[53] Because many of the flavonoids within a wine system contain a catechol B-ring, there can be a rapid oxidation of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, cyandin-3-O-glucoside, petunidin-3-O-glucoside, delphinidin-3-O-glucoside, and more. For the flavan-3-ols, this can lead to advanced browning of a wine, while the anthocyanins will become discolored leading to a loss of red wine color.

Susceptibility to various infections from fungal pathogens has been linked to the regulations of phenylpropanoid pathway. An American-hybrid variety known as 'Norton' has been shown to have significantly higher upregulation of the phenylpropanoid pathway in comparison to the European variety Cabernet Sauvignon.[54] When inoculated with *B. Cinerea*, Norton had a disease incidence of 7.5% while Cabernet Sauvignon had a disease incidence of over 90%.[55] These results highlight the importance of the phenylpropanoid pathway in pathogenic infection defense against *B. Cinerea* and other fungal pathogens which can impact wine and grape quality.

### 1.3.2: Abiotic Stress

Abiotic stress is caused by the local environment the plant is subjected to regarding atmospheric climate as well as soil conditions. Water status of plants is dynamic because it reflects multiple inputs – wind, humidity, temperature, leaf area, soil, and rootstock all impact the overall plant water status. Light and temperature are hard to unravel at times because the act of increasing light exposure intrinsically increases the temperature. Although excessive amounts of any abiotic stress can be deleterious, moderate amounts can be beneficial.

Chapter 2 below seeks to observe if any measured abiotic stress can be related to PA and/or anthocyanin concentration by evaluating the composition of many Cabernet Sauvignon berry samples from Napa Valley, selecting vineyards with good availability of abiotic stress measures.

#### 1.3.2.1: Water

The quote "Whiskey is for drinking, water is for fighting", generally attributed to Mark Twain, has never been as true as it is today. In a time of extreme weather, unprecedented deforestation, and increases in desertification, clean water is becoming scarce. In California, depending on the source, approximately 40% to 60% of the water is used for agricultural purposes with vine crops using approximately 1.9 acre-feet (619,177 gallons) per acre planted.[56] Although farmers have become more efficient in water use in the southwest, current environmental conditions suggest the problem of water availability will become worse in the southwestern United States.

It is an over generalization to say that grapevines are drought resistant. *Vitis sp.* have evolved in a variety of environmental conditions across millennia. Due to this, depending on where they evolved or how they were crossbred, they have different levels of drought tolerance, as well as differing mechanisms to combat dry conditions. In the wine industry, rootstocks have historically been used to prevent damage from the root louse phylloxera. Most grapes throughout the world are grafted with scion material from Europe, with rootstock from the United States. The common comparison on drought sensitivity would be between the drought tolerant *V. Rupestris* ("St. George"), naturally present from north Texas to the Chesapeake Bay, and the drought sensitive *V. Riparia* ("Riparia Gloire"), naturally present from Kansas to Maine.[57] A more in-depth review focusing on the history of rootstocks was done by Ollat et al., which is outside of the scope of this review.[58]

Although the history of rootstocks is not within the scope of this review, nonetheless how they respond to drought stress, the corresponding signaling of that stress, and the impact it can have on the phenylpropanoid pathway, and wine, is of importance.

Water stress occurs when the plants water demand exceeds water availability in the soil due to lack of soil moisture. When this occurs, ABA is biosynthesized in the root system and transported to other plant tissues to signal that there is a lack of available water. ABA acts as an anti-transpirant, interacting with the guard cells of the stomata and causing them to close and prevent excess water loss through transpiration.[59] If water is unavailable it can lead to cavitation and embolism within the xylem, decreasing the ability to function through lower hydraulic conductivity. If the plant cannot find water it will begin to shed leaves, and eventually die.

Regulated Deficit Irrigation (RDI), or Water Deficit Irrigation (WDI), is a technique used in the wine industry to a impose a specific amount of water stress onto a grapevine, without damaging the vine due to excess stress. Monitoring of water in a vineyard can be done by means of soil moisture probes, sap-flow sensors, porometers, and pressure bombs. Historically pressure bombs are the easiest to use and require the least amount of technical knowledge, however they can be difficult to use across large amounts of acreage. This is because midday water potential measurements are supposed to be taken at solar noon, leaving little time to travel from vineyard to vineyard.

Water deficits imposed at differing times can be used to achieve differing goals. When a water deficit is sensed by the plant, it tends to produce less vegetative matter. This can be visualized through the drying of the apical material at the shoot tips, leading to an end of the vegetative growth stage. If imposed pre-veraison, it can lead to decreases in berry size due to lower turgor in the skin tissue, preventing excessive expansion. This can lead to lower disease incidence through lower cluster compaction. Lastly, it is regularly used during the ripening phase to increase the production of secondary metabolites in the skin of ripening berries.

In **Section 1.4** research papers focusing on flavonoid production in the context of water deficits will be discussed along with timing and levels of water deficit imposed to illicit a plant response.[60-65]

### 1.3.2.2: Light

Light interception and carbon fixation are described as the light and dark reactions in plants. Photons of light are turned into chemical energy through photosystems I and II in the chloroplasts through 'light reactions', while carbon dioxide is turned into photosynthate through oxidation and reduction reactions. This is an incredibly simplistic view of a process that is the foundation of life on Earth. Light interception leads to carbon assimilation, leading to the production of biomass in the plant, which is then used, in our case, to make a delicious product known as wine from grapes (**Figure 1.15)**. Why is this under abiotic stress?

Although light is necessary, plants that have not adapted to excessive amounts of ultraviolet (UV) radiation can be overwhelmed by the light incidence, leading to the formation of reactive oxygen

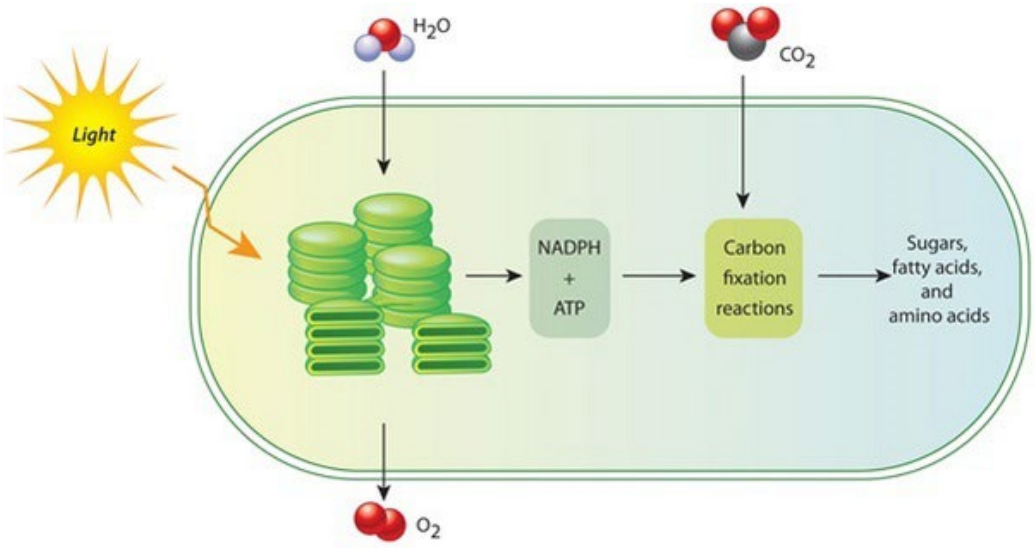


Figure 1.15: Light capture by plants

species (ROS) in the chloroplast. Overproduction of ROS species such as $H_2O_2$, $HO^{\cdot}$, and $O_2^-$ can cause oxidative stress in the leaves and fruits of plants. Generally photobleaching of plant material is not seen in vineyards, but in gardens or greenhouses when plants have not acclimated to an abundance of UV light it can lead to an overproduction of ROS species and chlorophyll bleaching. In vineyards light related scorching can occur, particularly in areas of high UV radiation, such as high-altitude vineyards. Although excessive light interception can cause bleaching or scorching that is not heat related, light can have positive impacts on the plant and fruit, in terms of wine quality.

Grapevines are just that – *vines.* They are climbers by nature; in riparian zones they can be seen climbing the canopies of trees, searching for sunlight. Differentiation of buds in the nodes of grapevines is only induced when the plants reach the top of the canopy, where increases in light incidence signal for the differentiation of the buds to produce inflorescence, and therefore progeny.[66] This evolutionary trait can be seen in vineyards as well. Increases in light incidence, particularly in the spring, can impact bud fertility for the following year. Furthermore, early light incidence can lead to increases in the thickness of the epidermal layer, enhancing the ability of fruit to deal with increased light exposure later in the growing season.[67, 68]

Although increases in light interception generate ROS species in the chloroplasts, it can also upregulate parts of the phenylpropanoid pathway. This allows for fruit acclimation to the increased incidence, decreasing the sunburn incidence of exposed fruit. For this reason, light exposure is carefully managed in high quality vineyards.

**1.3.2.3: Temperature**

Humans are currently witnessing one of the greatest threats to biodiversity and life on Earth in real-time. Abundant greenhouse gases in the atmosphere is leading to unprecedented increases in

ambient temperatures. These increased temperatures are leading to more extreme weather patterns, increased fire damage around the world, and a decrease in the already-strained water supply. This section is to briefly introduce the reader to how plants deal with heat events, and how they act as an abiotic stress to plants. Although there are extreme cold events taking place throughout the world as well, this will mainly focus on impacts of increased heat in the vineyard. Below, in **Section 1.4**, a discussion of how management techniques can impact the outcome of these heat events in the context of flavonoids and the phenylpropanoid pathway is provided.

Heat events and elevated temperature can cause the degradation of photosystem II, which is relatively sensitive to elevated temperatures, leading to a decrease in the electron transport chain efficiency and overall lower carbon assimilation.[69] Furthermore, sunburn and necrosis occur when the plant's ability to deal with generated ROS species is exceeded by the amount of ROS present. Plants have multiple ways of dealing with such species, such as heat shock proteins, enzymatic activity like superoxide dismutase, the xanthophyll cycle, ascorbate, glutathione, phenolics, and more. In a recent study on Syrah, extreme heat led to a decrease in soluble solids in exposed control when compared to shaded treatments during an extreme heat event in the 2013/2014 growing season, which the control never fully recovered from.[70]

Plants can cool themselves down through evaporative cooling, which happens as water loss through the stomata during transpiration, leading to a cooler microclimate around the canopy. However, differences in evolution have left different varieties with or without a coping mechanism for heat events. An example of this is in a paper published looking at the photosynthetic activity of Semillon under a heat event, wherein photosynthesis was inhibited by as much as 60% at 45 °C.[71] Another study looking at the stomatal control between different varieties showed, through thermal imaging, that leaf temperature was dramatically different between the varieties. The

authors also showed that there were differences in stomatal conductance showing that there is likely a variety-level control on stomatal closure. In this work Touriga Nacional was resistant to the closure of stomates, leading to a decreased canopy temperature through increased evaporative cooling.[72]

Although adaptation to heat events is an important parameter to consider when planting a vineyard, in recent history there have been devastating heat events occurring in areas of the world where established varieties have been planted for centuries. In July of 2019 a heatwave hit France causing a significant amount of sunburn to red Pinot noir grapes. In parts of Burgundy temperatures reached 42 °C, 16 °C higher than normal daytime temperatures in July. This, along with extreme frost conditions in the same year, led to a 40% reduction in yield in some areas of Burgundy.[73]

Sunburn and sun necrosis are caused by the exposure of clusters to light along with high ambient temperatures. Which can lead to devastating losses in yield and quality. A 2017 study on mitigating the impacts of heat events showed that temperatures in the Napa Valley were above 40 °C five times in a single season. In this study exposed fruit had 24% of the clusters damaged by sunburn and necrosis, while shaded fruit had 2%.[74]

### 1.4: Understanding the Impact of Vineyard Management on Flavonoids

Variables controlling the production of secondary metabolites from the phenylpropanoid pathway are controlled by factors such as variety, abiotic, and biotic stressors. Abiotic stressors such as temperature, water stress, and ultraviolet radiation can have significant impacts on flavonoid production, and can, to some extent, be manipulated through vineyard management practices. While biotic stressors in a vineyard are something to be prevented or eliminated because of the nearly universal negative impacts they can have, abiotic stressors can have positive impacts if managed properly. In the previous section the focus was on three types of environmental variables

that can impact the phenylpropanoid pathway. These factors, along with economic goals for yield, targeted market, and style also play a role in choices in vineyard management that will affect the synthesis of flavonoids. Management decisions dealing with abiotic stressors are like levers, for a viticulturist, which can be pulled or left alone depending on variety, local environment, wine style, economics, or overall risk. These levers, when pulled, can increase the quality of the fruit, but can also have serious, destructive, consequences if implemented at the wrong time. Timing of these decisions, and knowledge of what is occurring at that plant stage, determine the outcomes.

It has not been reported as to why certain environmental factors have variable impacts on the phenylpropanoid pathway depending on the timing or development stage of the vine. However, it can be speculated that this is due to variations in phytohormones, and how they specifically interact with parts of the phenylpropanoid pathway, thus causing environmental interactions. An example of this has been shown by spraying forchlorfenuron (CPPU), a cytokinin-like molecule, on Thompson Seedless grapes after full-bloom, which caused tannin concentrations to increase 4-fold. This increase in tannin was still present in the final product at commercial harvest, causing significant changes in the astringency of the table grapes.[75] Furthermore, abscisic acid (ABA) has been shown to clearly impact the production of anthocyanins. The exogenous application of ABA after veraison will cause all the fruit to increase the production of anthocyanins rapidly.[76] It is also known that the practice of deficit irrigation can increase ABA signaling. Understanding the timing of these hormones, the causation of upregulation, and how their upregulation impacts flavonoid production is a tool for viticulturists to positively impact grape components and wine qualities.

### 1.4.1: Yield Impacts on Flavonoids Concentrations

Impacts of yield in grapevines is seemingly a tense subject matter to discuss with viticulturists. This is because it is generally used to describe an innate sense of wine quality that can only be

achieved in vineyards with little fruit load. Rather than discuss whether yield per acre is a determining factor of overall fruit or wine quality. The focus of this section will be on the impacts of overall yield and crop thinning on secondary metabolite concentrations and composition in the fruit and subsequent wine.

Overall vine yield is a parameter that is defined by the size of the vine. A grower's determination of goals for yield can be based on disease potential, vineyard aesthetics, climate and economics - however, within a square meter of ground, only so much fruit can be produced. Climate and disease potential are of relative importance. In cooler climates, where attaining sugar ripeness may be difficult, cluster thinning can be an exercise to enhance the rate of ripening. Multiple studies have shown that decreasing the overall fruit load on a vine can increase the sugar loading in the remaining fruit.[77-79] Furthermore, depending on the variety, cluster thinning can decrease the severity of disease by opening the fruiting zone. Although thinning may increase air flow and decrease disease severity, it can also increase berry size. In varieties with compact clusters, a grower may *enhance* the amount of bunch rot through overly compact clusters due to increases in berry size (size compensation) post-thinning, when conducted too early in the growing season.[80] Therefore, it is important for growers to assess the positives and negatives before deciding on thinning.

A thinning experiment in Victoria, Australia on Cabernet Sauvignon had a control that had an estimated yield of 23 tons/acre, while the thinned treatment was farmed at 10 tons/acre. Thinning was conducted by hand and machine post-set when fruit was pea-size. This experiment showed a clear separation between the thinned vines and the control in anthocyanin and phenolic concentrations as well as increases in soluble solids.[81] In contrast, an experiment in Chile looked at the impact of cluster thinning Syrah. In this instance the control was farmed at 3.25 tons/acre

while the treatment yielded 1.62 tons/acre. Overall, there were no differences in this experiment concerning total anthocyanin concentration in the skins, however there were significant differences in the proportions of anthocyanins accumulated. Similar to the results seen by Guidoni in Nebbiolo, decreasing yield through thinning shifted the phenylpropanoid pathway to increase the proportion of dihydroxylated anthocyanins rather than trihydroxylated anthocyanins such as malvidin.[79, 82] The results of these two experiments may suggest a law of diminishing returns when contrasting the differences in the control and treatment yield levels. Where there was a clear difference in anthocyanin synthesis in the Australian experiment, there was little impact in the Chilean experiment which started with a much lower fruit load.

Work looking into the impact the overall impact on wine quality due to fruit thinning trials shows that thinning has utility in improving phenolics. A trial conducted looking at the impact on wine phenolics and aromatics following a thinning trial on Syrah (2.32 kg/vine vs 1.68 kg/vine) showed significant differences in phenolics and aromatics after fermentation and after seventeen months. The results include increases in overall phenolics, anthocyanins, and aromatics. A multivariate analysis performed from the results of gas chromatography clearly separated the control and treatments based on aromatic composition.[78] Another trial looking into thinning of Pinot noir in New Zealand showed similar results. In this experiment two levels of thinning (moderate and intense) were conducted just before veraison over three vintages. Although the lowest fruit load was approximately 1.5 tons/acre while the highest was 3.5 tons/acre there were still significant differences. Increases in wine polyphenols included significant differences in PA concentrations, with up to 36% increase in the intense thinning treatment. There was also a treatment effect on anthocyanin concentrations, wherein the thinning treatments led to increases in wine anthocyanin concentration every vintage. Furthermore, sensory trials on the wine showed significant

differences where wines from the thinned treatments were evaluated as being more fruity and sweet, while those from the control were determined to be herbaceous and acidic.[83]

Although these experiments do not factor in the economics behind decreasing yield to increase secondary metabolites, yield management can be used as a tool to have a positive impact regarding the concentration of anthocyanins in berries and wine. In cooler climates yield management can help enhance the rate at which sugar accumulation occurs in the fruit, allowing for ripeness to occur earlier, before plant senescence. Lastly, there can be a decrease in bunch rot incidence when cluster thinning is applied. However, in some varieties berry compensation can occur if cluster thinning is conducted before the end of periclinal cell division, leading to increased berry size and compact clusters. In cases of compact varieties, it is not recommended to conduct cluster thinning or yield reduction until lag phase.

### 1.4.2: Water Deficit Impacts on Flavonoid Concentrations

Water is a scarcity in farming. The advent of drip irrigation allowing for the precise management of volume and timing of application has created an entire area of research focused on irrigation management in vineyards. A vineyard management process known as Water Deficit Irrigation (WDI) is commonplace in the industry today, wherein the water stress of a vine is monitored through stomatal conductance or stem water potential to evaluate when to irrigate. In years when there isn't a drought, which are seemingly becoming rarer in California due to climate change, the soil profile has water which will sustain the vine through the initial part of the season. This is important because vines are under little to no abiotic stress in the early part of the season, which is also during the development of PAs.

Environmental factors impacting the phenylpropanoid pathway during ripening phase, such as water stress, have different impacts on the phenylpropanoid pathway prior to ripening phase,

during tannin production. Although there is clear evidence for the increases in accumulation of anthocyanins through early and late WDI treatments, this is not the case for PAs.[84-87] This suggests that the environmental drivers enhancing the synthesis of anthocyanins do not necessarily impact the phenylpropanoid pathway in the same way concerning PA synthesis.

Soil drying and water deficits lead to the release of ABA by the roots and leaves through impacts on transpiration. When soil water availability is unable to keep up with the transpiration of the plant, ABA is released in the leaves causing a closure of the stomata to prevent excessive evaporation.[88] Research evaluating the impacts of WDI on the metabolic pathways in Cabernet Sauvignon showed that there was nearly a 2-fold increase in the concentrations of ABA within the berries, which was correlated to increases in sugar and anthocyanin concentrations.[89] This same effect has been shown in multiple other studies looking at other varieties as well as multiple rootstock/scion combinations.[84, 87, 90-92] Therefore there is clear evidence to support the use of WDI in the context of increasing anthocyanin concentrations, with little to no evidence to suggest it impacts the phenylpropanoid pathway the same way pre-veraison. This is likely due to the signaling from the root system. Earlier it was discussed that exogenous spraying of cytokinin-like molecules (CPPU) can have significant impacts on the concentration of PAs in grape skins. It is likely that water deficit does not have a large impact on PAs due to the release of ABA rather than cytokinin's. Considering that ABA promotes anthocyanin production, it is clear why water stress can also increase the concentrations of these flavonoids.

Taken together this suggests that in irrigated vineyards, imposing an early season deficit will impact yields through decreases in berry size, while a deficit after veraison will have impacts on anthocyanin accumulation within the berries. Multiple studies suggest that an early and long-lasting water deficit leads to a larger effect on the phenylpropanoid pathway in terms of overall

anthocyanin synthesis. WDI taken too far can have catastrophic impacts on the health of the grapevine, but when done in a controlled manner, it can increase grape quality and decrease vegetative growth depending on the timing of application. From a viticulture management perspective there is a clear, positive, impact on red grape and wine quality from the use of less water. In the context of climate change, this should be a consideration for growers when balancing out yield objectives for specific blocks in terms of stylistic wine objectives.

### 1.4.3: Fruit Sun Exposure on Flavonoid Concentrations

The management practice of increasing sun exposure comes with the risk of increasing abiotic stress beyond the plant's capacity to deal with it. Within the abiotic stress section, the formation of ROS species in the cell was discussed, along with the incidence of sunburn or scorching from both heat and UV light interception. In addition, it was noted that timing of thinning impacts sunburn severity due to acclimation of fruit to light attenuation. In this section the management practices associated with the increase in exposure and their impacts on secondary metabolite synthesis will be addressed.

In the previous section WDI was discussed as positively impacting the production of anthocyanins through increases in ABA during the ripening phase. In this section most of what will be discussed in pre-veraison impacts on the phenylpropanoid pathway through cluster light attenuation by increasing leaf layers in the fruiting zone. Post-veraison impacts on exposure will also be discussed concerning the production of flavonols and anthocyanins in the skin of grapes. In **Section 1.2**, the phenylpropanoid pathway was shown and discussed as having variable functions depending on the period or phase of growth the plant is in. This is due to up or downregulation of transcriptional factors leading to enhanced activity of different parts of the phenylpropanoid pathway.

An experiment looking into light exposure impacts on Cabernet Sauvignon in young berries showed significant differences in PA concentration and composition. Fruit that was more exposed to light had lower levels of galloylation in the skins, increased levels of trihydroxylated subunits (epigallocatechin), a higher degree of polymerization, and an overall increase in PA concentration. Furthermore, there were 4-fold increases in flavonol concentrations in the exocarp of the berries. Real-Time PCR analysis was used to look at mRNA regulation of the phenylpropanoid pathway and how it was impacted by exposure on clusters. Namely, there were significant differences in leucoanthocyanidin reductase (LAR), chalcone synthase (CHS), anthocyanidin reductase (ANR), flavonoid 3'5' hydroxylase (F3'5'H), and flavonol synthase (FLS). Put simply, increases in FLS explain the increases in concentration of flavonols. LAR and ANR are directly related to the synthesis of PAs, therefore are directly linked to the increase in concentration of PAs. In terms of subunit composition – F3'5'H is directly related to the hydroxylation pattern of the B-ring on flavonoids. Therefore, the increases in trihydroxylated PA subunits in exposed fruit is directly linked to F3'5'H upregulation.[93] Similarly, another study conducted by the same group looking at impacts on the phenylpropanoid pathway during the ripening phase showed significant differences. In this study, multiple treatments of exposure were conducted pre- and post-veraison to look at the impacts on flavonoids. There were significant increases in anthocyanins and flavonols of the exposed fruit. Correspondingly, there were also transcriptional differences in both FLS and anthocyanidin-3-O-glucosyltransferase (UFGT) which stabilizes anthocyanidins through the addition of a glucose ester at the 3-position.[94] In some ways this is contradictory to results from other studies. Studies looking at exposure in red wine grapes during the ripening phase have shown clear increases in flavonols, as seen in the Koyama and Goto-Yamamoto 2008 work along with other studies.[94-96] However this is not always true for anthocyanin concentrations. Although Cortell

and Kennedy saw a 32% difference in anthocyanin concentration between exposed and shaded fruit, it was not significant with a 95% confidence interval.[96] Similar results from other authors exploring the influence of exposure on anthocyanin concentration, where the relationship between exposure and anthocyanin synthesis is not directly correlated, like that of flavonols.[95, 97, 98] Oddly, in the work by Dokoozlian and Kliewer in 1996, it was shown that light in the pre-veraison stage of berry development impacted the maximal amount of pigment production possible in wine grape skins, suggesting that early exposure can impact color development.[99]

*Why* light exposure impacts the phenylpropanoid pathway has been addressed above, but *how* this is implemented over the course of the growing season was only superficially discussed at the end of the last paragraph. When discussing exposure and leaf layers from a viticulture management perspective the discussion must be row orientation and mesoclimate. Row orientation can have clear impacts on exposure due to topographical limitations and the practicality of farming. This can lead to overexposure of one side of the canopy, leading to the scorching of fruit if managed improperly. Furthermore, regional climate needs to be taken into consideration when applying leaf thinning treatments in real-world scenarios. Many of the cited papers above have tried to separate light and temperature interactions using blowers in the vineyard. This is clearly not practical in commercial vineyards and therefore care must be taken because increased exposure will lead to significant increases in berry temperature.

Leaf thinning is commonplace in a vineyard as part of canopy management strategy. Depending on the timing and extent of this thinning, the effects on secondary metabolites can differ. Work

conducted looking at Cabernet Sauvignon, Nero D'Avola, Raboso Plave, and Sangiovese showed no impact on anthocyanin concentrations when leaf thinning was conducted at veraison, and in some cases there were lower levels of anthocyanins present in the skins. There was also a significant increase in the incidence of sunburn when leafing was done at this stage, particularly in Raboso Plave and Sangiovese. Furthermore, there was an impact on the ratios of di and trihydroxylated anthocyanin ratios depending on the amount of exposure and variety. Suggesting that leaf removal at veraison can impact the hydroxylation patterns of anthocyanins regardless of impact on concentration.[100] **Figure 16** is an example of the amount of sun necrosis that can occur when leaf thinning is conducted too late in the growing season.

Figure 1.16: Sun necrosis due to overexposure from a late season leafing pass in Cabernet Sauvignon

In contrast, a pre-bloom leaf thinning trial on Sangiovese showed opposite results. This treatment showed significant increases total phenolics and total anthocyanins when compared to the control, suggesting that early leaf thinning can have impacts on fruit quality in later stages of development.[77] Another study looking at defoliation timing of 5 different varieties showed that pre-flowering leaf thinning had the most significant impact on anthocyanins in wine.[67] These

works are in agreement with the earlier work by Dokoozlian and Kliewer suggesting that the timing of leaf thinning is important to secondary metabolite impacts.[99]

A common technique for preventing overexposure is using shade cloth in the fruiting zone to decrease light incidence and temperature. Recent work by Martinez-Luscher et al. showed that the application of a black shade cloth that blocked 40% of light incidence increased the total anthocyanins present in the skins by fresh weight. Their argument for the results is that the light-temperature continuum in real-world situations leads to degradation in overexposed berries, causing a degradation of anthocyanins if over exposed.[101] These results are in a way an echo of those presented by Bergqvist et al. in 2001, where exposure in a hot climate, the San Joaquin Valley, was experimented on with Cabernet Sauvignon and Grenache. This work showed that in an east-west orientation, increasing exposure on the north side of the canopy led to increases in anthocyanin production. However, overexposure on the south side of the canopy led to an overall decrease in anthocyanin concentration.[102]

Although there are clearly positive impacts on the phenylpropanoid pathway due to increases in light exposure, a grower must proceed with caution when determining to leaf thin. The greatest impacts of thinning will be had through earlier removal of leaves. If this technique is used later, closer to lag phase, there is a greater risk of sunburn incidence due to lack of grape acclimation to light exposure. Temperature impacts on flavonoids and the phenylpropanoid pathway will be discussed in the next section. When reading the impacts of temperature, the reader should combine light and temperature effects to understand the implications for a management strategy. As extreme heat events become more frequent due to global warming, the removal of leaf matter in the fruiting zone presents increased risk of sunburn.

Based on intriguing results in the first phase of this research, Chapter 3 below reports on an investigation into the effect of sun exposure on flavonoid composition in Cabernet Sauvignon fruit.

### 1.4.4: Temperature Impacts on Flavonoids in Grape Skins

As discussed above, increases in temperature can lead to significant production of ROS species within the cell, causing damage to the cuticle layer, exocarp layer, and in some cases causing stem necrosis and total loss. Although the section above was describing yield losses due to the stress constraints of the plant, here the focus will be on the phenylpropanoid pathway. Unlike the other variables discussed in **Section 1.3**, temperature is hard to manage outside of overhead sprayers, under vine misters, large irrigation regimens, particle film treatments, or shade netting.

Additionally – from a research perspective the separation of light exposure and fruit temperature can be difficult to separate. In a perfect situation a vineyard would have full exposure without overheating the fruit on the vine. As discussed in the previous subsection, light can be beneficial to the production of anthocyanins, flavanols, and flavonols. However, light can dramatically increase the surface temperature of red wine grapes. In the previous subsections the idea of decreasing irrigation and increasing exposure suggest that these are paramount in grape and wine quality, however, both taken to extremes can increase leaf and fruit temperature, leading to deleterious effects on quality. Furthermore, complete destruction of fruit through scorching is not required to have these effects. Over-exposure leading to increases in berry temperature has been shown to have negative effects on berry anthocyanin concentrations at harvest in Cabernet Sauvignon during a shade cloth study.[101] Excessive temperature can lead to the loss of anthocyanins in red grapes causing the pinking of fruit and an overall decrease in wine quality. Research on cyanidin-3-glucoside degradation to protocatechuic acid in black rice (*Oryza sativa* L.) has shown that it can also be sensitive to temperature increases.[103]

The negative impacts of temperature can also have significant impacts if a heatwave occurs in the early part of development. Early season scorch or sunburn can impact the fruit's ability to mature and produce color. In **Figure 1.17** uneven ripening can be seen between the eastern and western exposed bunches.[104] In this case heat damage to the fruit caused significant amounts of uneven ripening based on canopy direction. This highlights the importance of leafing decisions based on row-direction, trellis design, and understanding everything that encompasses growing grapes on a specific site.


Figure 1.17: Uneven ripening induced by early season sunburn on Cabernet Sauvignon. Photo credit: Gambetta et al. 2021

Outside of the degradation of anthocyanins elevated temperatures can impact the phenylpropanoid pathway through changes in transcription factors. An experiment on Pinot noir with two treatments increasing temperatures throughout the daytime (30 °C and 35 °C) showed that as temperature increased there was a decrease in F3'5'H and an increase in F3'H. This led a decrease in trihydroxylated anthocyanins such as petunidin-3-O-glucoside, malvidin-3-O-glucoside, and delphinidin-3-O-glucoside. Furthermore there was a decline in total anthocyanins at the end of the experiment, suggesting a degradation of anthocyanins in the heated treatments.[105] A similar experiment

conducted by another group also looked at anthocyanin synthesis across the growing season under elevated temperature conditions. In this experiment there was a significant difference between the total anthocyanins produced in berries at 20 °C then at 30 °C. There was also significantly more ABA present in the skins at 20 °C when compared to the 30 °C treatment.[106]

Increases in nighttime temperatures can be a common occurrence in mountainous regions that are susceptible to inversion layers, as a stream of cool air pushes underneath an area of warm air. This can lead to excessive nighttime heat in mountainous areas during the grape growing season, particularly during heat events.

In 2005 Mori et al. designed an experiment looking at the impact of elevated nighttime temperatures on anthocyanin biosynthesis. As the nighttime temperature was increased to 30 °C and compared with a 15 °C nighttime temperature, there was a decrease in anthocyanin synthesis. This led to significantly lower anthocyanin concentration in the higher nighttime treatment. The data was backed up by a gene expression analysis showing that PAL, CHS, F3'H, DFR, LDOX, and UFGT were all upregulated with a lower nighttime temperature compared to the treatment.[107] These results suggest that cooler days and nights are better to produce anthocyanins in wine grapes. These studies also showed the degradation of anthocyanins occurring when exposed to elevated temperatures during the day.

Impacts of day and night temperatures on PAs is less clear. Work conducted by Cohen et al. investigated heating, cooling, and reduced diurnal shifts and how these treatments impacted PA synthesis. Although there were differences in the rate at which fruit ripened, which was enhanced by diurnal compression, there was an increase in dihydroxylation substitution, on the B-ring, due to increased F3'H expression. Outside of these findings there was little impact on PA synthesis having to do with day and night temperatures.[108] Similar to the impacts discussed on light incidence

and water stress – the timing of these inputs appears to have an impact on how they alter the synthesis of flavonoids.

## 1.5: The Extractability of Proanthocyanidins

The extractability of flavonoids into a wine solution is important to the outcome of any red wine. Historical work has shown that overall tannin in grape skins decreases throughout the ripening period while the lignification of the seed coat decreases the extraction of seed tannin. Surprisingly, it is still not possible to predict wine tannin composition from fruit tannin composition. Recently a model has been built to estimate the extraction of polyphenols in a fermentation vessel with some success.[109] Although this may have utility for the wine industry in the future, there are challenges that need to be addressed concerning differences in cell wall material (CWM) and protein concentrations. Recent work has shown a direct correlation between the amount of PA extracted into the system and some specific pathogen related proteins in the skins.[110] This can have deleterious impacts on wine quality as the protein-PA interactions leads to precipitation of the PA material.

Anthocyanins have been suggested as impacting the extractability of PAs into a wine solution. Initial work regarding this theory was conducted in 1992 by Trousdale and Singleton wherein PAs and anthocyanins were added to white grape fermentations and fermented as a red wine. The results showed that as the concentration of anthocyanins was increased, there was an increase in the amount of PA material in the system. The authors postulated that this was due to incorporation and increases in solubilization of the PA material, leading to higher levels remaining in solution. The idea of anthocyanins impacting the amount of PAs extracted, or remaining, in a wine system was investigated by Oberholster et al. in 2009. This work showed that the addition of anthocyanins to a must has significant impacts on the mouthfeel characteristics of a wine following 6 months of

aging. Although there were significant differences in sensory aspects of the wine through the addition of anthocyanins, there was not a significant difference in PA concentration when looking at the white wine fermented on pomace with or without anthocyanins added.[111] The idea of anthocyanins impacting the extraction of PA material was explored by Kilmister et al. in 2013 where grapes with various levels of high or low PA and high or low anthocyanins were used for winemaking. The results from this experiment suggest that anthocyanins were a determining factor in the amount of PAs that were extracted into the wine. Grape samples that had low skin tannin levels but high anthocyanin levels appeared to have a similar amount of tannins in the finished wines as the grapes which had higher tannin and anthocyanin levels, possibly through the increase in solubilization of PAs through chemical interactions.[112]

Further work looking into the impact of PA extractability enhancement through the presence of anthocyanins was explored by Bindon and Bautista-Ortin.[113-115] These investigations confirmed that there is an enhancement to the extraction of PAs in a dilute ethanol solution, however, it also led to more questions about why anthocyanins impacted the extraction of skin PA extractability in certain situations, but not others. In the work by Bautista-Ortin it was not only shown that anthocyanins did enhance the extraction of PAs after interaction with CWM, but also that the PAs were lower in molecular mass. This suggests that the lower molecular weight PAs were more easily released from the CWM due to anthocyanin-CWM interactions, that anthocyanins were modulating the absorption of larger molecular weight PAs, or that the PAs were changing the molecular weight by their reaction with the anthocyanins.[116]

Cell wall material has shown to change throughout the course of the growing season. These changes include the deesterification of the galacturonans in the pectic polysaccharides in the non-cellulosic fraction of the skin CWM.[117, 118] This deesterification is of importance because the

formation of galacturonic acid can induce partial negative charges via the carboxylic acid functional groups at the typical pH of a wine or must, 3.5. Recent work has shown that changes in the esterification of the galacturonic acids, particularly if they have side chains, impacts anthocyanin interactions and stabilization. These results looking into the impacts of anthocyanin-CWM interactions may explain the enhancing impact seen in the previously described works when discussing PA extraction.[119] Nevertheless, this is a highly complex problem which also involves the affinity for CWM by both PAs and anthocyanins.

Isothermal titration calorimetry was conducted using isolated apple PAs with varying degrees of polymerization were studied in concert with homogalacturonans that had varying levels of esterification. The results from this experiment showed that there were increases in the thermodynamic interactions between larger PAs and homogalacturonans with higher levels of methyl esters.[120] In the context of ripening grapes, fruit that is riper will have a smaller proportion of methyl esters, and therefore likely have weaker interactions with PA material extracting during the maceration period. So, while the availability of PAs during ripening decreased as noted above, the extractability would appear to increase, leading to higher levels of PAs in wine made from riper grapes.

As a result of the sun exposure study described in Chapter 3, the next phase of my research directly investigates the effect of anthocyanin addition on tannin extraction in winemaking, and by using complementary tannin measures, attempts to resolve some of the complications noted above, as reported in Chapter 4.

# Chapter 2: Napa Valley Cabernet Sauvignon Proanthocyanidin Changes During Fruit Ripening: A Multi-Appellation Survey

## 2.1: Abstract

In 2015 an experiment was designed to investigate the distribution and variance of changes in flavonoids across the ripening phase of fruit development in Napa Valley. This Cabernet Sauvignon experiment was intended to evaluate the polyphenol differences across the Napa Valley in order to understand parameters controlling 'tannin activity'. This relatively new method had shown promise in understanding proanthocyanidin (PA) astringency based on size distribution, pigmentation, conformation, and composition. Results from the whole berry partial extractions showed that seed PA material was driving activity early in the ripening phase, while the formation of pigmented polymer was leading to a decrease in activity later in the growing season. A multivariate analysis showed that the main drivers of changes in activity across the ripening phase were the molecular mass of PAs and the amount of pigmentation. Given the high amount of variability seen in the experiment between sites in such a small geographical area, the results suggest that manipulation of activity may be possible in the vineyard, possibly explaining variations in wine mouthfeel attributes between locations. These results can be used to develop further, more controlled, experiments targeting the variables responsible for activity changes.

## 2.2: Introduction

Proanthocyanidins (PAs) are a class of compounds that are ubiquitous in the plant kingdom. It has been shown that in plants, they play an important role in plant defense against fungal, herbivore and microbial attack. Although PAs are considered defense compounds, they can have considerable impacts on grape and wine quality due to their ability to form stabilized pigments allowing for the aging of red wine. Furthermore, they contribute to the mouthfeel characteristics of red wine during gustation, enhancing the sensorial aspects of the wine drinking experience.

Historical research in the field of PAs and their impact on mouthfeel characteristics in wine has focused on their composition, size, pigmentation and concentration.[16] There is clear evidence that these factors have impacts on the mouthfeel of a wine, however the impact of the combination of these factors on mouthfeel is unclear. Recent work has focused on a new high-performance liquid chromatography method focused on capturing the thermodynamic properties of PA interaction with a hydrophobic surface.[121, 122] This concept of "tannin activity" was based on isothermal titration calorimetry using isolated PAs from wine of various ages. Observations of the interactions with poly($_L$-proline) showed that PAs extracted from plant tissue had an observed increased energy of interaction over PAs isolated from young and aged wine. Presumably changes in PA molecular shape and hydrophobicity explained this reduction in interaction energy.

Tannin activity is a relatively new concept in polyphenolic research. The idea behind the methodology is that structural variations in PAs have differing binding affinities, leading to variation in astringency during gustation. Astringency in red wine comes from the binding to proline rich proteins found in the saliva as well as association with the oral mucosa.[123] PAs associate with these proteins forming precipitates with a concomitant loss in oral lubrication, leading to wines being described as 'dry' and 'course', among other descriptors. Although there have been sensory studies on the effects of PAs using trained panels, there is a question of the subjectivity in organoleptic sensations. The difference in salivary flow among individuals is large and well documented.[16, 124] Therefore, it is reasonable to assume that astringency may be highly subjective in nature. The development and use of an objective methodology for measuring tannin activity would be of great use in understanding astringency in general, as well as specific questions that persist such as the effect of matrix composition on the perception of foundational tannin activity and how tannin structural change over time impacts corresponding activity.

Previous research that gathered tannin activity information focused on the maceration process and differences among aged wine.[125] In the current study, the goal was to understand the effect of fruit ripening and other vineyard management variables on partially extracted tannin activity. It has been shown that PA is synthesized between flowering and the onset of color development within the chloroplast.[18, 23] Fruit ripening, when flavan-3-ols are no longer being synthesized, is of interest because changes are likely to occur to the tannin. The importance of this is developing an understanding of changes in tannin leading up to commercial ripeness and providing knowledge to the industry to manage harvesting decisions. Lastly, understanding the farming variables that impact PA throughout development may lead to greater control of grape and wine qualities.

## 2.3: Materials and Methods

### 2.3.1: Instrumentation and Chemicals

Agilent models 1100 and 1260 (Santa Clara, CA.) HPLC systems were used to conduct analyses on grape extracts. Chemstation software was used for all chromatographic analysis. Chemstation software was used for all chromatographic analysis. All solvents were HPLC grade. Acetonitrile, acetone, acetic acid, anhydrous sodium acetate, L-(+)-ascorbic acid, phloroglucinol, hydrochloric acid, lithium chloride, *N,N*-dimethylformamide, ethanol, and o-phosphoric acid were all purchased from VWR (Radnor, PA). Potassium metabisulfite, (-)-epicatechin (≥98% purity by HPLC), malvidin-3-*O*-glucoside (≥95% purity by HPLC) were purchased from Sigma-Aldrich (St. Louis, MO.) as quantitative standards.

### 2.3.2: Vineyard Sampling

During the 2015 growing season 75 Cabernet Sauvignon blocks were monitored from the onset of color to commercial harvest. Sampling sites were in 9 of the 16 sub-appellations within Napa Valley – Atlas Peak, Calistoga, Howell Mountain, Mount Veeder, Oakville, Rutherford, St.

Helena, Stags Leap District, and Yountville (**Figure 2.1)**. Sampling was conducted approximately 5 times across the 2015 growing season, each sampling consisted of approximately 120 berries. Prior to veraison an area of 100 vines were flagged for berry sampling per block. Both sides of the canopy were sampled as well as the top, middle, bottom and back of sampled clusters. Berries were placed into Ziploc bags and kept at 4 °C until processed. Berry processing and extraction occurred within 48 hours of sampling. There were no replicated extractions from within a site, as it was assumed that the scope of the project would allow for determination of trends in polyphenol changes throughout maturity.

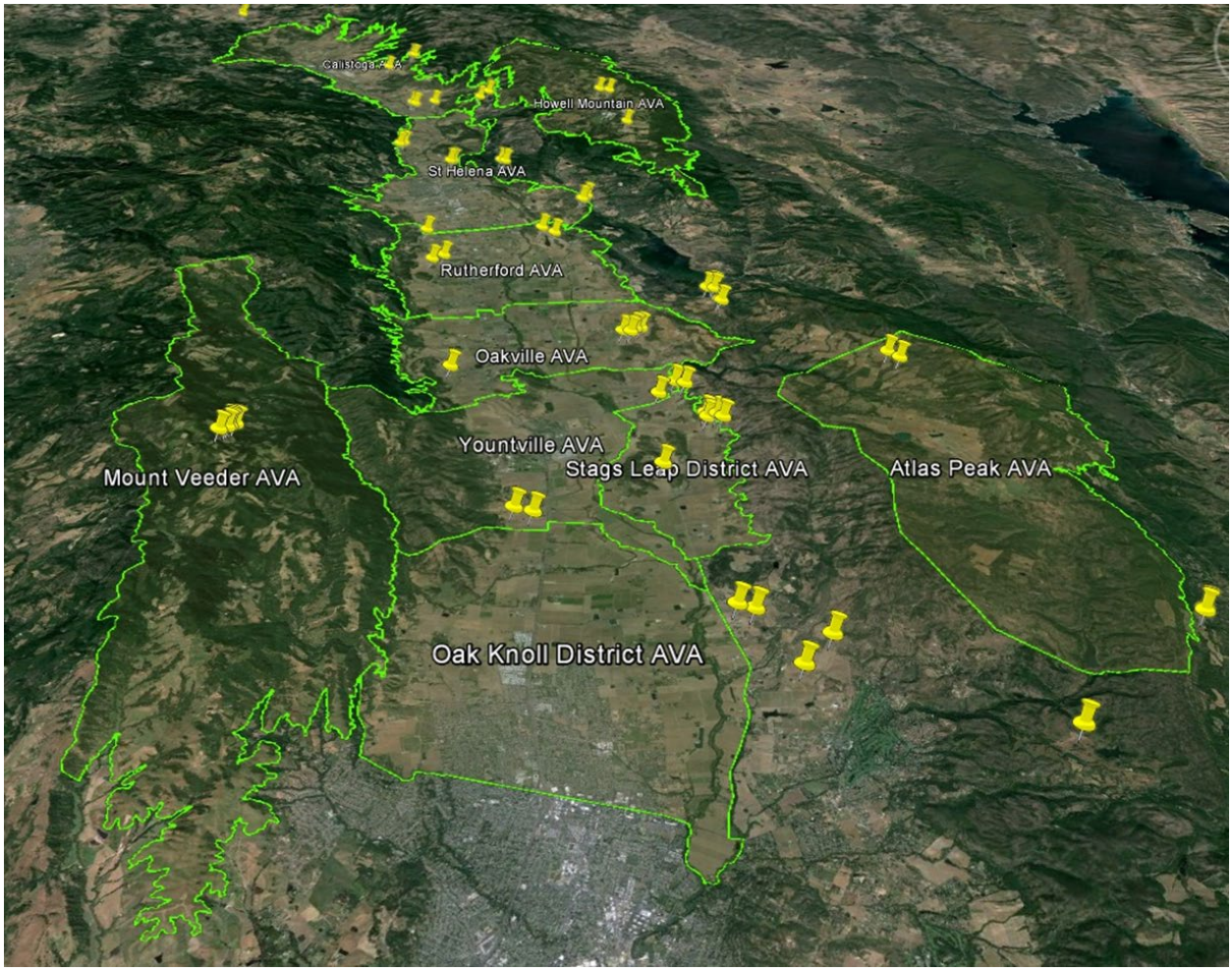


Figure 2.1: Map of the Napa Valley and American Viticultural Areas (AVAs). Yellow pins represent locations of blocks sampled during the 2015 vintage. Screen capture taken using Google Earth

### 2.3.3: Extraction

Partial extractions were completed using whole berries that were crushed for 30 seconds in polyethylene bags using a Seward Stomacher 400 Circulator (West Sussex, UK). After crushing, berries were transferred into a graduated cylinder where a dilute alcohol solution containing 200 mg/kg SO2 was diluted to 16% v/v ethanol (200mL). The mixture was transferred to glass jars, blanketed with nitrogen gas, sealed, and placed on an orbital shaker table, set at 100 RPM, for 48 hours. Extractions were conducted at ambient temperature. The temperature within the lab was set at 20 °C throughout the experiment.

After the samples were removed from the shaker table, solids were separated from solution through a ceramic Buchner funnel which was covered in polyethylene to press liquid from skins. Applied pressure on the pomace was approximately 0.1 MPa. The filtered solutions were then placed in 50 mL Falcon tubes, blanketed with nitrogen and kept at -20 °C.

### 2.3.4: Tannin Activity

The HPLC method for measuring the activity of wine tannin has been described previously.[121, 122, 125] Briefly, the HPLC method used a polystyrene divinylbenzene reversed-phase column (PLRP-S, 2.1 × 50 mm, 100 Å, 3 μm, Agilent Technologies, Santa Clara, CA) protected with a guard column (PRP-1, 3 × 8 mm, Hamilton Co., Reno, NV), with DAD detection at 280 nm. The mobile phases consisted of 1.5% (w/ w) o-phosphoric acid in water (180 mM, mobile phase A) and 20% (v/ v) mobile phase A in acetonitrile (mobile phase B) with a flow rate of 0.3 mL/min. The linear gradient was as follows (time in min (%B)): 0 (14), 12.6 (34), 12.6-13.3 (34), 15.1 (70) 15.1-16.8 (70), 19.6 (70), 19.6 (14), and 19.6-28 (14).

To determine thermodynamic information, samples were run at four column temperatures (25−40 °C, 5 °C increments), and temperatures were converted to Kelvin for calculations. Chromatograms

at 280 nm were baseline subtracted using water as a blank injection and were integrated as previously described.[121] Briefly, a baseline was drawn at 0 mAU and with the resulting area clipped at 5 and 28 min for total tannin ($Tannin_{Total}$); partial tannin ($Tannin_{Partial}$), respectively. $Tannin_{Partial}$ was the peak area eluting between 16.8 and 28 min. For each chromatogram an alternative retention factor for the tannin ($k_{alt}$) was calculated as follows:

$$k_{alt} = \frac{tannin_{Total}}{tannin_{Total} - tannin_{Partial}}$$

is related to thermodynamic information as follows:

$$lnk_{alt} = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} + ln\text{ø}$$

where ΔH° and ΔS° are the enthalpy and the entropy (respectively) of the interaction, R is the gas constant (8.3145 $J{\cdot}mol^{-1}{\cdot}K^{-1}$), ø is the mobile phase ratio, and T is the temperature of the experiment in Kelvin. The enthalpy was calculated by the slope from the Van't Hoff plot (i.e., ln $k_{alt}$ versus the reciprocal of the column temperature in Kelvin at each of the four temperatures).

Grape skin standard was prepared as previously described (mDP = 39.0 (by phloroglucinolysis) and served as an activity standard.[121] Activity standards were prepared by dissolving tannin in an aqueous solution (5 g/L) containing methanol (15% v/v), 40 mM sodium acetate, and 20 mM HCl.

### 2.3.5: Gel Permeation Chromatography

The size distribution of tannin isolates was determined using the previously described method of Kennedy and Taylor.[126] Briefly, the high-performance GPC method used to analyze tannins consisted of 2 PLgel (300 × 7.5 mm, 5 μm, 1000 by 500 Å) columns connected in series and protected by a guard column containing the same material (50 × 7.5 mm, 5 μm), all purchased from Agilent (Santa Clara, CA). The targeted sample injection amount was 40 μg. The isocratic

method utilized a mobile phase consisting of *N,N*-dimethylformamide containing 1% v/v glacial acetic acid, 5% v/v water, and 0.15 M lithium chloride. The flow rate was maintained at 1 mL/min with a column temperature of 60 °C, and elution was monitored at 280 nm.

Calibration curves were constructed using fractionated pre-veraison grape skin proanthocyanidins (PAs) prepared as previously described, and by correlating their average molecular mass (determined by phloroglucinolysis) with their retention time at 50% elution. Initial PA (*Vitis vinifera* L. cv. Cabernet Sauvignon) isolation was accomplished according to the method described by Kennedy and Jones.[5] For preparation of standards, 1.0 g of PA was dissolved in 50 mL of 60% (v/v) HPLC grade methanol containing 0.05% v/v TFA and then applied to (~18 mL/min) a glass column (Kimble Kontes Chromaflex, 4.8 × 15 cm, Vineland, NJ) containing Sephadex LH-20 chromatography resin (Amersham, Uppsala, Sweden) to an approximate bed volume of 200 mL. The column was previously equilibrated with 60% v/v methanol containing 0.05% v/v TFA. The applied PA was fractionated using the solvent system described by Kennedy and Taylor, but formic acid was replaced with 0.05% v/v TFA.[126] Eluted fractions were concentrated under reduced pressure at 38 °C to remove organic solvents and then lyophilized to a dry powder. Individual isolates were then used to construct a calibration curve. Integration was done using the GPC software for Agilent Chemstation. Elution after 13 minutes was excluded to minimize the effects of residual monomeric anthocyanins on PA size distribution analysis.

### 2.3.6: Acid Catalysis in the Presence of Excess Phloroglucinol

Tannin subunit composition of tannin isolates were determined using the previously described method of Kennedy and Jones.[5] Briefly, the reversed-phase HPLC method consisted of two Chromolith RP-18e (100 × 4.6 mm) columns connected in series and protected by a guard column containing the same material (4 × 4 mm), all purchased from EM Science (Gibbstown, NJ). The

method utilized a binary gradient with water containing 1% v/v aqueous acetic acid (mobile phase A) and acetonitrile containing 1% v/v acetic acid (mobile phase B). Pump flow rate was kept at 3.0 mL/min with a column temperature of 30 °C and with elution monitored at 280 nm. The linear gradient was as follows: time in min (% B); 0 (3%), 4.0 (3%), 14.0 (18%), 14.01 (80%), 16.0 (80%), 16.01 (3%), 18.0 (3%). (−)-Epicatechin was used as a quantitative standard. Individual subunits and mDP calculations were made according to Kennedy and Jones.[5]

### 2.3.7: Low Molecular Weight Phenolics

Low molecular weight phenolics were analyzed using a previously described methodology by Ritchey and Waterhouse.[127] Briefly, a 4 mm × 250 mm, 5 μm LiCrospher C18 was used as the stationary phase with a constant temperature of 40 °C throughout analysis. The flow rate was kept at 0.5 mL/min. A diode array detector (DAD) was used to monitor 280 nm and 520 nm. Mobile phases: (A) 50 mM ammonium di-hydrogen ammonium phosphate in water adjusted to pH 2.6 with *o*-phosphoric acid, (B) 80% Acetonitrile with 20% (A), (C) 0.2 M *o*-Phosphoric Acid in water adjusted to pH 1.5 with 1.0 M sodium hydroxide. The gradient was as follows: time in min (%B;%C); 0 (0%;0%), 8 (8%;0%), 20 (14%;86%), 25 (16.5%;82%), 35 (21.5%; 78.5%), 70 (50%;50%), 75 (0%;0%), 80 (0%;0%). (-)-Epicatechin was used as the quantitative standard at 280 nm. Malvidin-3-*O*-glucoside was used as the quantitative standard for anthocyanins at 520 nm.

### 2.3.8: Statistics

Graphpad Prism 9 (San Diego, CA) was used for multivariate analysis. A Probabilistic Principal Component Analysis (PPCA) was conducted to determine the loading plot. The variables used to conduct this analysis were activity, subunit composition constituents, molecular mass, and pigmented polymer. The multivariate analysis contained 371 samples. This software was used for

creating figures with polynomial regressions containing 95% confidence bands and visualizations of the relationships between variables.

## 2.4: Results and Discussion

The foremost goal of this study was to understand the effect of fruit ripening on PA chemistry and corresponding activity under partial extraction conditions and to explore the potential for regional and management practice influence. Previous tannin activity investigations focused on wine analysis, in which the PA had likely undergone additional structural change that would be mediated by the winemaking technique.[128] A large number of samples was chosen so that even without control of vineyard management techniques, it would be possible to observe changes in PA structure and activity that were dependent on ripening, and, to see if any of the measured farming practices (i.e. irrigation) or sub-appellation in Napa affected PA qualities.

Although this experiment was conducted in a relatively small geographical location (Napa Valley), the differences in slope, distance to the San Pablo Bay, and elevation all contribute to large

Table 2.1: Changes in the primary metabolites of Cabernet Sauvignon through the ripening phase in Napa Valley. Values presented are mean and standard error of mean (SEM) within a given sampling point.

| Berry Weight and Primary Metabolites Throughout Maturation | | | | | |
|---|---|---|---|---|---|
| Sample Set | Sample Count (N) | Berry Weight (g) | Brix | pH | TA (g/L) |
| 1 | 75 | 0.64 ± 0.02 | 13.18 ± 0.30 | 2.74 ± 0.01 | 25.11 ± 0.72 |
| 2 | 75 | 0.82 ± 0.02 | 18.17 ± 0.20 | 2.97 ± 0.02 | 13.21 ± 0.54 |
| 3 | 75 | 0.91 ± 0.02 | 21.59 ± 0.20 | 3.20 ± 0.02 | 8.28 ± 0.33 |
| 4 | 69 | 0.89 ± 0.02 | 24.07 ± 0.18 | 3.36 ± 0.01 | 6.33 ± 0.24 |
| 5 | 56 | 0.85 ± 0.02 | 25.60 ± 0.19 | 3.46 ± 0.02 | 5.49 ± 0.22 |

variations in conditions at a vineyard level. Furthermore, Cabernet in Napa Valley is intensely farmed, with a high attention to detail, due to the high accolades the area has received historically. For the purposes of this chapter, environmental data looking into the effects at a sub-appellation level was left out. This chapter was designed to provide insight into how research was developed from a large dataset with no control, to a small dataset that was highly controlled.

**Table 2.1** and **2.2** below show the mean of primary and secondary metabolites, respectively, across sampling points. All data shown is using the standard error of mean (SEM) where N=sample count for each sample set. Due to early commercial harvesting of monitored blocks during the experiment, set 4 and 5 have fewer samples then the initial three sets. In **Table 2.1** there was an increase in pH and decrease in total acidity throughout the ripening period, with an increase in berry weight until sampling set 5, where dehydration likely decreased berry weight across samples. In **Table 2.2** secondary metabolites are shown by mean and SEM per set. During the ripening phase there was an observed increase in total anthocyanins on a per berry basis until the final sampling point, where there was an observed decrease. This coincides with a decrease in berry weight from **Table 2.1** likely associated with dehydration. Given how late in the season this was, this decrease in total anthocyanins was likely from degradation which has been shown to occur in berries that have been overexposed.[101, 102]

PA concentration also showed a trend across the growing season, wherein the highest concentrations occurred at veraison, with a general decline afterwards. For the last sample set,

there was a minor uptick in the concentration of PA material extracted, suggesting a possible uptick in skin extractability at later periods of ripeness.

In terms of PA molecular mass (MM) and mean degree of polymerization (mDP), there was an inverse relationship between PA MM and mDP. As fruit ripening progressed there was an observed increase the in mDP of the extracted PA, likely due to decreases in the proportion of seed PA extracted as well as increases in anthocyanin incorporation into the PA structure, which have been shown to increase PA mDP likely through the displacement of terminal subunits.

Table 2.2: Changes in secondary metabolites of Cabernet Sauvignon across Napa Valley through the ripening phase in 2015. Values presented are the mean and standard error of mean.

| Secondary Metabolites Throughout Maturation | | | | | | |
|---|---|---|---|---|---|---|
| Sample Set | Sample Count (N) | Anthocyanin Concentration (mg/b) | Proanthocyanidin Concentration (mg/b) | PA mDP | PA Molecular Mass (g/mol) | Tannin Activity (-J/mol) |
| 1 | 75 | 0.15 ± 0.01 | 0.21 ± 0.009 | 4.61 ± 0.09 | 15077 ± 546 | 6639 ± 173 |
| 2 | 75 | 0.73 ± 0.03 | 0.19 ± 0.006 | 4.07 ± 0.13 | 10585 ± 548 | 4723 ± 192 |
| 3 | 75 | 1.11 ± 0.03 | 0.17 ± 0.006 | 4.91 ± 0.17 | 8354 ± 375 | 3648 ± 152 |
| 4 | 69 | 1.16 ± 0.03 | 0.16 ± 0.005 | 7.04 ± 0.38 | 7814 ± 226 | 3294 ± 128 |
| 5 | 56 | 1.10 ± 0.04 | 0.18 ± 0.008 | 11.24 ± 0.66 | 7460 ± 260 | 3444 ± 147 |

### 2.4.1: Changes in Anthocyanin Concentration per Berry

Anthocyanin biosynthesis followed a trend of rapid accumulation after the onset of color. Rapid biosynthesis of pigmented material increased until approximately 22 °Brix, where a maximal plateau was reached. Based on the standard error of the population, there was no change in concentration after the third sampling point. However, it is apparent when looking at **Figure 2.2** that large variation exists. Given the lack of control in this experiment, it is likely that large variations in exposure, yield, row orientation and irrigation management were affecting anthocyanin concentrations at this stage of maturity. Multiple studies have shown the impact of light exposure on the accumulation of anthocyanins throughout maturation.[96, 99, 129] It is also

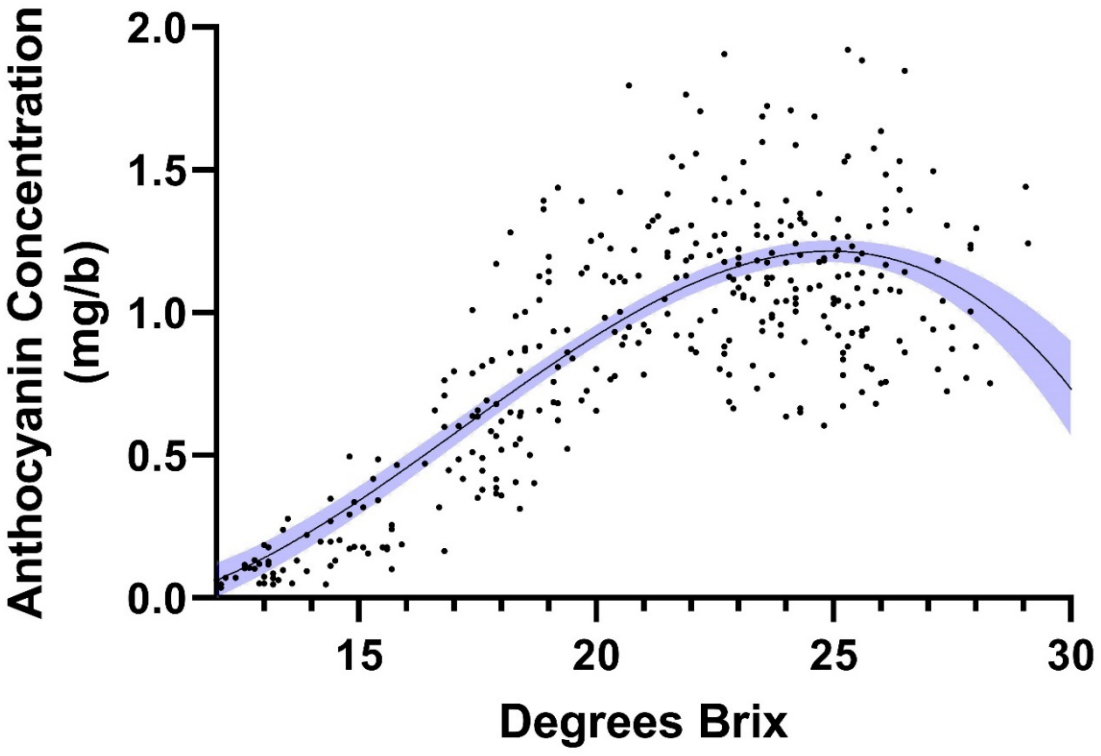


Figure 2.2: Changes in anthocyanin concentration across all samples (N=371) in 2015. The cubic regression analysis, with 95% confidence bands, was fit to the data. (Y=1.836–0.4585*X+0.03377*X^2–6.6E-4*X^3);R^2=0.7075

apparent that some sites had a decrease during the last period of maturation before commercial harvest. This decrease in anthocyanins is likely due to both the slowing of anthocyanin synthesis from the berry itself along with overexposure causing excess temperature in the berry. When this occurs, it has been shown that peroxidases are formed, causing the degradation of anthocyanins.[105, 130]

How vineyard location effects phenolic synthesis has long been of interest within the wine community. This study allowed for the analysis between vineyard sites based on location within the Napa Valley.

Although there were differences in diurnal temperature range as a result of vineyard elevation there were no differences based on location when looking at appellations (Data not shown).[108] There are a few possible explanations for this – one is that there may be a difference, but due to the size and scope of the project, lacking vineyard control, these differences were indiscernible. It is also possible that regional climate, along with vineyard management practices, are more impactful than small differences in local climate.

### 2.4.2: Change in Proanthocyanin Concentration and Composition

Proanthocyanidin concentration was measured by acid-catalysis in the presence of excess phloroglucinol. At the onset of color, the concentration of PAs inside each berry was its highest. This has been discussed in other work, where both seed and skin PAs decreased after veraison.[131, 132] In this project seed PA extraction likely impacted the PA concentration prior to complete hardening of the seed, which has been shown to have higher extraction levels, in dilute ethanol, at the early stages of ripening.[131] The results suggest that there is a slight increase in PA concentration at final timepoint of the experiment (**Table 2.2**). Possibly due to senescence of the grape skins at higher maturity, leading to increases in the extractability of PAs. Variation in PA on a per berry

basis can be seen in **Figure 2.3** where the $R^2$ shows that maturity only explains 14 percent of the variation. Early season variation can be explained by inconsistencies in the number of seeds per berry (data not shown). Work done by Harbertson et al. showed that there is little variation in the PAs in a seed, but large variation in PAs per berry based on the number of seeds.[133] In the later part of the project, it is likely that the graph is more representative of skin PA variations between

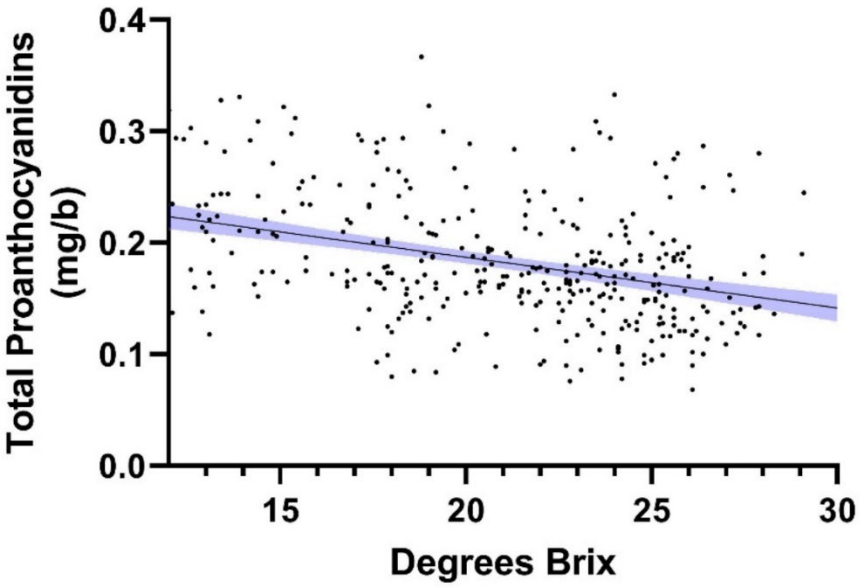


Figure 2.3: Changes in total PAs across the ripening phase in Cabernet Sauvignon from Napa Valley in 2015. The equation of the linear regression is (Y=0.2780-4.55E-3*X) with 95% confidence bands; R^2=0.1445; N=371

vineyard sites. Skin PA synthesis, occurring between flowering and veraison, is partially driven by light incidence on the growing berry.[18] Locations which had lower light incidence during this time would likely have lower PA concentrations within the skin.[99] To further complicate variation, it has been shown that vigor also influences composition and concentration, where lower vigor sites tend to have higher proportions of (-)-epigallocatechin when compared to sites of higher vigor.[134] Based on this, it is likely that there are multiple factors having to do with vineyard management that may have caused the variation observed. When looking at the linear regression

with 95% confidence bands, the trend observed is in agreement with previous research on berries during maturation.[133, 135, 136] However, there are other studies where there are increases in the content of proanthocyanidins extracted over the ripening phase, particularly at commercial harvest.[91, 113]

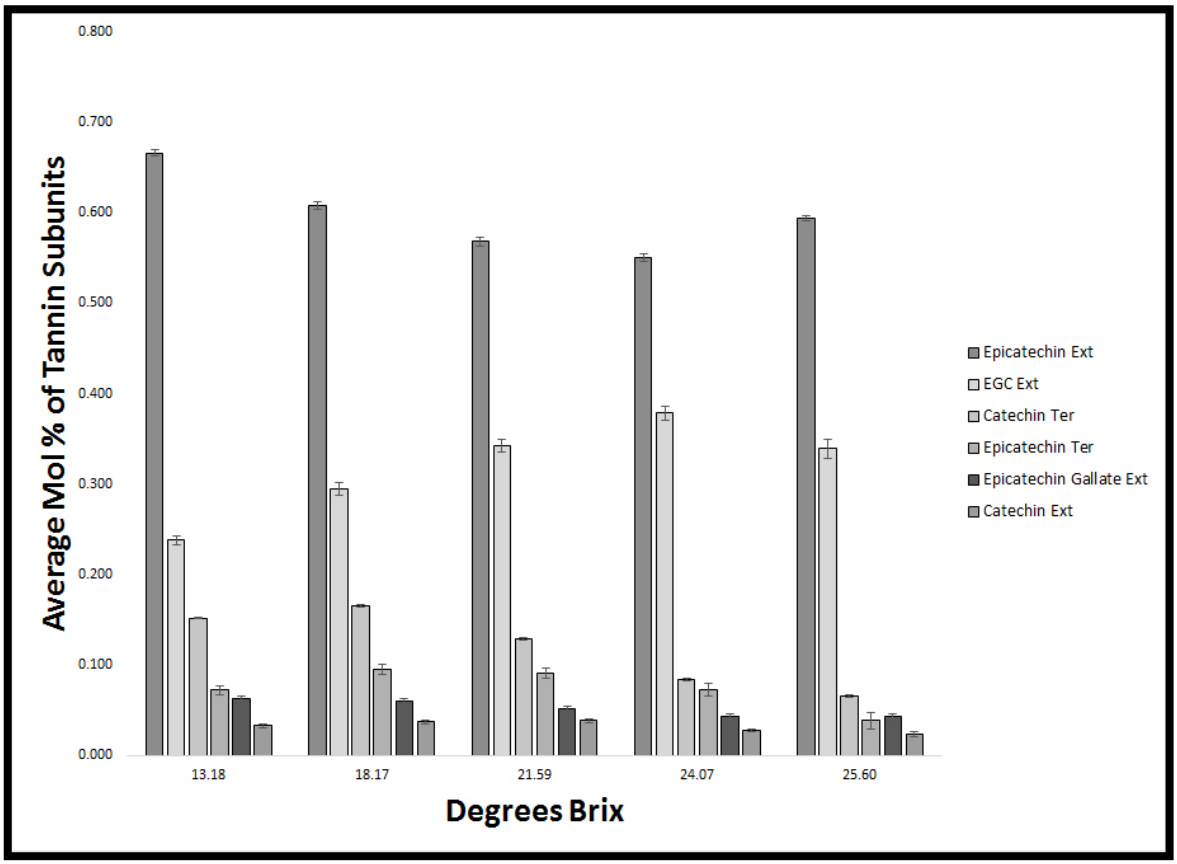


Figure 2.4: Changes in the subunit composition of PAs over time after analysis by acid-catalysis. Error bars are standard error of mean. Number of samples can be found in Table 1 with corresponding °Brix. EGC is Epigallocatechin; Ext is Extension subunit; Ter is Terminal subunit

Proanthocyanidin subunit compositional analysis can provide information about the *type* of PA extracted due to differences between skin and seed. In the current experiment whole berries were used in the partial extractions, leading to a mixture of seed and skin PAs. Initially this led to higher levels of (-)-epicatechin, (+)-catechin, and (-)-epicatechin-3-*O*-gallate extension subunits. These subunits decreased as maturation progressed, likely due to decreases in the extraction of seed PAs

as hardening of the seed coat occurred.[131, 137] Furthermore, there was an increase in (-)-epigallocatechin proportions at the latter stages of maturation, suggesting increased levels of skin extraction compared to seed extraction (**Figure 2.4**). In order to visually show the changes in skin and seed PA proportions throughout maturity linear regressions were made by evaluation the proportion of (-)-epigallocatechin, which is not present in seed PAs. These two regressions were superimposed to show the decreasing levels of PAs extracted from seed, with the increasing levels of PAs extracted from skins (**Figure 2.5)**. This change in extractability of the seed material in part explains the decrease in total PAs extracted, as well as the changes in subunit composition across maturity.

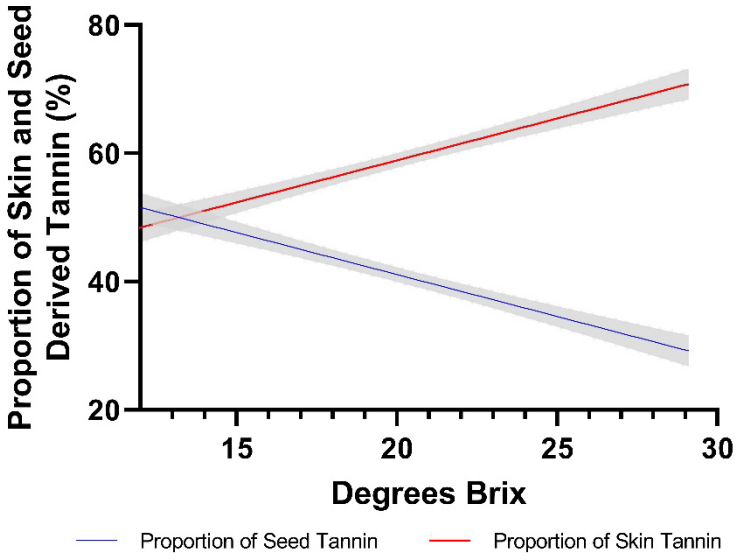


Figure 2.5: Representation of the increase in proportion of skin tannin extracted throughout maturity compared to that of seed tannin extracted. Linear regressions were made by deriving the proportion of skin PAs through (-)-epigallocatechin.

### 2.4.3: Average Molecular Mass

Although there was a higher amount of seed PAs extracted earlier in maturation, based on the proportions of (-)-epicatechin-3-*O*-gallate$_{(ext)}$ and (+)-catechin$_{(ter)}$, the PA size was larger than

expected. This is confounding when compared to previous research on the size of seed PA, however work comparing model wine extractions and exhaustive, acetone, extractions showed that the mild ethanol extractions had significantly higher mDP levels at veraison.[131, 138, 139] One explanation for these results is that the calibration curve for gel permeation chromatography was based on skin-derived PA. Due to the larger contribution of galloylated PA at the onset of color development, this likely affected early analysis. Seed derived PAs have a higher hydrodynamic volume at the same degree of polymerization due to the gallate ester at C-3. This leads to inaccurate measures of molecular mass by GPC when using a curve derived from skin PAs. However, the downward trend in size distribution would be maintained as the expected result based upon the percent of galloylated subunits throughout the maturation profile. A second possible explanation

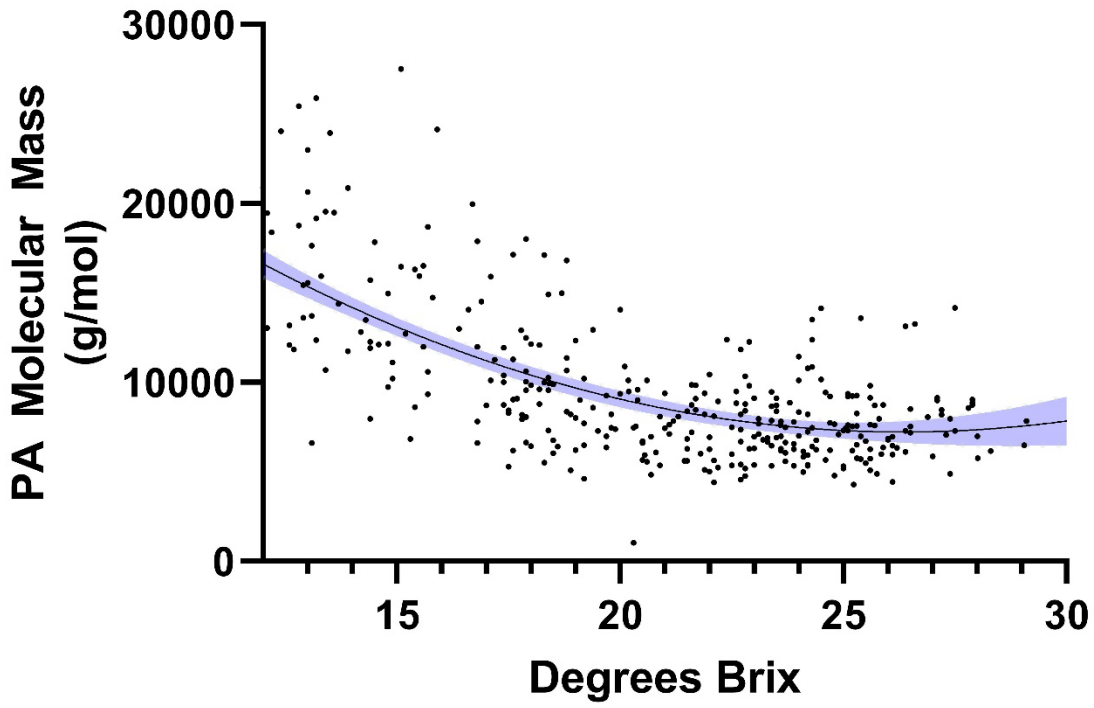


Figure 2.6: Results from gel permeation chromatography. Changes in PA molecular mass through the ripening phase in Cabernet Sauvignon from the Napa Valley in 2015. N=371; R^2=0.4551; (Y=38928-2407*X+45.68*X^2) with 95% confidence bands in blue.

for these results is that at this stage in maturation, there may fewer interactions between cell wall material (CWM) and larger PA material, leading to a larger sized fraction being extracted into solution compared with material later in ripening. This would hypothetically increase the size distribution within the solution at earlier stages of ripeness (**Figure 2.6)**.

A hypothesis for why this may occur is that as the grape progresses through fruit ripening, anthocyanins may impact the adsorption process of PAs through their own interactions with CWM. A study in 2013 has shown a relationship between the degree of methylation in pectin and the binding of PAs.[120] Furthermore, another study has shown the adsorption and desorption of anthocyanins from polysaccharide isolates, releasing lower molecular weight PAs from cell wall material.[115] Given the positive charge of anthocyanins in the flavylium form, as well as a partial negative charge of polysaccharide side chains due to galacturonic acid, at wine pH, it may be possible for monomeric anthocyanins to impact the desorption of PAs. In such a case the formation of a salt between the anthocyanin flavylium and pectin carboxyl group is plausible, possibly allowing for the disruption of hydrophobic and hydrogen bonding interactions.[140] Recent work has shown that there are interactions which stabilize the flavylium form of anthocyanins when interacting with demethylated pectin in acidic solutions, suggesting that these interactions between polysaccharides and anthocyanins are indeed occurring.[119] In regards to whether these interactions may impact the extraction of skin PAs, work conducted looking into Syrah fermentations showed that wine made from fruit with higher levels of anthocyanins corresponded to higher levels of PA material extracted into the wine, regardless of if the fruit had lower or higher levels of PAs to begin with.[112]

### 2.4.4: Mean Degree of Polymerization

The mean degree of polymerization suggests that the size of PA is relatively stable throughout fruit ripening until later in maturity, where there is a clear increase, which is in line with previous work (**Figure 2.7)**.[91] However, there have been instances contradictory to this trend, showing decreases in mDP throughout fruit ripening.[96, 132] In this experiment both phloroglucinolysis and gel permeation chromatography were used to estimate PA MM. Recently, work has been done to show that sulfonate groups may act as nucleophiles, and interact with PAs, at wine pH.[4] Based on the

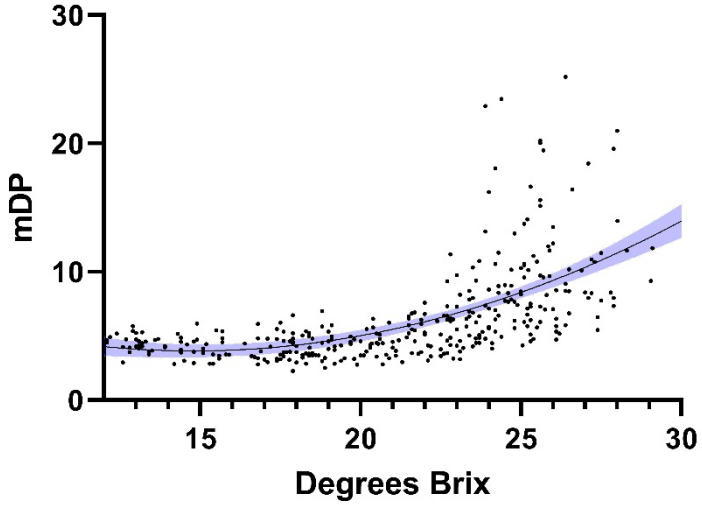


Figure 2.7: Non-linear regression of mean degree of polymerization changes in extracted tannin across ripening. N=371; R^2=0.2433; (Y=-1.625+0.3868*X) with 95% confidence bands.

work, hypothetically the (4β→8)-Interflavan linkage is cleaved through acid catalysis and a sulfonate addition occurs at C-4. The new terminal subunit would be a flavan-3-ol with a sulfonate adduct at the 4-position, which had not been previously identified in the acid catalysis methodology. This recent finding could explain the discrepancy in PA MM when measured by phloroglucinolysis and GPC. Although thiols tend to be good nucleophiles, it is likely that the

amount of sulfonated flavan-3-ols would be negligible in this experiment due to concentrations. Furthermore, following the acid-catalysis reaction in a solution with 20 molar equivalence of phloroglucinol would likely make the amount of sulfonated subunits negligible. This is assuming that these subunits stayed on the C18 column during solid phase extraction, which has not been explored in the context of an interference with this method. Given this, it is likely ruled out that this is causing an interference in the results presented.

Although the mDP has a low variability below 23 °Brix, there is clearly an increase in the spread of the data at later sampling points (**Table 2.2**). There are a few possible explanations for the results seen in **Figure 2.7** - the first is clear when looking at **Figure 2.5**. At 20 °Brix, more than 40% of the PA material extracted originated from seed material, while at 25 °Brix the proportion was less than 35%. Therefore, an increase in mDP is expected based on previous work showing that PA material derived from grape skins is larger.

Another possible explanation for the results is the formation bonds more resistant to acid-catalysis. Although it is unlikely that oxidative changes were made to the polymers during the extraction process based on work looking at micro-oxidation techniques, the possibility for pigmented polymer formation is within the realm of possibility.[128] Work has shown that the formation of a 4β →8 linkage between an extension subunit and an anthocyanin can lead to an oligomer that is more resistant to cleavage in an acid solution.[8] Although there was a clear increase in anthocyanin concentration above 20 °Brix, and work has shown that anthocyanin additions can impact mDP calculations, one would expect an increase in mDP that was more linear as anthocyanin synthesis occurred if that was the case. Given this, the results in **Figure 2.7** are likely a combination of multiple factors leading to a steep increase towards the end of the growing season - mainly due to

the tissue the material was extracted from and the confluence of anthocyanin synthesis leading to pigmented polymer formation.

### 2.4.5: Tannin Activity

The measurement of 'tannin activity' has been researched over the last decade looking at correlations between composition, size, and concentration in wines. Furthermore, recent work has looked at 'activity' during sensory work, showing the highest correlation to the astringency of wines.[141, 142] Previous vineyard work has explored the impacts of timing and ripening on phenolics.[91, 138, 143, 144] Generally, this work has focused on transcriptional factors and the upregulation of areas within the phenylpropanoid pathway as well as overall phenolic changes. In comparison to concentration or size distribution, this measurement is built on determining an effect of those various factors and how PA material interacts with a hydrophobic surface.

The previous works described above have shown that PA 'activity' is an intensive property, wherein additions of PA material do not change observed activity.[122] In the 2013 work by Barak and Kennedy there was a clear relationship between the MM of PAs and their corresponding activity.[121] Furthermore, this work showed that seed PAs are more exothermic at a similar size than those of skin PAs, likely due to compositional differences arising from increased galloylation.

Lastly, this method was used to look at changes in wine throughout maceration to understand the differences in maceration length on PA composition, size and corresponding activity. The results from this work confirmed that PA size distribution and levels of galloylation helped drive the activity, and therefore the astringency, of the wines made.[125]

In this experiment PA activity decreased throughout the growing season, reaching an average minimum at approximately 24 °Brix (**Figure 2.8**). Early samples contained green seeds, which

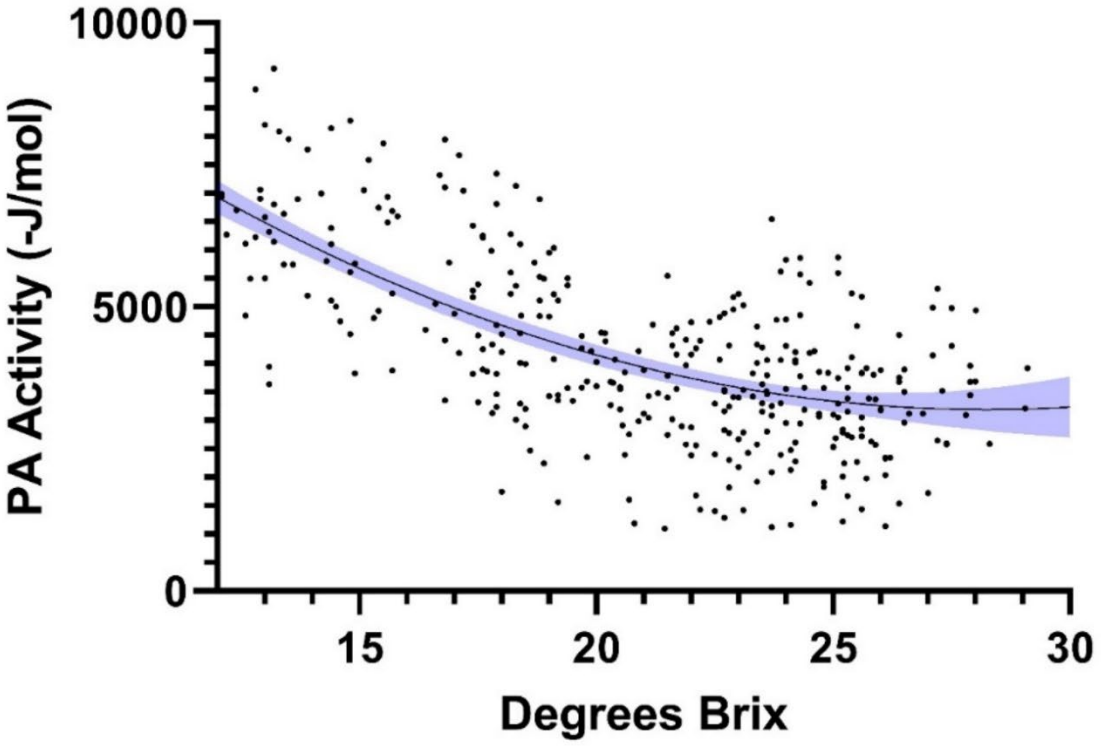


Figure 2.8: Non-linear regression of changes in PA activity in Cabernet Sauvignon across the 2015 growing season in Napa Valley. N=371; R^2=0.5101; (Y=14512-802*X+14.21*X^2) with 95% confidence bands in blue.

have been shown to readily extract in ethanolic solutions.[131] It is unknown whether the decreasing activity throughout the growing season is due to a decrease in seed-derived PA extraction as the fruit matured or that smaller PAs were extracted as ripening progressed, leading to an overall decrease in activity. Although there is a significant amount of variability, each block followed a similar trend, decreasing through the first half of the sampling period and maintaining a relatively flat trend before commercial harvest. Although there are publications looking into activity, it is hard to speculate on the results, as other experiments have focused on wine analysis, rather than understanding the changes in berry development. Regardless of the lack of publications, the results suggest that the high amount of variability seen in the extracts, even after significant seed PA

extraction had stopped, leads to the conclusion that manipulation in the vineyard is possible. If manipulation in the vineyard is indeed possible through higher factors causing the effect of decreases activity, than it can be suggested that astringency management starts during the vine cultivation.

### 2.4.6: Multivariate Analysis

The PCA (**FIGURE 2.9)** shows the relationship between subunit composition, activity, and size of PAs, as well as pigmented polymer concentration across the entire dataset (N=371). This multivariate analysis is similar to that of Yacco et al. which looked at the impact of maceration time on proanthocyanidin composition and corresponding tannin activity in Cabernet

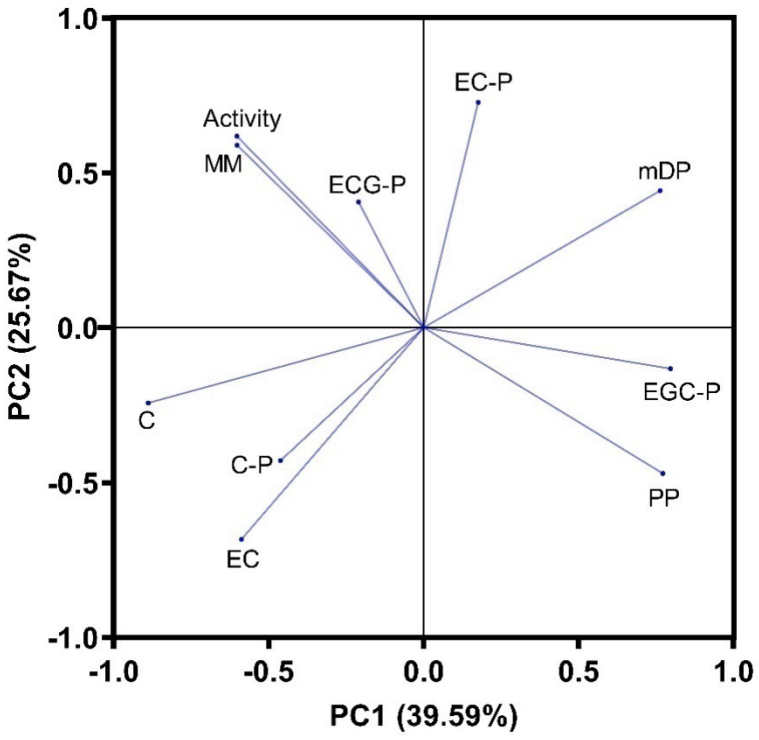


Figure 2.9: : Principal component analysis across PA and anthocyanin parameters. The entire data set was used for this PCA (N=371). PAs=total proanthocyanidins; MM=molecular mass; C=catechin; EC=epicatechin; EGC=epigallocatechin; ECG=epicatechin-3-O-gallate; mDP=mean degree of polymerization; PP=pigmented polymer; TAC=total anthocyanin concentration

Sauvignon.[125] In that work, tannin activity was found to be correlated with molecular mass, as well as (-)-epicatechin-3-*O*- gallate extension subunits, similar to the present study. However, in the study of Yacco, no correlation between pigmented polymer and tannin activity was observed. This finding is inconsistent with the present study where pigmented polymer was inversely correlated with tannin activity. This collective finding suggests that tannin activity management by means of pigmented polymer formation is more effective in the vineyard than during maceration. In the present study and across the entire dataset, it was also found that pigmented polymer concentration was most strongly correlated with total anthocyanin concentration (r2=0.70) suggesting that pigmented polymer formation (and associated effects on tannin activity) are correlated with anthocyanin management practices.

The inverse relationship between tannin activity and pigmented polymer could be explained by the incorporation of anthocyanins into the proanthocyanidin pool either as a nucleophile (hemiketal form) during acid catalysis resulting in a reduction in proanthocyanidin molecular mass (as observed); or by the incorporation of anthocyanins through an unknown alternative reaction pathway (i.e.: oxidative incorporation). Observations by Barak and Kennedy suggest that pigmented polymers at an equivalent molecular mass, have a lower tannin activity than unpigmented proanthocyanidins.[121] This presumably suggests that anthocyanins can alter tannin activity by modifying polymer size as well as by modifying the ability of the polymer to intermolecularly interact with other polymers.

## 2.5: Discussion and Relevance of Findings

The size of this experiment allowed for a clear anthocyanin synthesis curve to show periods of accumulation and degradation. Furthermore, it shows that although seed PA extraction was impacting the results during the earlier sampling points, the overall slope suggests little PA change

between the beginning and end of the ripening phase. In terms of size distribution and PA activity, there were large changes between the beginning and end of the experiment, suggesting that although there were stable tannin concentrations, the composition and size of the tannin extracted impacted the activity results.

The principal component analysis at the end of the results section clearly shows that there is a relationship between the GPC, PA concentration, subunit composition and activity results. There also appears to be an inverse relationship between proanthocyanidin activity, average molecular mass, and the concentration of anthocyanins. Although it cannot be confirmed within the context of this experiment, this suggests that anthocyanins are possibly modifying the activity or molecular mass of the PAs.

Lastly, in nearly all scatter plots there is a considerable amount of variation that can be seen. In the PA plot there is clearly variability. Similarly, although there was degradation of anthocyanins at the end of the growing season, there was approximately a 1 mg/berry difference between the minima and maxima at 25 °Brix. This variability across the experiment, and within the analysis conducted, suggests that viticulture management techniques may have large impacts on flavonoids synthesis in the vineyard. Future experiments need to continue to understand how site-to-site variability is being driven in the context of secondary metabolites in order to construct management schemes directed at maximizing fruit quality.

The results here are like previous works, however different in that the formation of pigmented polymer appear to be driving changes in activity. This suggests that changes in anthocyanin concentration during vineyard management practices may have profound impacts on the activity of PAs during the initial stages of maceration, while longer maceration lengths may lead to changes seen in the Yacco et al. work that were driving more by variations in composition due to higher

levels of galloylation.[125] This work provides evidence for the variability and possible management of a PA property known to be correlated with the astringent nature of PAs. Future work should focus on management practices driving the variability in this project to better understand how to manipulate this attribute in the future.

## 2.6: Acknowledgements

The authors would like to thank the American Vineyard Foundation for the funding of this project. We would also like to thank all the wineries participating in the project for your enthusiasm and willingness to help. Lastly, we want to thank Thibaut Scholasch and the entire team at Fruition Sciences for the ability to collaborate on this project.

# Chapter 3: Evaluating the Impact of Post-Veraison Shade on Anthocyanins and Proanthocyanidins

## 3.1: Abstract

Historically shading experiments have on wine grapes have investigated the impact of light incidence on proanthocyanidin, flavonol, or anthocyanin concentration. The current experiment was designed to look monomeric anthocyanins, but also the changes in proanthocyanidin structure and composition through ripening. Activity is a new methodology for assessing structure-activity relationships of proanthocyanidin material due to molecular mass and compositional differences. In 2016 a shade cloth study was imposed on Cabernet Sauvignon on Mt. Veeder. A control, which was exposed, and two treatments consisting of 40% and 80% shade were applied at the onset of veraison. Results from the experiment showed that there were significant differences in the hydroxylation pattern of anthocyanins, as well as the concentration throughout ripening. Compositional differences in the proanthocyanidin material were observed in the percent of galloylation, where shaded treatments had a significantly higher proportion of galloylated subunits than the exposed control. The molecular mass of the extracted proanthocyanidin material was significantly smaller in the exposed control than in the shaded treatments. These factors led to a lower measured activity of the proanthocyanidin material in the exposed control. This work suggests that manipulation of canopy architecture not only impacts the concentration and composition of monomeric anthocyanins but can also lead to structural differences in the proanthocyanidin material which has implications for wine quality.

## 3.2: Introduction

Proanthocyanidins and anthocyanins are two of the most important secondary metabolites synthesized in the berry during wine grape production. Although they are both produced from the phenylpropanoid pathway, the timing of their synthesis during the growing season differs. Synthesis of proanthocyanidins occurs from flowering until veraison in wine grape skins, while

anthocyanin production is the visual cue that the fruit is finished with lag phase. Environmental factors, such as water stress and light incidence, differentially impact transcriptional factors regulating the synthesis of these flavonoids, depending on the stage of fruit development.[1-2]

Following lag phase red wine grapes begin to develop color. This is called *veraison* and is the onset of what is considered fruit ripening, when sugar loading in the fruit accelerates. Although proanthocyanidin synthesis has stopped, it is still considered a parameter in which harvest decisions are made. Terms that the authors have heard described by viticulturists are 'phenolic ripeness' or 'tannin maturity'. These terms are outside of the historical parameters for ripeness such as pH, titratable acidity, or total soluble solids. Instead, this is based on an idea that the quality of proanthocyanidins in the skins is impacted during the ripening phase leading to differences in wine quality and structure.

Experiments focused on light incidence and attenuation during the ripening phase have focused primarily on the changes in monomeric anthocyanins and flavonols. The production of flavonols has been shown to be significantly enhanced by increasing cluster exposure, while experiments quantifying anthocyanins have had varied results.[1, 3-9] Although there our differences in the results seen between some of the experiments regarding cluster exposure, it is clear that increasing exposure leads to higher concentrations of flavonols and changes in the hydroxylation patterns of both flavonols and anthocyanins. This is due to an upregulation of the *F3'H* hydroxylase compared to *F3'5'H* hydroxylase, leading to higher proportions of di-hydroxylated flavonoids. Furthermore, most of the experiments looking at these changes are conducted using exhaustive extraction techniques to understand total synthesis, rather than model wine extractions. Although there may be differences when extracting flavonoids with an aggressive solvent, these results may not be representative in terms of the flavonoids extracted from a skin under mild ethanol, or wine-like,

extractions. Bindon et al. showed that anthocyanins extracted with 50% ethanol had nearly twice the concentrations extractions using 10% ethanol when using the same fruit. Furthermore, proanthocyanidins extracted in 70% acetone were 4-fold higher than those extracted in 10% ethanol.[10]

Results of extraction experiments such as Bindon's are of importance because the final product from research looking into grape maturity and development is wine. Although it is of interest to understand the total synthesis of compounds in some regards, it is also of interest to understand how model-wine extracts impact the apparent results as well. In the case of this experiment, the goal was to take the model-wine extraction results from the 2015 Cabernet Sauvignon survey seen in Chapter 2 and see if the results would be similar in a smaller controlled experiment. Furthermore, due to the high amount of variability seen in the 2015 experiment, a shade cloth trial was implemented to understand if variations in exposure in vineyards may have led to the large distribution in the results.

## 3.3: Materials and Methods

### 3.3.1: Experimental Design

In order to understand the impacts of shading during post-veraison maturation on multiple secondary metabolites, such as anthocyanins and proanthocyanidins, a shade cloth trial was implemented. The trial was setup up in a randomized complete block design (RCBD) on Mt. Veeder at the onset of color development in 2016; per treatment there were 5 replicates. Vines were planted in 2007 to Cabernet Sauvignon, clone 169. The rootstock was 420A with a spacing of 2.13 m × 1.22 m. Row orientation of the vines was 56 degrees off true north, with a vertical shoot positioned trellis design. A weather station was located 4 rows from the RCBD to calculate growing degree days. Treatments for this experiment were 0% (C), 40% (T1), and 80% (T2) shade.

Shade cloth was purchased from Green-Tek (Dinuba, CA). Shade cloth was placed on both sides of the canopy; in order to prevent the wind from blowing the cloth around it was tied at the corners to stakes that had been hammered into the soil; nylon ties were used to secure the shade cloth to the fruiting wire to prevent vertical light intrusion on the fruiting zone. **Figure 3.1** shows a picture of the shade cloth on the vines.

Figure 3.1: Photo of the shade cloth after it was installed. On the left side of the picture the thicker 80% (T2) cloth can be seen, while the thinning 40% (T1) cloth can be seen on the right.

Li-Cor 190R sensors were placed at fruiting zone height within the canopy, one on each side of the canopy – 2 per treatment, and connected to a Campbell Scientific (Logan, UT) Datalogger. The water status of the treatments was checked weekly by conducting predawn leaf water potential measurements (PDLWP) using a pressure chamber made by PMS Instruments (Corvallis, OR). 3 measurements were taken per replicate per treatment.

### 3.3.2: Vineyard Sampling

100 berries were taken from each replicate 5 times throughout the growing season, 50 from each side of the canopy. 60 of these berries were used to get primary metabolite information, while 40 were used for phenolic information. Sampling was conducted every 2 weeks starting; the first fruit sampling was conducted the same day that the shade cloth was installed, at 1078 Growing Degree Days (GDD), therefore this point is T=0 and fruit was expected to have no differences at this point. Yield per vine (kg/vine) was analyzed at the end of the growing season, along with pruning weights for Ravaz index information. Estimated yield per acre was calculated per treatment based on the yields per vine.

### 3.3.3: Extraction

The 40 grapes were taken per replicate for partial extraction. Skins were removed from pulp and seed by hand. Partial extractions were conducted in solutions containing 16% ethanol (v/v) for 72h in the dark. The extraction was placed on an orbital shaker at 150 rpm and 25 °C. Following partial extraction, skins were removed from solids by a Buchner funnel. Solids were pressed under vacuum with a polyethylene bag to collect the extract. The extract from the partial extraction was then frozen at −80 °C until analysis.

### 3.3.4: Instrumentation and Chemicals

An Agilent model 1100 (Santa Clara, CA.) HPLC system was used to conduct phenolic analyses of extractions. Chemstation software was used for all chromatographic analysis. Acetonitrile, acetone, acetic acid, anhydrous sodium acetate, L-(+)-ascorbic acid, hydrochloric acid, lithium chloride, *N,N*-dimethylformamide and *o*-phosphoric acid were all purchased from VWR (Radnor, PA) and were HPLC grade. (-)-epicatechin, oenin chloride, and kaempferol-3-*O*-glucoside were purchased from Sigma-Aldrich (St. Louis, MO.)

Primary metabolites were evaluated through the use of a Metter Toledo (Columbus, OH) auto titrator for total acidity and an Thermo Fisher Scientific (Waltham, MA) Orion Star pH Meter. Soluble solids (°Brix) was analyzed with a digital benchtop refractometer.

### 3.3.5: Tannin Activity

The HPLC method for measuring the activity of wine tannin has been described previously.[121, 122, 125] Briefly, the HPLC method used a polystyrene divinylbenzene reversed-phase column (PLRP-S, 2.1 × 50 mm, 100 Å, 3 μm, Agilent Technologies, Santa Clara, CA) protected with a guard column (PRP-1, 3 × 8 mm, Hamilton Co., Reno, NV), with DAD detection at 280 nm. The mobile phases consisted of 1.5% (w/ w) o-phosphoric acid in water (180 mM, mobile phase A) and 20% (v/ v) mobile phase A in acetonitrile (mobile phase B) with a flow rate of 0.3 mL/min. The linear gradient was as follows (time in min (%B)): 0 (14), 12.6 (34), 13.3 (34), 15.05 (70), 16.8 (70), 19.6 (14), and 28 (14).

To determine thermodynamic information, samples were run at four column temperatures (25−40 °C, 5 °C increments), and temperatures were converted to Kelvin for calculations. Chromatograms at 280 nm were baseline subtracted using water as a blank injection and were integrated as previously described.[121] Briefly, a baseline was drawn at 0 mAU and with the resulting area clipped at 5 and 28 min for total tannin ($Tannin_{Total}$); partial tannin ($Tannin_{Partial}$), respectively. Which corresponded to polymers, was the peak area eluting between 16.8 and 28 min. For each chromatogram an alternative retention factor for the tannin ($k_{alt}$) was calculated as follows:

$$k_{alt} = \frac{tannin_{Total}}{tannin_{Total} - tannin_{Partial}}$$

is related to thermodynamic information as follows:

$$lnk_{alt} = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} + ln\phi$$

where ΔH° and ΔS° are the enthalpy and the entropy (respectively) of the interaction, R is the gas constant (8.3145 $J \cdot mol^{-1} \cdot K^{-1}$), and T is the temperature of the experiment in Kelvin. The enthalpy was calculated by the slope from the Van't Hoff plot (i.e., ln $k_{alt}$ versus the reciprocal of the column temperature in Kelvin at each of the four temperatures).

### 3.3.6: Gel Permeation Chromatography

The size distribution of tannin isolates was determined using the previously described method of Kennedy and Taylor.[126] Briefly, the high-performance GPC method used to analyze tannins consisted of 2 PLgel (300 × 7.5 mm, 5 μm, 1000 by 500 Å) columns connected in series and protected by a guard column containing the same material (50 × 7.5 mm, 5 μm), all purchased from Agilent (Santa Clara, CA). The targeted sample injection amount was 40 μg. The isocratic method utilized a mobile phase consisting of *N,N*-dimethylformamide containing 1% v/v glacial acetic acid, 5% v/v water, and 0.15 M lithium chloride. The flow rate was maintained at 1 mL/min with a column temperature of 60 °C, and elution was monitored at 280 nm.

Integration was done using the GPC software for Agilent Chemstation. An additional cut was made at approximately 13 minutes to minimize the effects of residual monomeric anthocyanins on PA size distribution analysis.

### 3.3.7: Acid Catalysis in the Presence of Excess Phloroglucinol

Tannin subunit composition of tannin isolates were determined using the previously described method of Kennedy and Jones.[5] Briefly, the reversed-phase HPLC method consisted of two Chromolith RP-18e (100 × 4.6 mm) columns connected in series and protected by a guard column containing the same material (4 × 4 mm), all purchased from EM Science (Gibbstown, NJ). The method utilized a binary gradient with water containing 1% v/v aqueous acetic acid (mobile phase A) and acetonitrile containing 1% v/v acetic acid (mobile phase B). Pump flow rate was kept at

3.0 mL/min with a column temperature of 30 °C and with elution monitored at 280 nm. The linear gradient was as follows: time in min (% B); 0 (3%), 4.0 (3%), 14.0 (18%), 14.01 (80%), 16.0 (80%), 16.01 (3%), 18.0 (3%). (−)-Epicatechin was used as a quantitative standard.

### 3.3.8: Low Molecular Weight Phenolics

Low molecular weight phenolics were analyzed using a previously described methodology by Ritchey and Waterhouse.[127] Mobile phases: (A) 50 mM ammonium di-hydrogen ammonium phosphate in water adjusted to pH 2.6, (B) 80% Acetonitrile with 20% (A), (C) 0.2 M O-Phosphoric Acid in water adjusted to pH 1.5. The gradient for this methodology is reported by the authors.

### 3.3.9: Statistics

Graphpad Prism 9 (San Diego, CA) was used for multivariate analysis. A Probabilistic Principle Component Analysis (PPCA) was conducted to determine the loading plots. This software was also used for visualizations and Multiple Comparison Analysis of Variance. All significance between treatments is at 95% with Fishers LSD.

## 3.4: Results and Discussion

This study was conducted to gain a better understanding of the impact of shade implementation on anthocyanins and proanthocyanidins (PAs), when applied post-veraison. In a previous survey in Napa Valley Cabernet Sauvignon, it was seen that there was an inverse relationship between the PA activity and the concentration of anthocyanins. The goal for this project was to see whether manipulating the anthocyanin concentrations using shade impacted the PA activity throughout the growing season. This may provide insight into viticulture techniques and the impact that they have on secondary metabolites throughout ripening.

### 3.4.1: Berry Primary Metabolites, Weight, Vine Yield, and Ravaz Index

Berry weight hasn't been shown to be affected by increased light attenuation above the fruiting zone during the ripening phase; although it may be impacted by earlier shading.[94, 96, 101] In this study there were no significant differences in berry weight between treatments. Total soluble solids (TSS), pH, and titratable acidity were measured at each sampling date to look for differences in accumulation or degradation of primary metabolites (**Table 3.1**). Total soluble solids were significant different between the control and treatments in the final two timepoints, suggesting a

decrease in carbon assimilation in the shaded treatments, possibly due to the lower leaves being covered to the first wire.[145] Titratable acidity was significant different between the Control and Treatment 2 from 1219 to 1471 GDD, however at the final timepoint there were no significant differences between the control and treatments. This is likely due to a heatwave that occurred at the end of September, in 2015. Between September 13$^{th}$ and 27$^{th}$ there were 5 days above 38 °C. This may have led to the spike in pH seen at 1621.

Yield per vine, estimated yield per acre, and Ravaz Index (RI) were evaluated at harvest and pruning. When vines were pruned all shoots were placed in a pile next to the treatment and were weighed by digital scale in the vineyard. Yield per vine was also evaluated per treatment by harvesting all the fruit per treatment to weigh the clusters. Estimated yield per vine was calculated based on the vine spacing and yield per vine. Based on the results in **Table 3.2** there was no impact or differences seen due to shade implementation. This suggests any effects seen were not due to source-sink relations or fruit load differences, which have been shown to impact the production of anthocyanins during cluster thinning trials.[79, 81, 82]

Table 3.1: Changes in primary metabolites in Cabernet Sauvignon throughout ripening. Superscript letters show significance with 95% confidence by ANOVA. Letters only represent significant differences within a GDD point. N=5. Mean and Standard Error of Mean are shown.

**Primary Metabolites and Berry Weight through Ripening**

| Treatment | Growing Degree Days | Berry Weight (g) | Soluble Solids (°Brix) | pH | Titratable Acidity (g/L) |
|---|---|---|---|---|---|
| C | 1078 | 0.94 ± 0.04 | 13.28 ± 0.20 | 2.64 ± 0.01 | 19.96 ± 0.41 |
| T1 | " | 0.91 ± 0.03 | 13.32 ± 0.15 | 2.65 ± 0.01 | 20.60 ± 0.46 |
| T2 | " | 0.92 ± 0.02 | 13.40 ± 0.18 | 2.64 ± 0.01 | 20.77 ± 0.38 |
| C | 1219 | 0.91 ± 0.03 | 18.22 ± 0.15 | 2.95 ± 0.02 | 11.29 ± 0.25 **b** |
| T1 | " | 0.90 ± 0.05 | 17.80 ± 0.12 | 2.97 ± 0.02 | 11.85 ± 0.27 **ab** |
| T2 | " | 0.96 ± 0.03 | 17.34 ± 0.11 | 3.00 ± 0.01 | 12.65 ± 0.26 **a** |
| C | 1345 | 0.90 ± 0.03 | 20.42 ± 0.31 | 3.17 ± 0.03 | 7.75 ± 0.15 **b** |
| T1 | " | 0.89 ± 0.03 | 20.12 ± 0.15 | 3.20 ± 0.03 | 8.34 ± 0.09 **ab** |
| T2 | " | 0.91 ± 0.04 | 19.60 ± 0.20 | 3.22 ± 0.01 | 9.14 ± 0.15 **a** |
| C | 1471 | 0.91 ± 0.04 | 22.40 ± 0.27 **a** | 3.33 ± 0.02 **a** | 5.29 ± 0.15 **b** |
| T1 | " | 0.90 ± 0.04 | 21.88 ± 0.17 **ab** | 3.26 ± 0.03 **b** | 5.70 ± 0.15 **ab** |
| T2 | " | 0.92 ± 0.03 | 20.94 ± 0.23 **b** | 3.18 ± 0.01 **c** | 6.27 ± 0.22 **a** |
| C | 1621 | 0.90 ±0.03 | 25.68 ± 0.58 **a** | 3.80 ± 0.02 **a** | 3.41 ± 0.05 |
| T1 | " | 0.90 ±0.04 | 22.90 ± 0.42 **b** | 3.70 ± 0.04 **b** | 3.72 ± 0.07 |
| T2 | " | 0.91 ± 0.04 | 23.60 ± 0.65 **b** | 3.74 ± 0.03 **ab** | 3.62 ± 0.05 |

Table 3.2: Yield characteristics and Ravaz Index were evaluated at harvest and pruning. There were no significant differences between the control and treatments regarding yield per vine, estimated yield per acre, or yield to pruning weight. N=5. Mean and Standard Error of Mean are shown.

**Yield Characteristics and Ravaz Index at Harvest**

| Treatment | Growing Degree Days | Yield per Vine (kg) | Estimated Yield per Acre (t/acre) | Ravaz Index |
|---|---|---|---|---|
| C | 1621 | 3.95 ± 0.74 | 1.39 ± 0.26 | 7.06 ± 0.64 |
| T1 | " | 4.46 ± 0.53 | 1.57 ± 0.19 | 7.91 ± 0.67 |
| T2 | " | 4.11 ± 0.42 | 1.45 ± 0.15 | 7.79 ± 0.70 |

### 3.4.2: Impact of Shading the Fruit Zone on Water Potential ($\Psi_{PDLWP}$)

The use of a water deficit to stress vines has been shown to have a significant impact on the plant's synthesis of anthocyanins during the ripening phase, as well as variability in the hydroxylation patterns seen in flavonoids.[84, 85, 87] As a grapevine senses drought conditions in the root system, there is a production and release of ABA, leading to an increase in total anthocyanins.[88] Water deficit irrigation is purposely providing the vine enough water to survive, while also applying some stress to the vine to improve fruit quality. Throughout the experiment, Predawn Leaf Water Potentials ($\Psi_{PDLWP}$) were evaluated weekly. Results from this can be seen in **Figure 3.2**. Across the growing season there were no significant differences between the exposed control and the shaded treatments in terms of water stress.

The relationship between $\Psi_{PDLWP}$ and stem water potential ($\Psi_{STEM}$) was done in 2002 and there was a strong correlation between these two values. However, $\Psi_{STEM}$ generally has a higher water

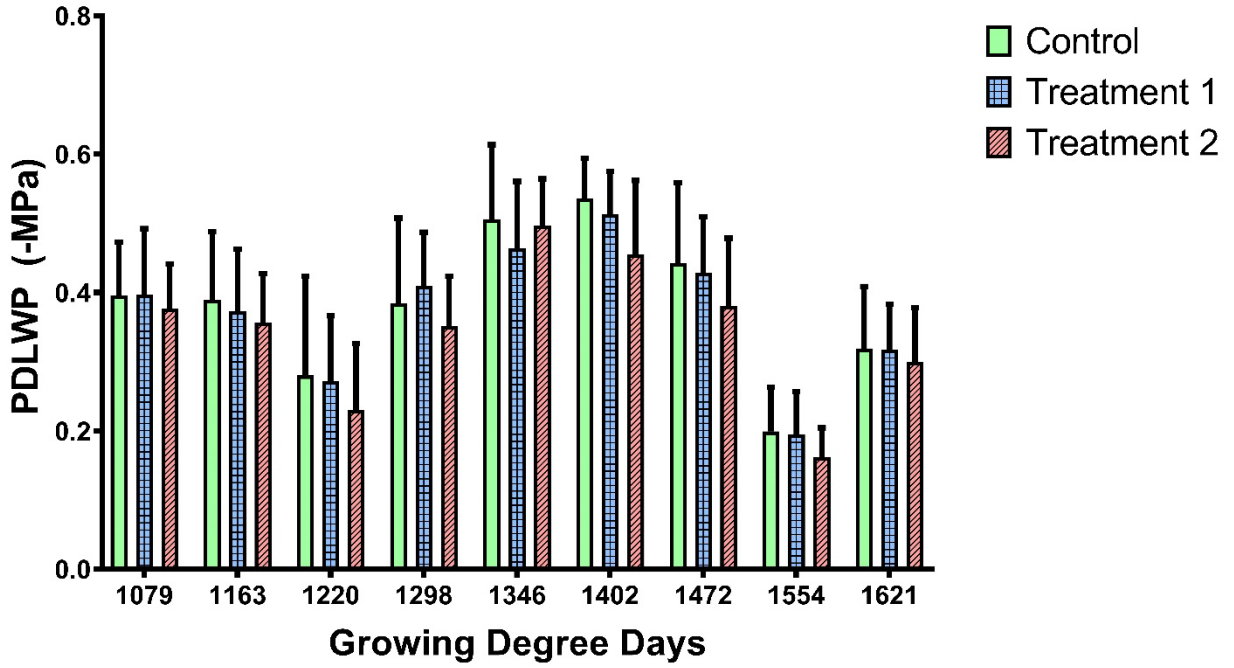


Figure 3.2: Plant water status measured by predawn leaf water potential over the course of ripening. N=15 per treatment; 3 leaves were evaluated per replicate, 15 total per treatment. A multiple comparison ANOVA was conducted to evaluate statistical significance between treatments – no significance was found. Error bars are SEM.

tension than that of $\Psi_{PDLWP}$ so the relationship of the values must be understood. The linear regression by Williams and Araujo was $y = -0.679 + 1.095x$ with $\Psi_{PDLWP}$ to estimate $\Psi_{STEM}$ in comparison to $\Psi_{PDLWP}$.[146] Regardless of the relationship or the type of water measurement, the data from this experiment suggests that while there was variability within treatments, none of these treatments were significantly different from one or another.

### 3.4.3: Changes in Anthocyanin Concentration and Composition

Anthocyanins were monitored throughout the ripening phase for concentration and compositional differences between treatments. There have been multiple studies on the effects of shading during the ripening phase showing that overexposure and temperature can lead to the degradation of

anthocyanins.[101, 105] There is not a consensus on whether increased light incidence can increase total anthocyanin concentrations.[95, 96, 100]

Previous work has shown that in some cases fruit with increased light exposure has an increase in anthocyanin concentration, as well as an upregulation of transcription factors in Cabernet Sauvignon.[9] It has also been shown that increases in nighttime temperature can lead to decreased concentrations of anthocyanins in the skins due to lower synthesis.[107] These results combined suggest that there is a light-temperature continuum that is not solely related to light incidence or day and nighttime temperatures – but both.

The results from the current work can be seen in **Figure 3.3**; as expected the initial timepoint, when the shade treatments were applied, there was no significant difference, however, by the

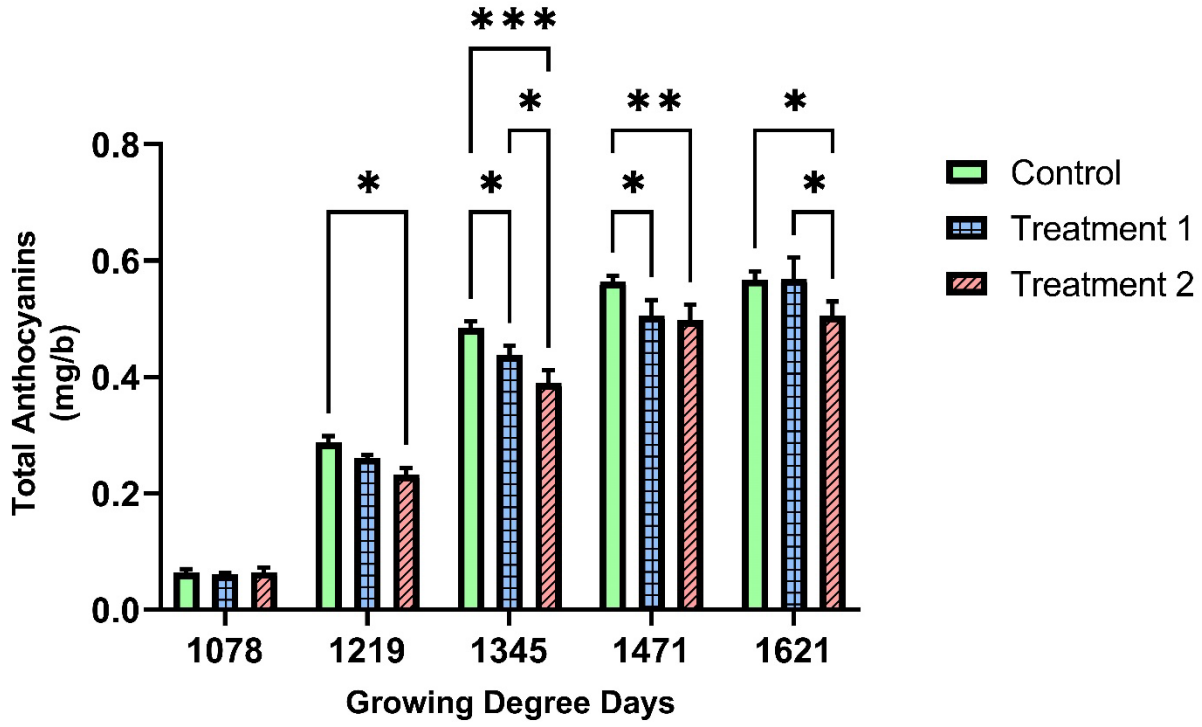


Figure 3.3: Changes in Total Anthocyanin Concentration (TAC) over the course of ripening in Cabernet Sauvignon during the 2015 vintage. *=0.05; **=0.01; ***=0.001; Multiple comparisons ANOVA was used to compare treatments; N=5; Standard Error of Mean was used for error bars.

second sampling date (GDD 1219) there is already a significant difference between the Control and Treatment 2. There is a clear treatment effect occurring at the third sampling date wherein all treatments were significantly different. However, as the experiment moved closer to commercial harvest, the effect began to wane. In the last timepoint (GDD 1621) there was no longer a significant difference between the Control and Treatment 1, yet both were still significantly different than Treatment 2. One possible explanation for this is that the Control is ripening faster than the treatments, however Treatment 1 had less soluble solids than Treatment 2 at the last time point, while having significantly more anthocyanins in the skins on a per berry basis.

Previous work has shown that changes in exposure can lead to variability in hydroxylation patterns, occurring due to differences in *F3'H* and *F'3'5H* which impact whether a flavonoid is di- or tri-hydroxylated on the B-ring. In terms of anthocyanins, there was a significant shift in the hydroxylation pattern seen after the implementation of the shade cloth. This can be seen in the far-right column of **Table 3.3**. At the second sampling point there is a shift for more exposed fruit to have a lower proportion of tri-hydroxylated substituents compared to the shaded fruit. The opposite trend can be seen in the dehydroxylated proportions, where there is an increase in the proportion of dehydroxylated anthocyanins. However, looking at the individual anthocyanins, there is a significant increase in the proportions of all other anthocyanins in the exposed fruit except that of malvidin-3-glucoside. This effect was mentioned in another study, where exposure to fruit may decrease methylation and acylation of individual anthocyanins. If it was solely a shift in the transcription of *F3'H* and *F3'5'H* due to exposure, the expected result would be a lower proportion of all tri-hydroxylated anthocyanins in exposed fruit, rather than just malvidin-3-glucoside. Acylated, coumarylated and caffeoylated anthocyanins were left off this table. They followed the same trend as seen in the monoglucosides below.

Table 3.3: Changes in monomeric anthocyanins between treatments over ripening. The table highlights the changes in hydroxylation pattens due to shade cloth implementation. P=0.05. N=5 with standard error of mean. (TAC: Total Anthocyanin Concentration; D3G: Delphinidin-3-O-Glucoside; C3G: Cyanidin-3-O-Glucoside; Pt3G: Petunidin-3-O-Glucoside; Pe3G: Peonidin-3-O-Glucoside; M3G: Malvidin-3-O-Glucoside)

**Changes in Anthocyanin Concentration and Composition**

| Treatment | GDD | TAC (mg/b) | D3G % | C3G % | Pt3G % | Pe3G% | M3G % | 3'4'-OH % | 3'4'5'-OH % |
|---|---|---|---|---|---|---|---|---|---|
| C | 1078 | 0.06 ± 0.07 | 2.56 ± 0.45 | 0.29 ± 0.01 | 4.69 ± 0.41 | 5.64 ± 0.29 | 51.0 ± 0.65 | 9.26 ± 0.66 | 91.0 ± 0.63 |
| T1 | " | 0.06 ± 0.00 | 2.71 ± 0.07 | 0.25 ± 0.03 | 4.78 ± 0.07 | 5.62 ± 0.10 | 51.0 ± 0.33 | 9.24 ± 0.15 | 90.8 ± 0.20 |
| T2 | " | 0.06 ± 0.01 | 2.43 ± 0.39 | 0.22 ± 0.08 | 4.47 ± 0.31 | 5.59 ± 0.28 | 51.2 ± 0.69 | 9.07 ± 0.66 | 91.0 ± 0.71 |
| C | 1219 | 0.29 ± 0.01 **a** | 4.85 ± 0.29 **a** | 0.70 ± 0.06 **a** | 6.17 ± 0.21 **a** | 5.72 ± 0.25 **a** | 51.4 ± 0.50 **b** | 10.9 ± 0.28 **a** | 89.2 ± 0.20 **b** |
| T1 | " | 0.26 ± 0.01 **ab** | 3.55 ± 0.26 **b** | 0.43 ± 0.03 **b** | 5.12 ± 0.21 **b** | 5.29 ± 0.10 **a** | 52.6 ± 0.47 **b** | 9.86 ± 0.09 **b** | 90.0 ± 0.00 **b** |
| T2 | " | 0.23 ± 0.01 **b** | 2.36 ± 0.34 **c** | 0.24 ± 0.04 **c** | 3.97 ± 0.33 **c** | 4.55 ± 0.20 **b** | 54.7 ± 0.44 **a** | 8.49 ± 0.41 **c** | 91.4 ± 0.51 **a** |
| C | 1345 | 0.48 ± 0.01 **a** | 6.42 ± 0.17 **a** | 0.71 ± 004 **a** | 6.72 ± 0.16 **a** | 5.14 ± 0.17 **a** | 50.7 ± 0.26 **b** | 10.6 ± 0.23 **a** | 89.6 ± 0.24 **b** |
| T1 | " | 0.44 ± 0.02 **b** | 4.98 ± 0.46 **b** | 0.44 ± 0.05 **b** | 5.59 ± 0.28 **b** | 4.55 ± 0.17 **b** | 52.2 ± 0.73 **b** | 9.58 ± 0.25 **b** | 90.2 ± 0.20 **b** |
| T2 | " | 0.39 ± 0.02 **c** | 3.00 ± 0.33 **c** | 0.21 ± 0.02 **c** | 4.13 ± 0.30 **c** | 3.87 ± 0.15 **c** | 54.6 ± 0.54 **a** | 8.22 ± 0.31 **c** | 91.6 ± 0.40 **a** |
| C | 1471 | 0.56 ± 0.01 **a** | 6.44 ± 0.17**a** | 0.60 ± 0.03 **a** | 6.80 ± 0.16 **a** | 4.57 ± 0.13 **a** | 52.5 ± 0.26 b | 9.76 ± 0.14 **a** | 90.4 ± 0.24 **b** |
| T1 | " | 0.51 ± 0.03 **b** | 4.87 ± 0.49 **b** | 0.35 ± 0.05 **b** | 5.47 ± 0.31 **b** | 3.94 ± 0.14 b | 54.0 ± 0.67 **ab** | 8.77 ± 0.25 **b** | 91.2 ± 0.20 **b** |
| T2 | " | 0.50 ± 0.03 **b** | 3.29 ± 0.38 **c** | 0.17 ± 0.03 **c** | 4.17 ± 0.32 **c** | 3.31 ± 0.11 **c** | 54.8 ± 0.69 **a** | 7.77 ± 0.24 **c** | 92.4 ± 0.25 **a** |
| C | 1621 | 0.57 ± 0.01 **a** | 6.26 ± 0.38 **a** | 0.50 ± 0.04 **a** | 6.60 ± 0.30 **a** | 4.06 ± 0.09 **a** | 53.3 ± 0.35 **b** | 9.07 ± 0.17 **a** | 90.8 ± 0.20 **b** |
| T1 | " | 0.57 ± 0.04 **a** | 4.44 ± 0.41 **b** | 0.30 ± 0.05 **b** | 5.25 ± 0.29 **b** | 3.61 ± 0.31 **ab** | 54.8 ± 0.49 **ab** | 8.50 ± 0.41 **ab** | 91.3 ± 0.33 **b** |
| T2 | " | 0.51 ± 0.02 **b** | 2.57 ± 0.50 **c** | 0.14 ± 0.04 **b** | 3.60 ± 0.44 **c** | 3.05 ± 0.18 **b** | 56.7 ± 0.82 a | 7.44 ± 0.41 **b** | 92.7 ± 0.33 **a** |

### 3.4.4: Proanthocyanidin Concentration and Composition

Proanthocyanidin synthesis has been shown to stop at the onset of color development.[94] Due to this, it was expected that the shade cloth application would have little impact on the concentration of PAs throughout the experiment. Proanthocyanidin changes over the course of the ripening phase can be seen in **Figure 3.4**. Although there were no dramatic treatment effects, such as those seen in the anthocyanins, there was a significantly more PAs in Treatment 2 at the final timepoint.

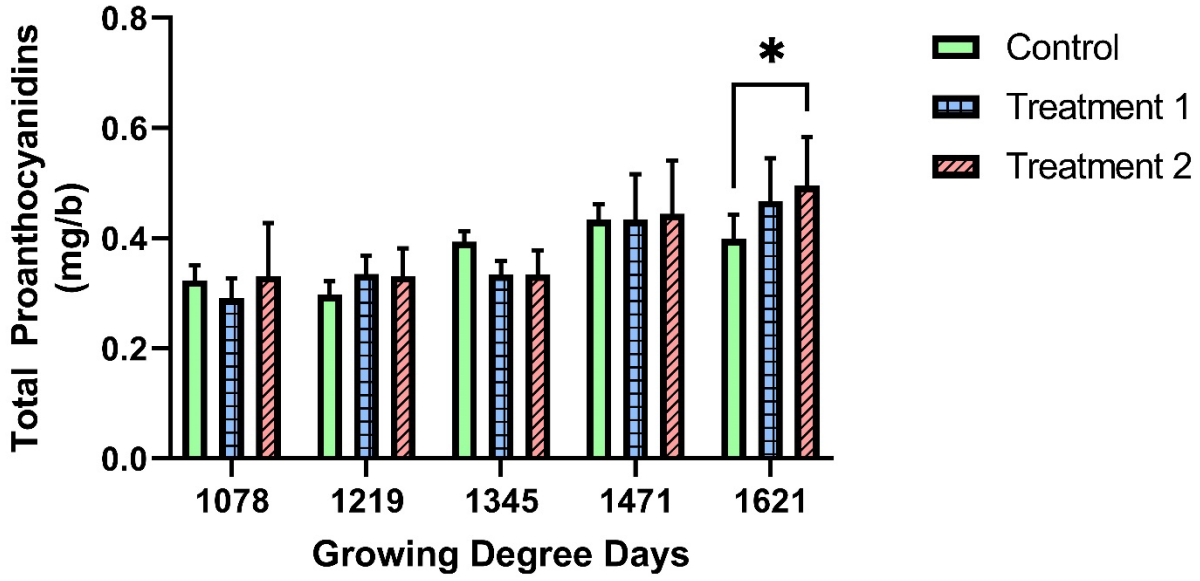


Figure 3.4: Changes in PA concentration over the ripening phase. *=0.05; N=5; Error bars are Standard Error of Mean; a multiple comparisons ANOVA was done to evaluate significance between the treatments

In terms of the last timepoint, the data does not support any clear explanation as to why there as a slight decrease in PA extracted compared to that of the shaded treatments. One possible explanation is that during the heat way between the last two sampling points, some of the PA was oxidized due to interactions with polyphenol oxidase (PPO), which can cause the oxidation of catechol groups leading to oxidative products. Phloroglucinol was used to quantify PAs in this experiment, and it is possible that if oxidation occurred due to heat stress, less PAs were quantified,

leading to this result. Regardless of the result at the end of the experiment, there appears to be no clear pattern having to do with the application of shade over the fruit at veraison.

Across all treatments there is a slight increase in the PAs extracted from the skins during the 72h extraction in ethanol. The results of this can be seen in **Table 3.4** where uppercase letters represent changes within a treatment. These results clearly suggest slightly increasing release of skin PAs as maturity is increase. One possible explanation is that as the cell wall loosens during advanced ripening, there was an increase in the extraction of PA compounds, leading to slight increases in extractability.[147] Another possible explanation is due to the influx of anthocyanins. Previous studies have suggested that interactions between anthocyanins and cell wall material (CWM) occurs.[112, 113, 115] If this is occurring, it is plausible that when anthocyanins interact with CWM, the decrease the likelihood of PAs being bound, which may be increases in PA material during an extraction. This experiment was not designed to understand the effects of extractability, or the impacts anthocyanins may have on the phenomenon, however the result does provide insight to future experimentation.

Regarding the subunit composition of the PA material extracted, there is little difference within the extension subunits EGC, EC, and C. Although the trend in the EGC composition suggest that the control had a slightly higher proportion, only the fourth sampling point had a significant difference in comparison to the shaded treatments. This relationship is also seen in the proportions of tri-hydroxylated extension subunits and di-hydroxylated, where significant differences were only observed at the fourth sampling point, with the control having higher levels of tri-hydroxylated and lower levels of di-hydroxylated PAs.

Table 3.4: Changes in PA concentration in mg/b, extension subunit composition, and hydroxylation pattern. In the concentration column capital letters represent changes overtime within a treatment. All lowercase letters identify significance due to treatment effects. EGC:Epigallocatechin, EC:Epicatechin, C:Catechin, ECG:Epigallocatechin. N=5; Error is presented in standard error of mean; significance is reported after evaluation from a multiple comparisons ANOVA

**Changes in Proanthocyanidin Concentration and Composition**

| Treatment | GDD | TPA (mg/b) | EGC-P % | EC-P % | C-P % | ECG-P % | 3'4'-OH % | 3'4'5'-OH % |
|---|---|---|---|---|---|---|---|---|
| C | 1078 | **BC** 0.32 ± 0.01 | 36.8 ± 0.95 | 56.8 ± 0.84 | 2.24 ± 0.20 | 4.24 ± 0.25 | 63.2 ± 0.95 | 36.8 ± 0.97 |
| T1 | " | **B** 0.29 ± 0.02 | 35.8 ± 0.59 | 57.2 ± 0.64 | 2.26 ± 0.23 | 4.69 ± 0.11 | 64.2 ± 0.59 | 36.0 ± 0.63 |
| T2 | " | **B** 0.33 ± 0.04 | 36.3 ± 0.26 | 56.5 ± 0.17 | 2.54 ± 0.31 | 4.74 ± 0.19 | 63.7 ± 0.26 | 36.4 ± 0.25 |
| C | 1219 | **C** 0.30 ± 0.01 | 36.3 ± 1.00 | 55.7 ± 0.93 | 2.53 ± 0.12 | 5.52 ± 0.18 **c** | 63.7 ± 1.00 | 36.2 ± 1.02 |
| T1 | " | **B** 0.34 ± 0.02 | 36.3 ± 1.11 | 54.2 ± 0.87 | 2.79 ± 0.31 | 6.73 ± 0.35 **b** | 63.7 ± 1.11 | 36.2 ± 0.97 |
| T2 | " | **B** 0.33 ± 0.02 | 35.5 ± 0.82 | 54.3 ± 0.76 | 2.70 ± 0.39 | 7.57 ± 0.18 **a** | 64.5 ± 0.82 | 35.6 ± 0.81 |
| C | 1345 | **AB** 0.39 ± 0.01 | 38.0 ± 1.46 | 53.5 ± 1.07 | 2.60 ± 0.30 | 5.90 ± 0.25 **c** | 62.0 ± 1.46 | 37.8 ± 1.50 |
| T1 | " | **B** 0.33 ± 0.01 | 37.5 ± 0.77 | 52.1 ± 0.81 | 2.38 ± 0.23 | 8.03 ± 0.25 **b** | 62.5 ± 0.77 | 37.8 ± 0.80 |
| T2 | " | **B** 0.33 ± 0.02 | 36.0 ± 0.42 | 52.2 ± 0.51 | 2.46 ± 0.13 | 9.37 ± 0.27 **a** | 64.0 ± 0.42 | 36.0 ± 0.55 |
| C | 1471 | **A** 0.43 ± 0.01 | 40.4 ± 0.76 **a** | 52.3 ± 0.51 | 1.97 ± 0.24 | 5.28 ± 0.33 **c** | 59.6 ± 0.76 **b** | 40.4 ± 0.75 **a** |
| T1 | " | **A** 0.43 ± 0.04 | 39.4 ± 0.63 **ab** | 51.1 ± 0.40 | 2.46 ± 0.27 | 7.11 ± 0.25 **b** | 60.6 ± 0.63 **ab** | 39.4 ± 0.68 **ab** |
| T2 | " | **A** 0.44 ± 0.04 | 37.8 ± 0.93 **b** | 51.0 ± 0.95 | 2.11 ± 0.11 | 9.08 ± 0.29 **a** | 62.2 ± 0.93 **a** | 37.6 ± 0.93 **b** |
| C | 1621 | **A** 0.40 ± 0.02 **b** | 40.4 ± 0.94 | 51.8 ± 0.67 | 2.16 ± 0.11 | 5.68 ± 0.24 **c** | 59.6 ± 0.94 | 40.4 ± 0.93 |
| T1 | " | **A** 0.47 ± 0.04 **ab** | 40.2 ± 0.83 | 50.6 ± 0.72 | 1.99 ± 0.15 | 7.27 ± 0.22 **b** | 59.8 ± 0.83 | 40.2 ± 0.74 |
| T2 | " | **A** 0.50 ± 0.04 **a** | 38.9 ± 0.36 | 50.7 ± 0.43 | 2.04 ± 0.15 | 8.43 ± 0.09 **a** | 61.1 ± 0.36 | 39.0 ± 0.45 |

Interestingly, there were large differences regarding the proportions of ECG, as seen in **Table 3.4**. Following the initial timepoint, when the shade was applied, there were significant differences at every other timepoint. The differences have a pattern which suggests that they are being impacted by the treatment and are significant across the entirety of the growing season. Previous work looking into the shading of fruit has shown impacts on significant impacts on extension subunit galloylation, however this was preveraison.[96] Recent work on tea leaves has shown that CsSCPL genes in *Camellia sinensis*, which suggests that Serine Carboxypeptidase-like Acyltransferases may impact the galloylation when the leaves are exposed or excluded from light.[148] The results are of interest as this is a post-veraison experiment, and subunit composition was not expected to be impacted in ways that would be expected of an experiment starting at flowering.

### 3.4.5: Tannin Molecular Mass and Mean Degree of Polymerization

In the 2015 survey project, discussed in chapter 2, there was a decrease in PA molecular mass associated with increasing ripeness. The initial extraction protocol from that project assigned whole berries for extraction rather than just skins, therefore it was assumed that seed PAs would be involved in the results. In this experiment the goal was to build upon the results from the 2015 Cabernet Sauvignon project while also eliminating variables. In this regard, it was not predetermined that the results from the GPC work would deliver the same results regarding the overall trend seen in 2015. However, it can be seen in **Figure 3.5** that there is a decreasing PA size throughout ripeness, like that seen in 2015. This suggests that although there was seed PA extraction occurring, which can be seen in the PA subunit composition in 2015, skin PAs alone follow a similar trend. Due to the first time point being taken at the application of shade cloth, it was expected that there would be no significant difference between treatments. As early as the

second sampling point (GDD 1219) there are significant differences between treatments concerning the size of the PAs extracted. Although there are significant differences within the experiment, and a clear treatment effect concerning the molecular mass of PAs in the context of the amount of fruit exposure, there were no significant differences at commercial harvest.

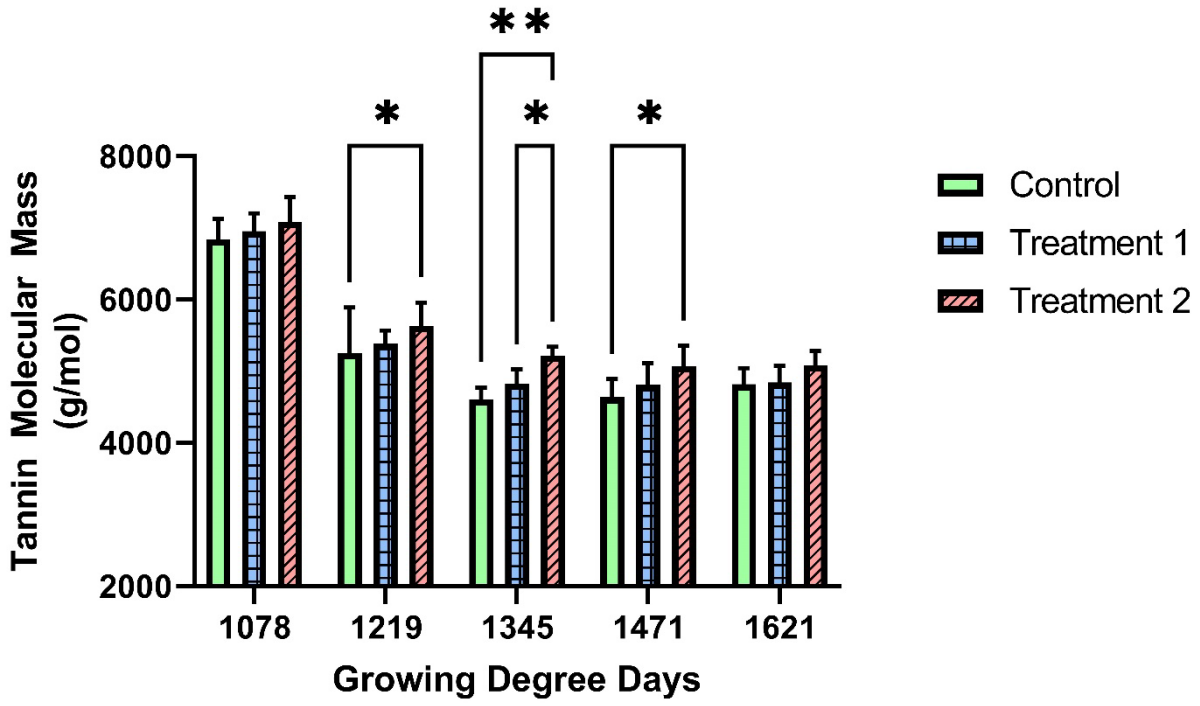


Figure 3.5: Changes in PA molecular mass, as characterized by gel permeation chromatography, throughout the 2016 growing season on Cabernet Sauvignon. *=0.05; **=0.01; N=5; Error bars are standard error of mean. Significance was evaluated by a multiple comparison's ANOVA.

In this experiment mDP was also measured through the quantification of extension and terminal subunits following acid-catalysis in the presence of a nucleophile. The results from this analysis suggest that PA size distribution is increasing throughout ripening (**Figure 3.6**) in all treatments, inverse to that of the GPC results. In the case of mDP, there is also a significant difference between the Treatment 1 and Treatment 2 at commercial harvest, however there is no difference between the Control and either Treatment. Although there is a difference between the two treatments at the

final timepoint, there is no pattern to suggest that the imposed shading impacted flavan-3-ols. The results for PA mean degree of polymerization also follow the trend seen in the non-linear regression from 2015 (**FIGURE 2.7**), although with a larger PAs, suggesting that data from the survey is likely similar to expected trends in controlled experiments on Cabernet Sauvignon in Napa Valley. The increase in mDP relative to those seen in 2015 is likely due to the removal of seed material from the experimental design, which is known to have smaller PAs when compared to the skin.

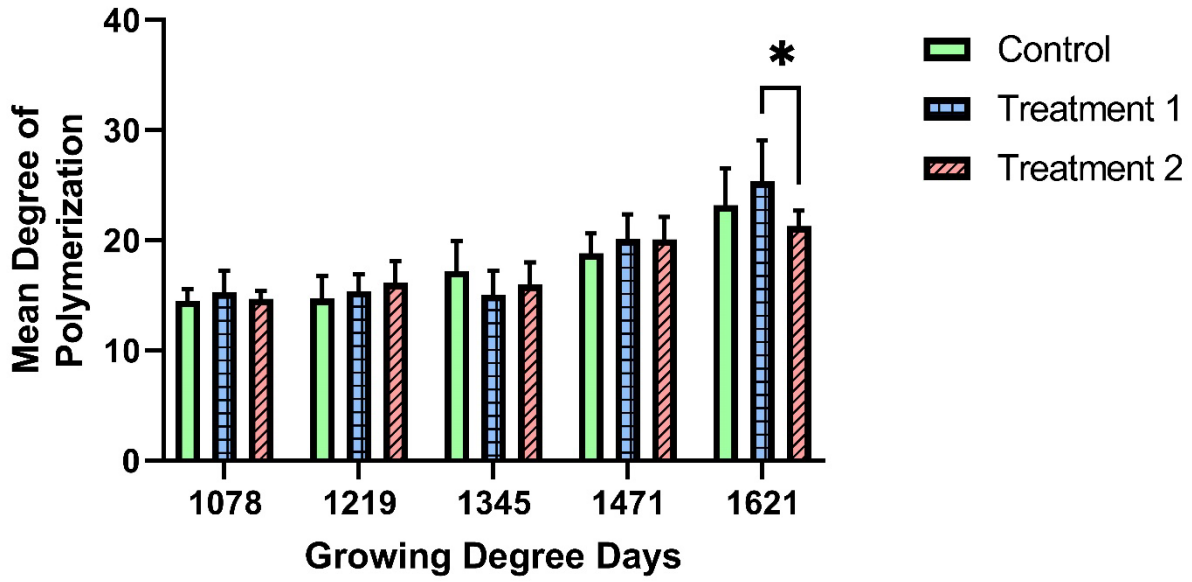


Figure 3.6: Mean degree of polymerization as calculated from acid-catalysis of PAs. *=0.05; N=5; Error bars shown are standard error of mean. Significance was evaluated by a multiple comparison's ANOVA at each timepoint.

Although both GPC and mDP are measures of polymer size, mDP is a calculation based on the ratio of extension and terminal subunits in a PA polymer. If a nucleophile that is not a flavan-3-ol was at the terminal position, it can lead to increases in mDP due to a smaller denominator in the calculation. Oxidation can also lead to problems with this methodology, as PAs become more resistant to acid-catalyzed cleavage, the mass balance of the analysis will decline, leading to

inaccurate assumptions on the actual polymer size. Although both analyses follow the trends seen from the 2015 survey in Chapter 2, it is likely that the GPC data is more representative of the polymer size extracted from grape skins in a model wine solution.

### 3.4.6: PA Activity

Activity is a relatively new methodology based on thermodynamic measurements using high-performance liquid chromatography (HPLC). This assay quantifies the variations in interactions between PAs and a polystyrene divinylbenzene column (PLRP-S) over multiple temperatures to derive an enthalpic value to address structure-activity relations occurring due to variations in PA size, structure, and composition. The results from this method are independent of PA concentration, as shown by Revelette et al. in 2014, where a serial dilution of a wine (thus changing PA concentration) did not affect the corresponding measuredactivity.[12] This work showed that activity is an intensive property, designed to quantify the strength of interaction between extracted

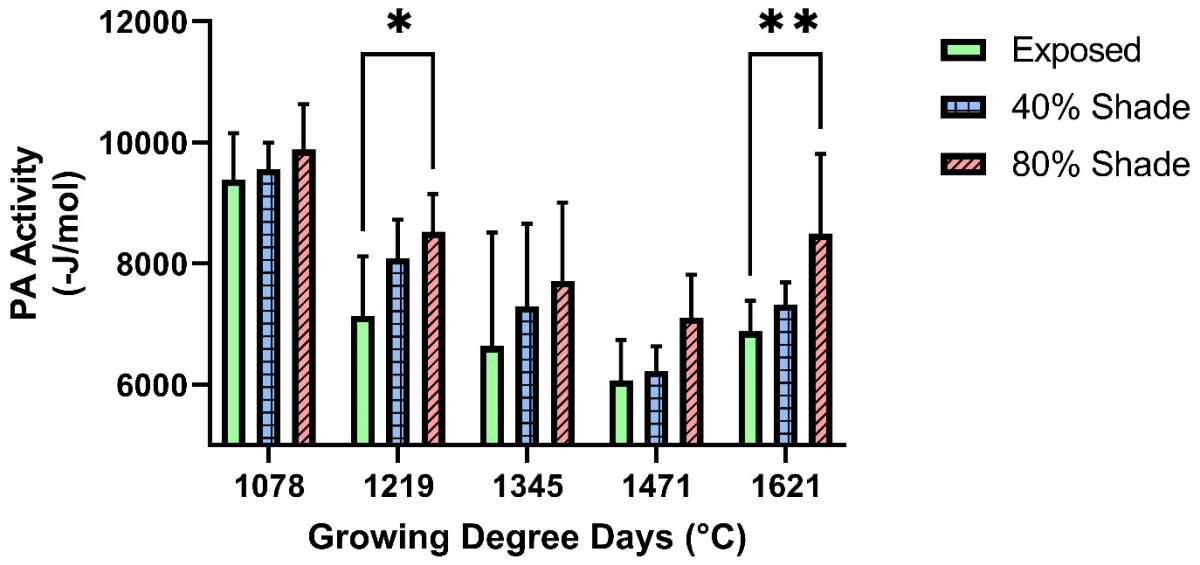


Figure 3.7: Changes in PA activity over the course of ripening in Cabernet Sauvignon. Activity decreased through ripening, like the 2015 survey in Chapter 2. N=5; *=0.05; **= 0.01; Significance was evaluated using a multiple comparisons ANOVA.

PAs and a hydrophobic surface. Recent sensory work has shown that, when comparing other PA quantification techniques, activity was the most correlated with the wine scores on astringency and dryness.[32]

The Cabernet Sauvignon survey project in Chapter 2 was the first time this HPLC method had been used to understand the changes in PAs occurring over the course of the ripening phase by using model wine extractions. In this experiment, a controlled viticulture study was conducted to understand if this property could be manipulated in the vineyard. **Figure 3.7** shows that there were significant differences in the activity of the PAs in control and treatment wines. Proanthocyanidins extracted from skins of the exposed control had a lower average activity and therefore would be expected, based on previous work, to be less drying when compared to the treatments. The results followed a similar pattern to that seen in Chapter 2, as well as PA molecular mass in **Figure 3.5**, however there was a significant difference in PA activity at harvest, which was not seen in PA molecular mass. The multivariate analysis from 2015 showed a strong relationship between PA size and activity, this positive correlation was also seen in this experiment. The relationship between PA size and astringency in wine-like solutions has been well documented and researched, showing that higher molecular weight PAs have a stronger binding affinity with proline-rich-proteins in mucus, leading to increases in astringency.[33-34]

Although size distribution has been shown to have correlations with PA activity and the feeling of astringency, the subunit composition of PAs has also been evaluated. The results from that work showed that increases in the proportion of (-)-epicatechin gallate (ECG) led to marked increases in the precipitation of salivary proteins. This suggests that not only the size of the proanthocyanidin, but also the subunits it consists of, is likely to impact the PA activity. In this experiment, there were significant differences found between treatments (**Table 3.4)**. Significant

differences occurred at the second sampling point (1219 GDD) and continued through commercial harvest. This find is of interest because it implies post-synthesis modification of the PA polymer due to the shading being imposed at veraison. Previous work has shown that increases in shading led to higher proportions of galloylation in grape skin PAs, however that work did not show any differences at commercial harvest, as seen in this work.[7]

The enzyme responsible for the galloylation of flavan-3-ol polymers in tea leaves has been shown to be an enzyme like those used for acylation. These enzymes, serine carboxypeptidase-like acyltransferases (SCPL) were shown to have galloylation activity towards (-)-epicatechin. Interestingly, this work shows that the genes responsible for the upregulation of SPCL enzymes are mainly driving by light response in tea leaves.[35] However, another study showed that increasing the exposure of tea leaves led to increases in PA galloylation, which is contradictory of the results seen in this experiment on grape skins.[36] Although the results differ, the possibility of post-synthesis modifications to PAs during the ripening phase, and whether exposure impacts the proportion of galloylation in PAs, is of interest.

A correlation between the galloylation of PA subunits and activity was seen in an experiment looking at extended maceration on activity.[125] As the maceration time of wine fermentations was lengthened, the concentration and composition of PAs changed, likely due to seed extraction. Grape seeds are known to have higher levels of galloylation, likely leading to variable compositions of PAs in the maceration experiment, depending on the length of maceration time. This led to increases in PA activity over the course of extended maceration. As was discussed earlier, because activity is an intensive property not controlled by concentration, and that seed PAs are a lower molecular mass than skin PAs, this change in activity is likely due to increases in compositional differences.

In **Figure 3.8** results from a principal component analysis are shown. Like the results from Chapter 2, there was an inverse relationship between the percent of pigmentation in the PAs and activity. Moreover, these was a clear relationship between the molecular mass of the PAs and the activity. The results from this controlled experiment suggest that anthocyanin incorporation into PAs was likely driving changes in the PA size distribution, leading to a significant decrease in molecular mass. Previous work has shown a clear relationship between the PA molecular mass and activity, which was likely contributing factor to the differences seen in the activity results, however because this experiment had other variables activity differences were likely driven by multiple factors.[11] Although there were compositional differences in terms of galloylation, this did not seem to have

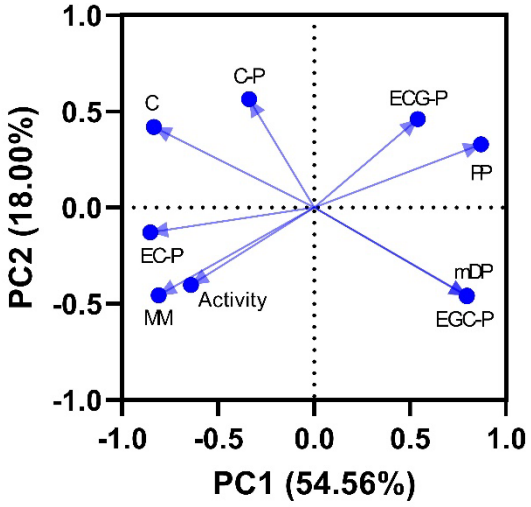


Figure 3.8: Principal component analysis looking at size and structural variables in PAs and their relationship with PA activity.

the same relationship as seen in previous work on activity, wherein increases in galloylation was positively correlated with activity. This inverse correlation may be due to the increasing proportion over ripening, with a decrease in molecular mass and activity. In this case it is likely that correlation does not equal causation but is likely correlated in this PCA due to ripening effects.

### 3.4.7: Implications

Not only does this work confirm the findings of the 2015 Cabernet Sauvignon survey work from the previous chapter, the results suggest that the variability seen within that project is in part due to variations in exposure. In this experiment there were clear impacts on variables that have been shown alter the activity of PAs, such as pigmentation and molecular mass. This led to significantly different activity values in model wine extracts from commercial harvest. Given the recent sensory evaluation of activity and its relationship with astringency, it is possible that these activity differences may lead to wines with variable types of astringency qualities.[32, 37-38]

Previous shade experiments have looked at the impact of light incidence on hydroxylation pathways, flavonol concentration, anthocyanin concentration, or volatiles. In this experiment we wanted to use a new method used to understand structure-activity relations in proanthocyanidins to look at changes over the course of a growing season in a controlled shade study. Results from this work suggest that increasing levels of shade increased the PA activity and molecular mass while decreasing the concentration of monomeric anthocyanins. Shade cloth also led to increases in the proportion of galloylation in the PAs, which would likely lead to a change in astringency quality in resultant wines.

## 3.5: Acknowledgements

The authors would like to thank Charles Thomas and Thomas-Hsi Vineyards for allowing us to use their vineyard. We would also like to thank the American Vineyard Foundation for financial support of this project.

# Chapter 4: Anthocyanin Addition Alters Tannin Extraction from Grape Skins in Model Solutions via Chemical Reactions

## 4.1: Abstract

Condensed tannin extraction and stable color formation are two of the cornerstones of red wine production. Without condensed tannin, red wine would lack the tactile feeling of astringency, and without the formation of modified pigments it would lack color stability for long term aging. To understand how malvidin-3,5-diglucoside, interact with condensed tannin under non-oxidative conditions, an experiment was designed conducting model-wine skin extractions of Sauvignon Blanc grapes harvested at various dates of maturity. Monomeric malvidin-3,5-diglucoside was isolated from color concentrate and added during these extractions. Following a 72-hour extraction, solutions were evaluated for recovery of monomeric anthocyanins, skin tannin concentration, skin tannin extractability and impact of anthocyanins on condensed tannin size. Anthocyanins showed a significant impact on the extraction of flavan-3-ol material in the early stages of ripening that declined in the latter stages of ripening. Furthermore, anthocyanins significantly decreased the size of the condensed tannin extracted. These results suggest that anthocyanins are not only enhancing the extractability of condensed tannin, but also readily incorporating into the polymeric material, leading to a decrease in the average molecular mass of the condensed tannin polymer. The extent of reaction in 72 hours suggest that the rate of interflavan bond cleavage may be higher than previously reported and merits closer scrutiny.

## 4.2: Introduction

The phenolics present in grape skin are some of the most important compounds in red wine production. Specifically, the color of red wine is determined by anthocyanins, and condensed tannins (CT) contribute to the tactile feeling of astringency. Condensed tannin derived from grapes varies depending on the location from which it was derived in the berry. Condensed tannin derived from seed generally have a higher mole proportion of galloylated and procyanidin subunits, with

prodelphinidin subunits being absent, while those from the skin have a higher proportion of prodelphinidin and procyanidin subunits. Furthermore, CT from the skins have been shown to have a larger mean degree of polymerization (mDP) in comparison to that of the seeds.[143]

It has been known for many years that anthocyanins and CT react in wine solution to form pigmented polymers.[149] These reactions are important in the production of red wine due to the color-stabilizing feature of pigmented polymer. Without these reactions, the color of red wine would not persist through the long aging cycle of some red wines.

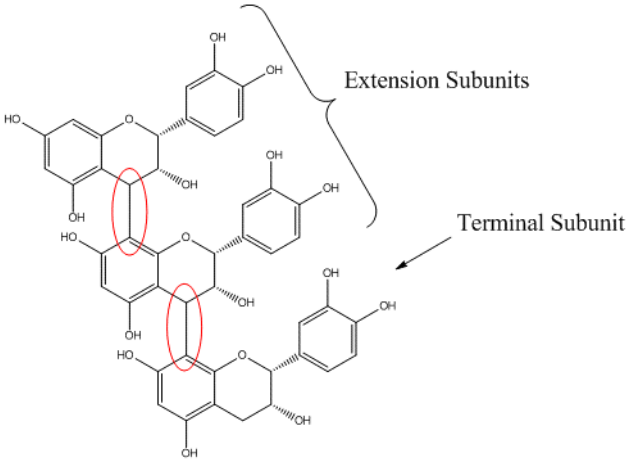


Figure 4.1: Condensed tannin trimer, showing extension and terminal subunits as well as the interflavan linkage (IFL) circled in red.

Condensed tannins are known to have an acid labile 4β→8 linkage, also known as an interflavan linkage (IFL), between the flavanol subunits that make up the polymer (**Figure 4.1**). Although CT has been shown to also have 4β→6 branching linkages (Procyanidin B7), previous kinetics work has shown that the cleavage of this type of linkage has a higher activation energy than that of the 4β→8 linkage. In acidic conditions, such as wine (pH ~ 3.5), these bonds will cleave – forming an intermediate that is readily attacked by a nucleophile in an $S_n1$ reaction.[7, 150] Historically, the cleavage of the IFL in the presence of an excess nucleophile, and subsequent analysis of reaction products, has been used to approximate the degree of polymerization (mDP) and subunit composition of CT material.[5, 10, 151-155]

Just as CT extension units have an electrophilic center at C-4 following cleavage, anthocyanins also can act as electrophiles. In this case flavan-3-ol monomers attack the C-4 position of an anthocyanin in the flavylium form, resulting in the formation of a flav-2,3-ene addition product.

A colorless dimer is formed through the formation of an IFL, however the extension subunit in this case is the anthocyanin.[36] Further modification of this product results from the bond formation between the hydroxyl at the 7-position of the terminal subunit and the unsaturated 2-position of the colorless anthocyanin extension subunit, resulting in the A-type dimer. Although this reaction has been shown to occur in wine, the equilibrium proportion of malvidin-3-O-glucoside (the principle anthocyanin in *Vitis vinifera* L.) in the flavylium form at wine pH (3.5) is under twenty percent; therefore, the rate of this product formation is likely lower than total anthocyanin amount would suggest.[156] It is known that malvidin-3-*O*-glucoside can act as a nucleophile while in the hemiketal form (**Figure 4.2**) in the reformation of CT after acid-catalyzed bond breaking events. Moreover, the pKa between the two dominant equilibrium forms of malvidin-3-*O*-glucoside, the flavylium and hemiketal forms, is approximately 2.6.[12, 156-158] Therefore, the addition of anthocyanins at wine pH increases the number of flavonoids that can act as a nucleophile.

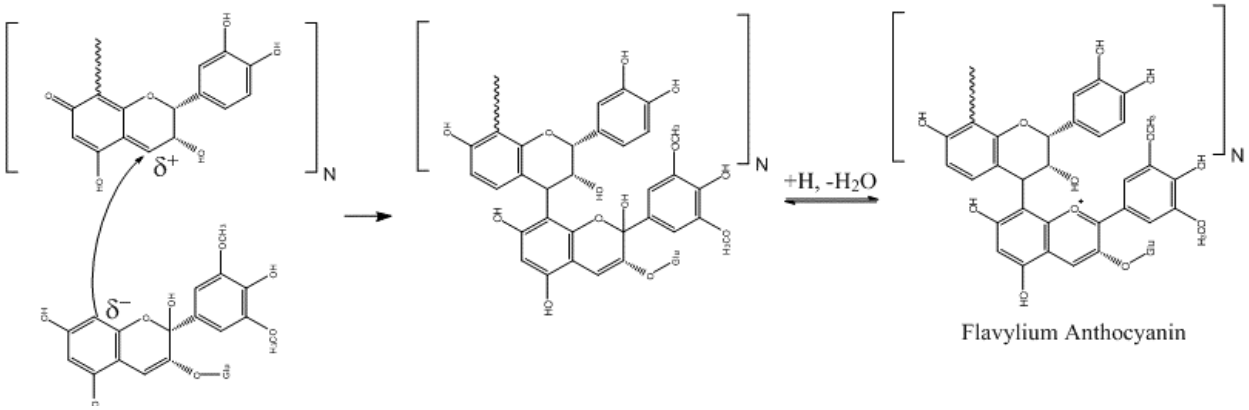


Figure 4.2: Nucleophilic attack of a condensed tannin cleavage intermediate by an anthocyanin in the hemiketal form to form a pigmented tannin product.

Pigmented CT can also form through acetaldehyde-mediated bond formation events. In one instance, acetaldehyde undergoes nucleophilic addition from the 6 or 8 positions of a flavan-3-ols and anthocyanins to form an ethylidene-bridged product.[159-162] Previous work has suggested that

the rate of anthocyanin incorporation into CT through acid-catalysis is slower than that of the oxidative pigmented tannin formation reactions occurring in the presence of acetaldehyde.[163] Additionally, investigations of CT cleavage at a pH of 3.6 and temperature of 20 °C indicated that the half-life of the IFL of a procyanidin B2 is approximately 200-400 hours, however recent work has suggested that it may be a much slower process.[6, 164] Despite this, the nucleophilic addition of an anthocyanin hemiketal form to a CT polymer cleavage intermediate remains a primary causal explanation for pigmented tannin formation during wine aging.[25] Furthermore, it has been shown in a young wine as possibly being the dominant form in which pigment is stabilized.[165]

Recently, research has looked at not only the formation of pigmented polymer, but also the possibility that anthocyanins may enhance the extraction of CT material from grape skins. Early work in this subject area looked at the impact of anthocyanins during white grape fermentations, indicating that such additions appeared to keep CT material in solution. The work concluded that the formation of pigmented polymer led to the ability to maintain CT in solution, while it precipitated out of the wines without anthocyanin additions.[166]

While *Vitis vinifera* contains only monoglucosides of the anthocyanidins, the addition of hybrid grape-based anthocyanin extracts that contain diglucosides is commonplace in US winemaking. The paucity of data on diglucosides and their impact on color development suggest that additional work would be of interest.

Condensed tannin interactions with cell wall material (CWM) has been well documented through isothermal titration calorimetry.[120] Given the location of CT in the vacuole of the cell in the skins of grapes, and the changes that have been visually annotated through scanning electron microscopy in ripening grape skins, they are likely to have a significant impact on tannin extractability.[113] A significant amount of work has been completed to understand the impact of changes in CWM over

the ripening phase in grape skins. More specifically, it has been shown that there is an increase in the proportion of water-soluble polysaccharides extractable from the skins from veraison until commercial harvest. This increase in water-soluble portions of CWM was reported to be due to possible modifications to galacturonan throughout development leading to increases in solubility in aqueous solutions.[117] These modifications are likely explained by the deesterifiation of methylated galacturonans leading to increases in solubility throughout ripening. The degree of esterification (methylation and acetylation) has been shown to decrease throughout ripening in multiple varieties, with variability in the percent decreased being varietal dependent.[147]

As stated above, CT interactions with CWM has been probed through isothermal titration calorimetry to better understand the types interactions of polysaccharide and CT.[120] The results of this work not only show that homogalacturonans with higher degrees of methylation interact more strongly with CT, but also that higher degrees of polymerization in CT favor CWM interactions. Furthermore, this work, and follow-up work, showed that the interactions were entropic in nature favoring hydrophobic and hydrogen bonding interactions between the macromolecules.[167]

Although major focus has been on CT and CWM interactions, there has been research on the absorption of anthocyanins to CWM as well. Based on work conducted using isolated CWM in the presence of anthocyanins and CT, it has been shown that there is an impact on the extraction of CT material from CWM when anthocyanins are in the same solution. This along with other work looking into the impact of anthocyanins on CT extraction, provide framework for the possible interactions between anthocyanins and CWM. This same work showed that the addition of anthocyanins to CWM isolates alone led to greater than 49% of anthocyanins in solution being absorbed onto the isolate material.[115] Recently, research has shown that the adsorption, as well as

the desorption, of anthocyanins to CWM is dependent not only on ethanol and temperature, but also the type of CWM isolate.[168]

This study was designed to better understand the impact of anthocyanins on the extractability of CT, as well as the formation of pigmented tannin in solution. Anthocyanin diglucosides were added in varying concentrations to model wine extracts containing grape skins (cv. Sauvignon blanc). The goals of this study were two-fold: Firstly, understand to what extent added anthocyanins influence the extraction of CT, and secondly, determine to what extent pigmented tannins could be formed at this early stage of wine production in the absence of fermentation metabolites.

## 4.3: Materials and Methods

### 4.3.1: Grape Material

Grapes used for this project were from the Anderson Valley in Mendocino County, California. Sauvignon Blanc was chosen for its lack of anthocyanins present in the skins. The grapes were sampled at 5 time points throughout the growing season starting at lag phase and ending at commercial harvest. Sampling was conducted by removing whole clusters from a single side of the canopy and placing bags of clusters on ice for transport back to the University of California, Davis.

### 4.3.2: Density Flotation

Density flotation was conducted as previously published.[169] Briefly, three solutions were made for targeted soluble solids concentration at each sampling stage. The solutions were bracketed at +/- 1 °Brix and checked using an Atago PAL-1 (Atago, Tokyo, Japan) prior to flotation to have uniform ripeness in collected berries. Berries were trimmed from rachis through the pedicel. Following trimming, berries were placed in the highest concentration sucrose solution and sinking

berries were discarded. Berries that floated in the first sucrose solution were then placed in the middle sucrose solution. Berries that floated in the middle sucrose solution were then placed in the lowest sucrose solution. Berries that sank in the lowest and the middle solution were collected for experimentation. Berries spent approximately 2 minutes or less in each solution.

### 4.3.3: Reagents

All solvents used in this study were HPLC-grade or higher. Glacial acetic acid, l-ascorbic acid, hydrochloric acid, methanol, phosphoric acid, sodium acetate anhydrous, citric acid, and sodium citrate were purchased from VWR International (Radnor, PA). Phloroglucinol, (−)-epicatechin, acetonitrile, ethanol, methanol, *N,N*-dimethylformamide, lithium chloride and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid was purchased from MP Biomedicals (Irvine, CA). Water was purified using an Milli-Q purification system (MilliporeSigma, Burlington, MA).

### 4.3.4: Monomeric Anthocyanin Isolation

Monomeric anthocyanins were derived from a hybrid color concentrate product. Additional purification was carried out by low pressure chromatography using Sephadex LH-20. Briefly, the column was swelled and packed in a solution of Milli-Q water acidified with 0.1% TFA. Concentrate was loaded onto a column of LH-20 with an approximate bed volume of 1 L. Anthocyanins were eluted from the column with 1:1 (v/v) methanol and water with 0.1% TFA. Fractions were collected based on visual inspection of the eluted material. Following elution, fractions were freeze dried and the powders were analyzed for purity by RP-HPLC as described below. The average purity of the powders was 62% malvidin-3,5-diglucoside by weight and 93% by peak area at 520 nm. Prior to isolation, the concentrate product was tested by acid-catalysis for the presence of CT. The results confirmed the absence of polymeric material.

### 4.3.4: Skin Extraction Process

Skin extractions were conducted using fresh, unfrozen berries, in a sequential manner. Skins were initially extracted in a model wine-like solution of ethanol followed by an acetone extraction, as described below. All extractions were conducted in triplicate.

#### 4.3.4.1: Partial Extraction

After flotation, the volume of the grapes was evaluated by placing them in a graduated cylinder and filling with water. Skins were removed from pulp and seed by hand. Skins were extracted in the same volume of solution as berry volume. Partial extraction solutions contained 16% ethanol (v/v) buffered at a pH of 3.3 with a 300 mM citric acid buffer for 72 hours. The extraction was placed on an orbital shaker at 150 RPM and 25 °C. These model wine solutions had purified anthocyanins added to them in order to make extraction solutions containing 0, 300, and 1000 mg/L of anthocyanins. Prior to extraction the exact concentration of anthocyanins from each solution was quantified by RP-HPLC so that anthocyanin recovery could later be evaluated. Following partial extraction, skins were removed from solids by Buchner funnel. Solids were pressed under vacuum with a polyethylene bag to collect the extract. The extract from the partial extraction was then frozen at -80 °C until analysis.

#### 4.3.4.2: Exhaustive Extraction

Skins recovered from the Buchner funnel in the partial extraction were ground under liquid nitrogen using a ball mill (Retsch, Haan, Germany) and placed in 66% aqueous acetone for 72 hours to extract phenolic material not removed during the partial extraction process. The extractions were placed on an orbital shaker at 150 RPM and 25 °C. After the acetone extraction process, solids were separated again by Buchner funnel, this time including a Whatman filter. Following filtration, the acetone was removed under reduced pressure at 30 °C, leaving a small

aqueous volume. This small volume was then brought to the original extraction volume using Milli-Q water. The extract from the exhaustive extraction was frozen at -80 °C until analysis.

### 4.3.5: Solid Phase Extraction

Solid phase extraction (SPE) conducted as published with slight modifications.[170] Briefly, Waters (Milford, MA) HLB Oasis Cartridges with 30 mg of sorbent material were used. The cartridge was equilibrated using 3 mL of methanol and washed with 3 mL of milli-q water. Following equilibration, 1 mL of extract was loaded onto the cartridge, then the cartridge was dried for 12 minutes under a stream of nitrogen. Following drying, the cartridge was washed with 40 mL of 95:5 (v/v) acetonitrile: 0.01 M hydrochloric acid to remove monomeric material. Polymeric material was then eluted using 300 µL of neat formic acid followed by 2700 µL of 95% methanol.

Initial samples were evaluated for the presence of monomeric anthocyanins following SPE. Based on visual inspection of a RP-HPLC chromatogram at 520 nm (discussed below), the polymeric material eluted by the formic acid and methanol was free of any monomeric anthocyanins.

### 4.3.6: RP-HPLC Analysis

#### 4.3.6.1: System

All RP-HPLC analysis were conducting using an Agilent 1100 (Santa Clara, CA) with a diode array detector (DAD), with the exception of gel permeation chromatography, which was conducted on an Agilent 1260 with a DAD.

#### 4.3.6.2: Gel Permeation Chromatography

Gel permeation chromatography was conducted to analyze CT size distribution as previously described with slight modifications.[126] Briefly, Agilent OligioPore® and MesoPore® columns (250 × 4.6 mm) were connected in series. Flow rate was 0.65 mL/min, with a runtime of 40

minutes. A calibration curve was created using fractioned grape skin and seed material from pre-veraison Cabernet Sauvignon grapes using Toyopearl-HW40. Fractions were collected as described in the original methodology and degrees of polymerization were characterized by acid-catalysis in the presence of a phloroglucinol. Monomers, dimers, and trimers of (-)-catechin were used as the low molecular weight end of the calibration curve. WinGPC software was used to analyze the cumulative size distribution of the samples.

#### 4.3.6.3: Acid-Catalysis in the Presence of Excess Phloroglucinol

Condensed tannin subunit composition and mean degree of polymerization (mDP) were determined using the previously described method with modified HPLC conditions.[5] Briefly, the reversed-phase HPLC method consisted of two Chromolith RP-18e (100 × 4.6 mm) columns connected in series and protected by a guard column containing the same material. The method utilized a binary gradient with water containing 1% v/v aqueous acetic acid (mobile phase A) and acetonitrile containing 1% v/v acetic acid (mobile phase B). Pump flow rate was kept at 3.0 mL/min with a column temperature of 30 °C and with elution monitored at 280 nm. The linear gradient was as follows: time in min (% B); 0 (3%), 4.0 (3%), 14.0 (18%), 14.01 (80%), 16.0 (80%), 16.01 (3%), 18.0 (3%). (−)-Epicatechin was used as a quantitative standard.

#### 4.3.6.4: Monomeric Anthocyanin Analysis

Anthocyanin analysis was conducted using a previously published method with modifications.[171] Briefly, a Phenomenex Kinetex F5 100 × 4.6 mm column was used with an injection volume of 5 µL and a temperature of 50 °C. Mobile phase A consisted of milli-q water with 0.2% TFA; mobile phase B consisted of acetonitrile with 0.2% TFA. Eluting peaks were monitored at 520 nm. Elution conditions were 2.0 mL/min; a linear gradient from 3% to 6% B from 0 to 10 minutes; a linear gradient from 6% to 20% B from 10 to 22 minutes. The column was then washed with 70% B for

5 minutes and re-equilibrated with 3% B for 3 minutes before the next injection. Anthocyanins were quantified by a malvidin-3,5-diglucoside standard curve (Extrasynthese, Genay, France).

### 4.3.7: Spectrophotometric Analysis

#### 4.3.7.1: Methyl Cellulose Precipitation (MCP)

Triplicate grape extracts were analyzed by methyl cellulose precipitation as previously published.[172] Briefly, 25 µL of extraction sample was placed into 1200 µL deep well plates and combined with 300 µL of 0.04% (w/v) methyl cellulose (Sigma Aldrich, St. Louis, MO, USA) solution or water (Control), and mixed on an Thermomixer-C (Eppendorf AG, Hamburg, Germany) for exactly 5 minutes at 1500 RPM and left to stand for 3 minutes. Following the mixing, 200 µL of saturated ammonium sulfate was added to the wells to prevent the re-release of tannin into solution following precipitation. Water was then added to the wells (475 µL for treatment and 775 µL for the control) and again it was mixed for 5 minutes. The deep well plate was then allowed to stand for 10 minutes before being centrifuged for 5 minutes at 2,272 × *g*. Both the treated and control sample were taken (200 µL) and placed in a 96 half-area well plate (Corning, Corning, NY, USA). 280 nm absorbance of both the treatment and control was taken simultaneously using a Synergy Neo2 Multi-Mode Reader (Biotek Instruments Inc, Winooski, VT, USA). (-)-epicatechin (Sigma Aldrich) was used as a quantitative standard. Tannin quantification was conducted by calculating Δ280 nm (Control – Treated).

#### 4.3.7.2: Pigmented Tannin

Pigmented CT was analyzed by applying a slight modification to the SPE described above, followed by a measurement at 535 nm. Briefly, the SPE was used to separate monomeric and low molecular weight material from the CT. The final elution solvent was altered from 95% methanol and water (v/v) to 95% methanol with 0.1 M HCl. Following elution, samples were then diluted

with 3000 µL of 0.1 M HCl 95% methanol for a final dilution factor of 6. The sample was then evaluated on a Shimadzu UV-1280 Spectrophotometer (Shimadzu, Kyoto, Japan) at 535 nm. The wavelength was chosen after looking at the $\lambda_{max}$ of malvidin-3,5-diglucoside during the creation of the calibration curve. By conducting a SPE prior to spectrophotometer readings, a bleaching step using $SO_2$ could be avoided, as these are used to prevent monomeric anthocyanin absorption during pigmented polymer analysis.

### 4.3.8: Statistics

All statistics and figures were prepared using GraphPad Prism 8 (San Diego, CA, USA). Statistics showing significance were all conducted by an analysis of variance (ANOVA) with 95% confidence intervals. A Tukey's HSD post-hoc analysis was used for multiple comparisons of means in order to evaluate significance within treatments between sampling points, these values are represented by lower case letters in bar graphs signifying changes within a treatment over the course of the growing season. A Tukey's HSD post-hoc analysis was also used for the two-way ANOVA, which can be seen as capital letters in the bar graphs. These represent significant differences between treatments at that specific sampling point.

## 4.4: Results and Discussion

The current study focused specifically on the addition of anthocyanins to white wine grape skins. It was expected that there would be some losses of anthocyanins through absorption to CWM as well as interactions and reactions with CT. In order to look at these losses, anthocyanin recoveries were analyzed to compare and contrast versus the formation of pigmented polymer. In order to determine the impact of anthocyanins on the extractability of CT, sequential extractions of skins were conducted to quantify the amount of CT released from skins in a hydroalcoholic solution and whether anthocyanins have an impact on extractability. Lastly, partial extractions were analyzed

for changes in CT, looking at the percent of pigmented tannin formation, concentration of malvidin-3,5-diglucoside incorporated, mean degree of polymerization, and molecular mass of CT by gel permeation chromatography. Although there are two differing measurements of the size of CT, there are implications having to do with the assay used. Mean degree of polymerization relies on depolymerization of the IFL bond, while GPC relies on a hydrodynamic volume of an intact tannin through a tortuous path in a gel column. The results below are not necessarily to be taken as what would be seen in a true wine environment, but likely help to illuminate a subset of the reactions taking place in that environment.

### 4.4.1: Recovery of Anthocyanins

Recovery of anthocyanins was evaluated by comparing the sum of the monomeric anthocyanins recovered from the partial and exhaustive extractions to the quantified amount added prior to beginning the partial extraction. It was assumed some monomeric anthocyanins would be lost during this process due to binding events with the skin cell wall material CWM.[173] Other losses of monomeric anthocyanins would likely be

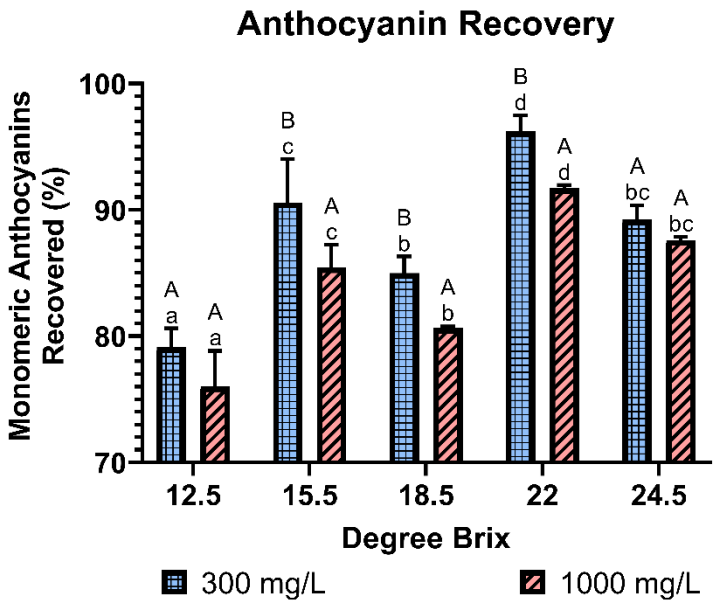


Figure 4.3: Monomeric anthocyanins recovered from the extracts after the completion of both extractions with different levels of added anthocyanins. Uppercase letters show significance between treatments while lower case letters show significance within a treatment at different ripeness levels (time). A T-Test was performed between treatments at each time point while an analysis of variance was conducted to understand significance within treatment over time

due to reactions with the CT polymer forming pigmented tannin. In **Figure 4.3**, as the concentration of monomeric anthocyanins in solution increase, the recovery of anthocyanins decreases at each stage of berry maturation. However, there were only significant differences between treatments for the three middle sampling points. But when looking at the data from each treatment together over time, the trendlines from each treatment had similar slopes, but the Y-intercepts were significantly different. This demonstrates that the two treatments had different recoveries overall **(Figure 4.4)**. Furthermore, there is a trend for anthocyanin recovery to increase throughout the ripening phase, so losses due to adsorption and/or pigmented polymer formation may decrease with grape maturity. This may be due to changes in CWM throughout the growing season, which has been shown to change in concert with berry softening, and recent work has shown that CWM composition can significantly impact anthocyanins adsorption and desorption.[173]

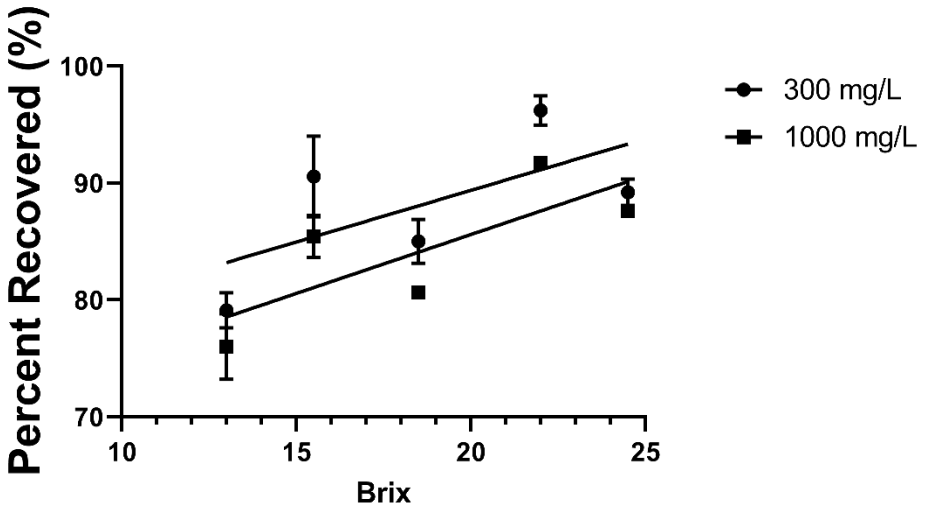


Figure 4.4: The data from Figure 3 is replotted as lines for each treatment. Monomeric anthocyanins recovered from the extracts after the completion of both extractions with different levels of added anthocyanins. These two lines have Y-intercepts that are different with >95% confidence.

### 4.4.2: Concentration of Skin Tannin Extracted and Tannin Extractability

The concentration of grape skin CT was evaluated through two extractions sequentially. The initial extraction (partial) was to quantify CT released in the presence of a model-wine like solution, while the second extraction (exhaustive) was to evaluate the residual CT that was not extracted in hydroalcoholic conditions.

In **Figure 4.5** there are three plots showing the results from acid-catalysis in the presence of excess phloroglucinol. There were significant differences seen between the treatments in partial extraction (**5A**) CT during the ripening phase, however, only a single treatment point, 300 mg/L at 24.5 °brix, showed significantly more CT in model-wine extractions compared to that of the control after 15.5 °Brix. Furthermore, when looking strictly at the control concentrations, it seems that later in the ripening stages more CT were released during the model-wine extraction. In the case of 15.5 °Brix it may be hard to say whether this result was an artifact of variation in skin tannin presence in the grapes, due to the

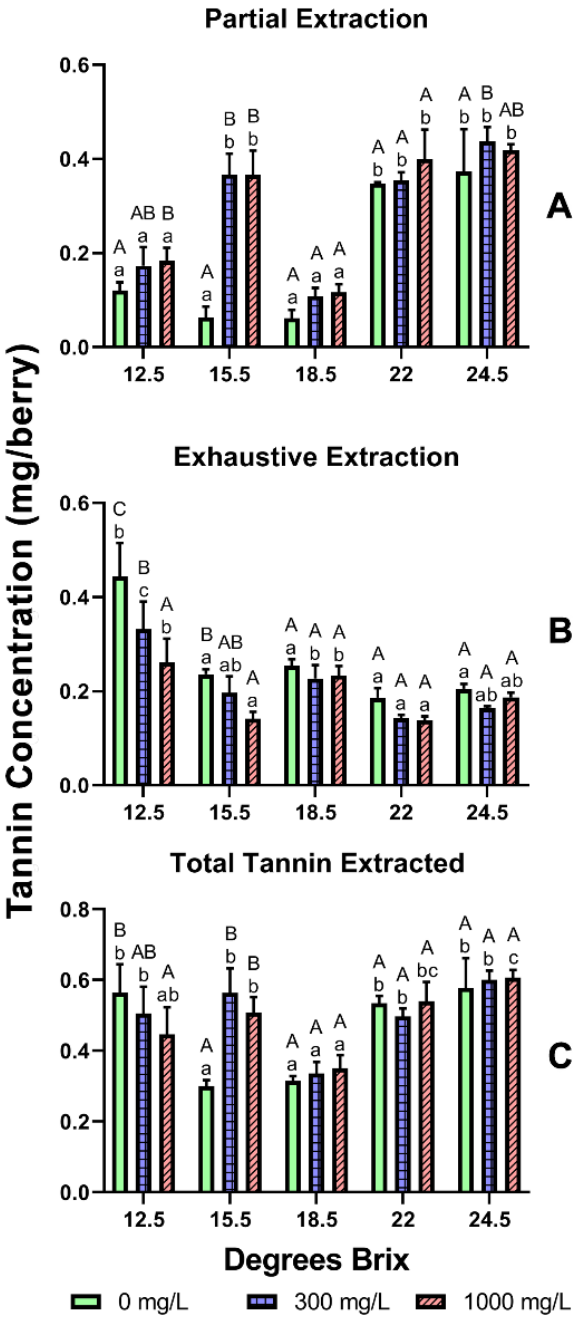


Figure 4.5: Results from the sequential extraction of grape skins by acid-catalysis after treatment with different levels of anthocyanins. Analysis of Variance with a Tukey's post-hoc was conducted to evaluate statistical significance. Uppercase letters show significance between treatments while lower case letters show significance within a treatment over time (°Brix levels).

higher total tannin concentration in treatment samples seen in **5C**. However, it should be noted that although there is a higher total tannin concentration at 12.5 °Brix in the control, there was still a higher concentration of tannin extracted in the partial extractions in the treatments. Therefore, the large discrepancy between treatments and control at 15.5 °Brix may be due to both an increase in extraction due to the presence of anthocyanins and the overall higher total tannin available to extract in the skins at that time point.

The exhaustive extractions (**5B**) only had significant differences between treatments in the first two sampling points, with the last three being similar across the treatments. In all cases there appeared to be higher levels of CT in the exhaustive extractions in the first two sampling points, with very little change occurring from 18.5 to 24.5 °Brix.

In the final plot (**5C**) the total CT values are shown. Even with the strict measures taken to control variability in samples, it appears that there was still significant variation in total CT levels in the first two sampling points. Following 18.5 °Brix, no significant differences in total CT was seen between treatments. Interestingly, there was a decrease in total CT between 12.5 and 18.5 °Brix, followed by an increase in CT from 18.5 to 24.5 °Brix. However, looking at the lowercase letters of significance within treatments overtime, there was only a significant difference between the first and last sampling point in the 1000 mg/L treatment. Estimated depolymerization yields were conducted using the methyl cellulose precipitation method. This was conducted to rule out possibility of problems with the phloroglucinolysis methodology across all sampling points. The MCP results correlated well with the phloroglucinolysis ($R^2$ = 0.82) results suggesting that depolymerization variability did not have a major impact on the results.

Previous work has focused on the changes in CT concentration over the ripening phase.[18, 95, 129, 133, 136, 143, 174-176] However, these works have been conducted with exhaustive extractions with either

methanol or acetone to remove CT from the CWM. In all cases there was a decrease in the amount of tannin extractable with acetone over the ripening phase, as the results in **5B** also show. Although this is informative from a perspective of the synthesis or degradation of secondary metabolites in the skins of wine grapes, it does not elucidate information on how much of this material is being released under model wine conditions.

Work conducted by Baustista-Ortin et al. in 2013 showed that there was also a decrease in tannin concentration with model-wine extractions throughout the ripening phase, which is contradictory to the results seen here in **5A**.[132] However, two other studies focusing on model wine extractions through ripening have shown similar results in agreeance with our findings from **5A**, wherein there is a trend to increases in the extraction of CT material through the ripening phase in model wine extractions.[139, 144]

The impact of anthocyanins on the extraction of CT from the skins of grapes has recently been discussed in depth.[112, 114, 115] These works have shown an increase in CT extracted as the concentration of monomeric anthocyanins increases. Recent work by Beaver et al. using Langmuir modeling suggest that binding is occurring in a monolayer at single locations on the cell wall interface without significant stacking through π-π interactions. This may not be the case for anthocyanins which would still have the potential for self-aggregation on CWM.[173] However, a logical hypothesis may be that anthocyanins are mitigating CT-skin cell wall interactions by participating in these interactions themselves.[177] To probe this phenomenon, we compared the amount of tannin extracted in model wine, our partial extract (**5A**), with total tannin extracted (**5C**) to derive tannin extractability. **Figure 4.6** shows the changes in extractability of CT from fresh grapes skins over the course of the growing season. Results show that the presence of anthocyanins had significant effects on the extractability of CT from fresh grape skins. However, although there

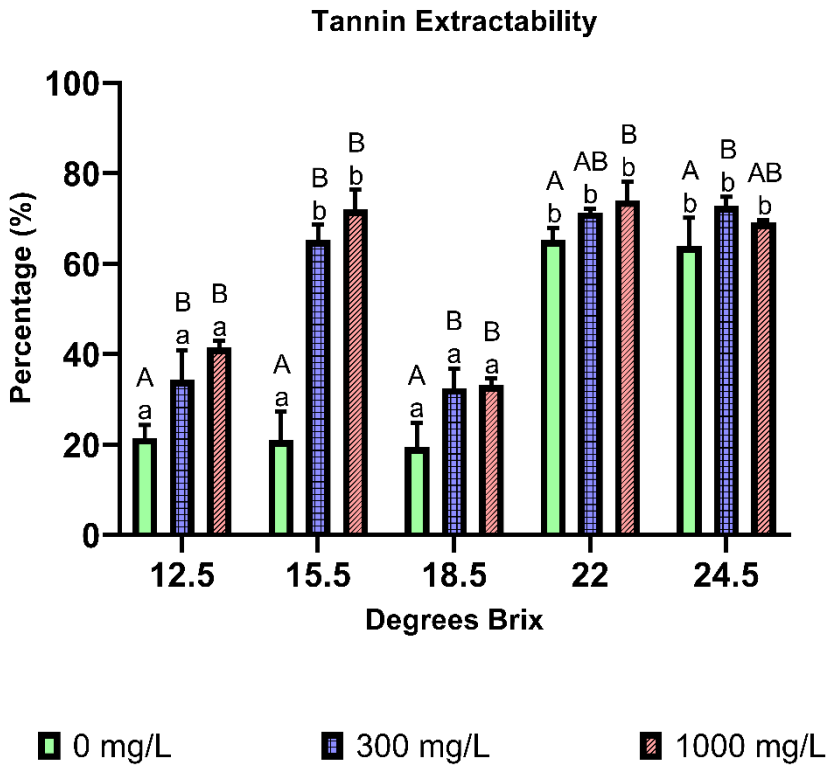


Figure 4.6: Condensed tannin extractability by acid-catalysis as affected by anthocyanin additions. This is the percentage of condensed tannin extracted in model-wine like extractions divided by that total combined tannin extracted from the sequential extractions. Multivariate Analysis of Variance with a Tukey's post-hoc was conducted to evaluate statistical significance. Uppercase letters show significance between treatments while lower case letters show significance within a treatment over time (°Brix levels).

were significant impacts at the end of the ripening phase, the magnitude of the effect seems to have decreased compared to the earlier stages of development. Furthermore, between 18.5 and 22 °Brix there was a trend towards an increase in the extractability of CT material from the control skins. This result suggests that changes in berry physiology may be leading to an ease in the release of CT material into model wine extractions. This finding agrees with the results seen by Allegro et al. in 2016, which showed an increase in the extractability of CT material throughout the ripening phase in Sangiovese.[139] Interestingly, although though there was a clear impact of anthocyanins on extractability, there was not a treatment effect in terms of added anthocyanin concentration. These results are partially in disagreement with previous work; in this work an effect on CT

extraction was seen at concentrations of anthocyanins as low 300 mg/L where in previous work it was suggested that the effect may only be seen above 1500 mg/L.[114] Although an effect is seen, this effect does not appear to be impacted by increasing concentration. Previous work has suggested that monomeric anthocyanins have little impact on tannin extractability even at very high concentrations (2 g/L).[113] However, in the latter case, the CWM was from ripe grape skins. The current data, shown in **Figure 4.6**, suggests that the ability for anthocyanins to facilitate increases in tannin extractability may be diminished at the later stages of grape ripening. Therefore, ripeness of the skins from where the CWM was derived may need to be considered when comparing confounding results. Furthermore, at the current time there is little understanding of how variety may impact the results. If variations in CWM content and composition vary between varieties, it would be expected that CT and anthocyanin extractability are likely variety dependent as well.

### 4.4.3: Pigmented Tannin Formation

The formation of pigmented polymer is generally regarded as a crucial step in red wine development. As discussed above, the two main mechanisms generally discussed are through the acetaldehyde-mediated ethylidene bridge formation (oxidation-induced) and the incorporation through IFL cleavage and subsequent nucleophilic attack. The latter occurs via 4β→8 cleavage, and as also noted above, prior work has indicated that the rate for these reactions is slow.[6]

The current study used a diglucoside as an anthocyanin, and with the absence of yeast and oxidation, the formation of pigmented polymer is expected via IFL reactions, as the formation of acetaldehyde in the extraction environment is unlikely.[128, 178] **Figure 4.7A** shows the results of percent of pigmented polymer in partial extractions after 72 hours of contact with white grape skins. The calculation for the proportion of pigmented polymer was calculated by dividing the

malvidin-3,5-diglucoside present (mg/L) by CT (mg/L EC equivalents) in partial extractions. Although there appears to be a large increase in the percent of pigmented polymer formed at the 18.5 °Brix sampling point, this is likely a function of the low amount of tannin extracted at this sampling point in all cases (**Figure 4.5A**). In **Figure 4.7B** the amount of malvidin-3,5-diglucoside present varies, but is relatively steady across all timepoints. Given that a subset of samples showed no monomeric anthocyanins following SPE, the results suggest that there is clearly substantial formation of pigment incorporation into the CT polymer within the 72-hour extraction time frame. Interestingly, there is a much larger concentration of malvidin-3,5-diglucoside present in the polymer in the 1000 mg/L treatments. Although there is 3.3× more anthocyanins present in the 1000 mg/L compared to the 300 mg/L treatment, there is 4-10× more malvidin-3,5-diglucoside that has incorporated into the polymer. This suggests that anthocyanin concentration was limiting in the case of the lower concentration. If there was the same proportion of pigmentation in both treatments, it would suggest that the anthocyanins were adequately abundant, in both cases, to react with the CT intermediate. While these results show that the reaction of the CT is likely faster than previous

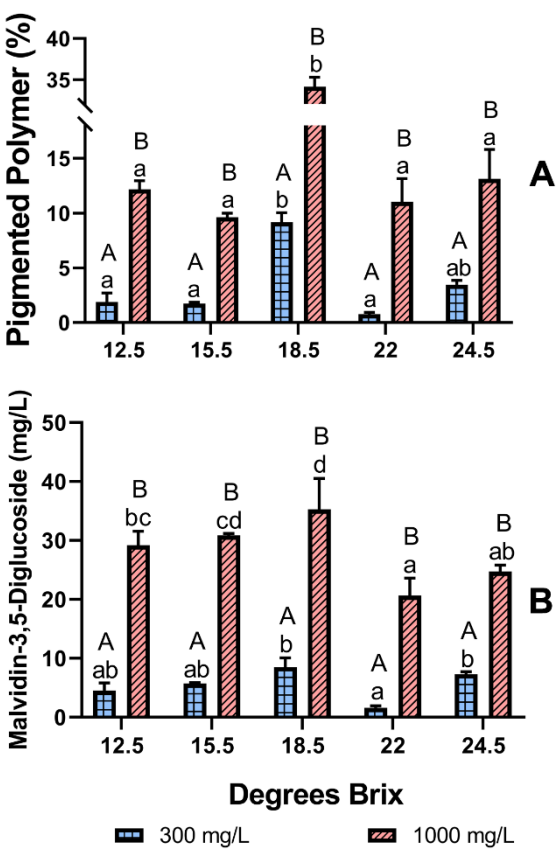


Figure 4.7: Changes in pigment incorporation into CT polymer at different times (ripeness levels) during 72-hour partial extraction at different levels of anthocyanins. (A) shows the proportion of malvidin-3,5-diglucoside when compared to CT in partial extractions. (B) Shows the concentration of malvidin-3,5-diglucoside incorporated into the CT

reports would suggest, it is clear that direct kinetic measurements are needed to clarify how anthocyanins affect this reaction.[6]

Lastly, **Figure 4.8** shows the relationship between the percentage of pigmentation and the average molecular mass of the CT as quantified by GPC. The lower line represents the molecular mass of an (-)-epicatechin dimer, while the upper dotted line represents the molecular mass of an (-)-epicatechin-4β→8-malvidin-3,5-diglucoside dimer. This non-linear regression suggests that as the system is saturated with higher amounts of subunits which can act as a terminal subunit, here anthocyanins, the average molecular mass of CT in the system decreases. Although the linear regression suggests that the material is approximately dimer in character, given the two threshold lines, it should be understood that the formation of a trimer of (-)-epicatechin has a lower molecular mass than that of an (-)-epicatechin-malvidin-3,5-diglucoside dimer. The results support the idea that the added anthocyanins are acting as a nucleophilic terminal unit after IFL cleavage, with the result that the average molecular mass would be decreasing.

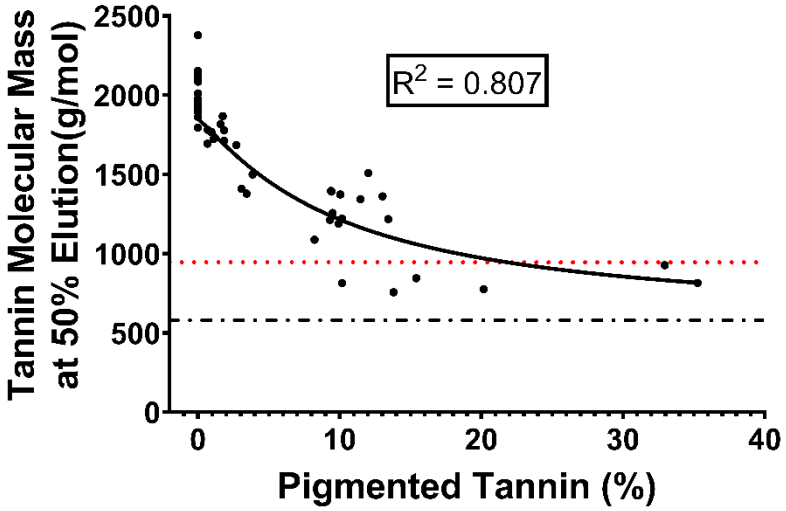


Figure 4.8: Non-linear regression of the relationship between CT molecular mass by GPC and the percentage of pigmentation. Upper line (red dots) signifies the molecular mass of a (-)-epicatechin-malvidin-3,5-diglucoside dimer. The lower (black dots) represents the molecular mass of an (-)-epicatechin-(-)-epicatechin dimer.

### 4.4.5: Changes in Tannin Size Distribution

Condensed tannin size is generally considered important because it is known to affect the astringency of red wine.[16] It is possible that the addition of anthocyanins functional groups could alter which tannins are solubilized, or how proteins might react with those tannins, and either effect may impact astringency. Condensed tannin mean degree of polymerization (mDP) is the calculated by the summation of all the subunits (extension + terminal) divided by the terminal subunits. Prior work has shown variations in the change in mDP through ripeness, with some suggesting that it is increasing with time, while others show little change over the ripening phase in grape skins.[96, 135, 143] It has also been shown that the CT extracted by model-wine solutions and acetone have differing size distributions.[135] This is likely due to larger CT having a higher affinity for CWM material which has been shown through isothermal titration calorimetry, and thus poorly extracted with the weaker model wine solvent.[120]

In this study, the partial extracts showed a decrease in mDP in the control samples between 12.5 and 18.5 °Brix, with an increase by commercial harvest (**Table 4.1**). These results are in line with the results from GPC (**Figure 4.9**), wherein there appears to be an increasing CT molecular mass extracted in partial extractions at the later stages of ripening. Both the 300 and the 1000 mg/L treatments, however, show significantly higher mDP values at all stages with the exception of commercial harvest. It is possible that variations in mDP may be due to the formation of pigmented tannin thereby impacting the calculations involved with mDP. If anthocyanins incorporated at the terminal position of a CT polymer, then there would be fewer flavan-3-ol terminal units to be observed by the analysis. Anthocyanins are known to occupy this position in CT - given the results from the acid-catalysis assay, terminal subunits may be underrepresented, leading to the possibility of misleading mDP calculations.[165]

If anthocyanins react as nucleophiles on acid cleaved IFLs and assume terminal unit positions, the overall effect will be a decrease in the size distribution. However, this is the opposite of the effect reported by the cleavage method (**Table 4.1**), where mDP increases. The acid cleavage method reports a larger mDP because there are fewer reported terminal units, as now many of the terminal units are "missing" since they are now anthocyanins. A similar effect on size is observed any time a large amount of any substitute nucleophile is added, such as toluene thiol, or phloroglucinol.

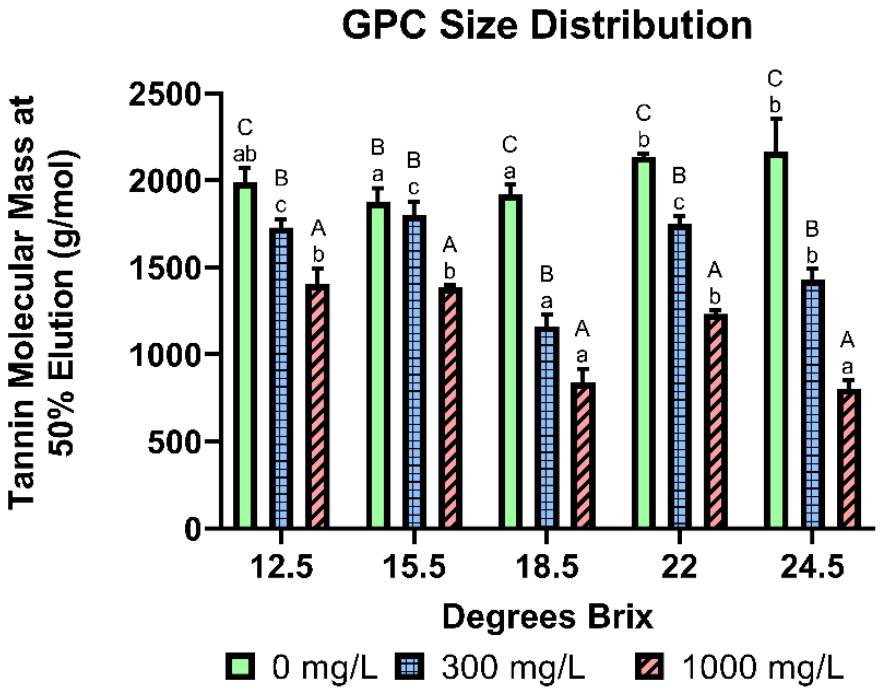


Figure 4.9: Variations in condensed tannin molecular mass, as measured by GPC, throughout the ripening phase in skin extractions, with different levels of added anthocyanins.

Table 4.1: Mean degree of polymerization values calculated for triplicate extractions within each treatment. Partial and exhaustive extractions were both evaluated to compare the tannin size removed from the skins in a hydroalcoholic environment versus an acetone extraction. Capital letters represent statistical differences between treatments while lower case letters represent statistical differences within a treatment over time (between sampling sets).

Table 4.1: Multivariate analysis of variance on the mean degree of polymerization (mDP) from acid cleavage on partial and exhaustive extracts

| Treatment | °Brix | mDP-P | mDP-Ex |
|---|---|---|---|
| 0 | | 9.11 ± 1.44 A b | 25.99 ± 0.83 A a |
| 300 | 12.5 | 11.30 ± 0.51 B b | 19.04 ± 1.41 B a |
| 1000 | | 12.42 ± 0.54 B b | 23.12 ± 0.98 AB bc |
| 0 | | 6.07 ± 0.69 A a | 23.70 ± 0.52 AB a |
| 300 | 15.5 | 13.33 ± 0.32 B bc | 27.65 ± 3.71 A b |
| 1000 | | 13.62 ± 0.14 B b | 22.54 ± 1.56 B bc |
| 0 | | 6.50 ± 0.99 A a | 20.59 ± 4.04 A a |
| 300 | 18.5 | 8.66 ± 0.44 B a | 16.48 ± 1.31 A a |
| 1000 | | 9.12 ± 0.22 B a | 16.33 ± 0.95 A ab |
| 0 | | 12.02 ± 0.16 A b | 21.49 ± 1.32 A a |
| 300 | 22 | 14.29 ± 1.88 B c | 16.66 ± 2.08 B a |
| 1000 | | 21.08 ± 0.60 C c | 13.34 ± 0.37 B a |
| 0 | | 14.07 ± 1.64 A b | 21.37 ± 3.20 A a |
| 300 | 24.5 | 13.20 ±0.28 A bc | 18.66 ± 1.02 A a |
| 1000 | | 13.78 ± 0.40 A b | 20.60 ± 0.38 A b |

Both analytical techniques agree, that in the partial extraction control samples, that CT size is increasing towards the end of ripening (**Table 4.1 and Figure 4.9)**. Previous work focusing on the impacts of ripeness on extractability also showed an increase in CT molecular mass with increases in ripeness between 22 and 26 °Brix.[113] This result suggests that, along with tannin concentration, higher molecular weight CT material is extractable with increased ripeness of fruit. Furthermore, looking at the mDP in the control between the partial and exhaustive extraction, there is a decrease in the exhaustive extraction coinciding with the increase in the partial extraction. Given the sequential manner of the extraction, the result suggests that larger molecular weight material is being extracted with increased ripeness levels.

When the same samples are analyzed by GPC, an opposite effect is observed, and the size decreases significantly (**Figure 4.9**) as increasing amounts of anthocyanins were added to the grape skins. In particular, the partial extractions yielded significantly "smaller" tannin in all cases. The results support the concept that anthocyanins are readily acting as nucleophiles on the CT during the model wine extractions and decreasing the size of CT by adding monomers (anthocyanins) to the distribution of oligomers. In all cases, there are significant differences between one or more treatments and the control, and the observed average size decreased by 30 and 60 percent in the 300 mg/L and 1000 mg/L treatments, respectively. A change in size distribution was also noted by Vidal when adding terminal subunits to fractionated CT material.[2] This decrease in polymer size following the addition of molecules which can act as terminal subunits suggests that tannin size is determined by the ratio of molecules which are acting as electrophile and nucleophiles. In this case, as in the Vidal paper, when the addition of molecules which can act as terminal subunits is increased, there is a clear decrease in the size distribution of the polymer, suggesting cleavage and reformation of smaller oligomers.

Furthermore, this result suggests that there is substantial incorporation of anthocyanins into CTs within the 72-hour period of tannin extraction. Further evidence of this incorporation can be seen in the gel permeation chromatogram (**Figure 4.10**); not only is there a shift towards lower molecular weight material in the chromatogram by gel permeation chromatography, but also an increase in absolute absorbance, suggesting an increase in flavonoid material absorbing at 280 nm. Considering that all starting skin material was the same, and that the total flavan-3-ol material extracted had no significant differences after the third sampling point, it is likely that the decrease in average size, and the increase in tannin concentration observed at 280 nm is due to anthocyanin incorporation.

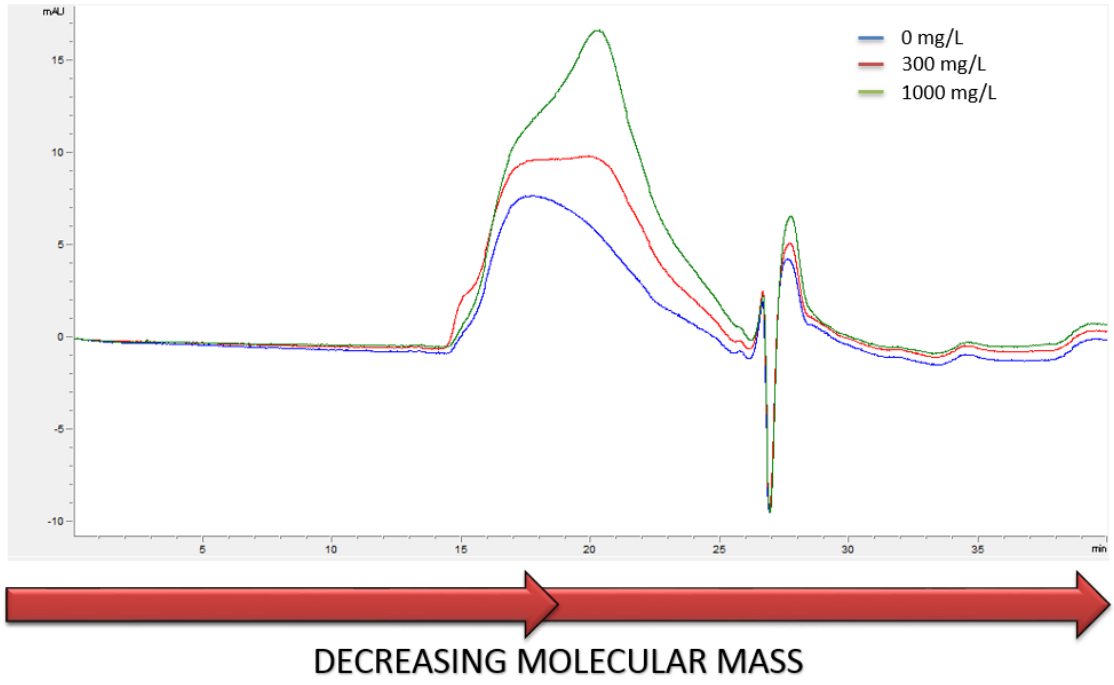


Figure 10: Example of an elution profile by GPC of an extracted tannin sample, showing not only a shift in the size distribution due to a later elution, but also an increase in absolute absorbance at 280 nm with increasing anthocyanin addition.

In summary, the addition of anthocyanins to white grape skins during a model-wine extraction appears to impact the extraction of flavan-3-ols in model wine solutions. In all cases sampled there was not any significant difference between the 300 mg/L and 1000 mg/L treatments in terms of flavan-3-ol material extracted. However, there was a substantial increase in polymeric flavonoid material in the system as can be seen by gel permeation chromatography. These results, along with the pigmented tannin results, suggest that pigmented polymer is forming readily, and likely at a faster rate than could be expected based on published data.[6] Additionally, there appeared to be an increase in the recovery of anthocyanins from the first to last sampling points, within treatments. Although this difference in recovery does not appear to be correlated between the amount of pigmented tannin formed and the anthocyanin recovery. This suggests that changes in CWM occurring during the growing season are likely impacting the recovery of anthocyanins. Although this work does not contain polysaccharide data, it can be inferred that there are structural changes occurring throughout the growing season.[118, 179]

The addition of anthocyanins also impacted mDP in the partial extractions. Given that mDP is calculated based on subunits quantified after cleavage, and that there was the opposite trend seen in GPC, it is likely that terminal subunits were not accounted for as they were anthocyanins. However, there was a clear impact on the size distribution of tannin when in the presence of anthocyanins. **Figure 4.8** adds further detail to this argument suggesting that the increases in proportions of anthocyanins into CT polymers is leading to decreases in molecular mass. This result suggests that under non-oxidative, acid conditions, the IFL is cleaved quickly and that the released electrophile is trapped by the nucleophilic/hemiketal form of anthocyanins, leading to increased flavonoid material in oligomeric form, decreased mDP and pigmentation of the CT.

Although there was a relatively short period in which these skins were extracted in comparison to a normal wine ferment, the presence of anthocyanins induced significant changes in CT. It appears that IFL breaking events and subsequent reformation are happening faster than previously thought in a mildly acidic solution. Given the discrepancy in the half-lives currently published on the acid-catalyzed cleavage of the IFL, the kinetics of this key reaction should be studied in detail. It should be noted that monoglucosides will react differently, perhaps even faster than the diglucosides studied here, so the chemical changes observed here may be a major factor early on in red wine color development. We are pursuing these kinetic investigations. Furthermore, a detailed look at CWM changes in the skin and how these changes might alter tannin extractability is needed.

## 4.5: Acknowledgements

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## Concluding Remarks

Determining when to harvest grapes to produce wine is a major decision in the life of the wine. The impacts of this decision can have a lasting impact on the quality and character of the wine produced. Understanding the environmental factors involved in grape growing allows for viticulturists to make informed decisions depending on the goals of the vineyard. The work in this dissertation explored the changes in phenolics of the course of the ripening phase with model-wine extractions.

In 2015 the goal of the experiment was to probe changes in 'tannin activity' in the vineyard rather than in the cellar. Previous experiments looking into activity focused on changes in wine due to increases in PA concentration, composition, or confirmation (wine age). Rather than look at how activity changes in a wine due to fermentation or storage conditions, this experiment explored how the thermodynamics of PA material changed over the course of the ripening phase in whole berry model wine extractions. The results of this experiment showed that activity decreases over the course of the growing season, as seed PA extraction decreases. The results also suggest that monomeric anthocyanin concentration is paramount in dictating the size distribution of the PA material, likely through pigmented polymer formation by means nucleophilic and electrophilic interactions.

The results from the 2015 work were used to design a shade cloth experiment in 2016. The thought process behind this experimental design was that the variability seen in the 2015 data was likely due, in some regards, to canopy management differences between the wineries in 2015, leading to large differences in the production of anthocyanins, and therefore changes in activity. Results from the 2016 experiment showed that light attenuation through the application of shade cloth led to significant changes in anthocyanin concentration, activity, PA molecular mass, and PA

composition. Not only did these results confirm those seen in 2015, but they also showed that the manipulation of light on the canopy after veraison can lead to significant changes post-synthesis in PA material extracted from skins. In order to confirm both the 2015 and 2016 work, a third experiment was designed to understand the impact of anthocyanins on these parameters.

In 2018 a Sauvignon Blanc experiment was conceived to eliminate the variability of anthocyanin synthesis by adding purified anthocyanins, at the same concentrations (0, 300, 1000 mg/L) throughout ripening, to model wine extractions of white grape skins. Furthermore, extra care was taken to prevent the interference of monomeric anthocyanins on analyses such as gel permeation chromatography. These results not only confirmed that anthocyanins are readily incorporating into the PA material within a 72-hour window, but that incorporation is leading to a significant decrease in the molecular mass of the PA material.

Further research is required looking specifically at the acid-catalyzed cleavage of the IFL. Previous work has looked at this cleavage at temperatures wherein the heterocyclic ring is likely to open, leading to questionable results. Furthermore, work has been done in polar aprotic solvents to prevent solvolysis by water, which would lead to a flavan-3,4-diol. Although this leads to more accurate results due to the lack of solvolysis interference, it likely leads to much slower rate constants due to the lower solvent polarity to stabilize the formation of the quinone methide (carbocation) following bond cleavage.

These experiments were designed in a linear fashion to understand the role in which monomeric anthocyanins, PA molecular mass, and the formation of pigmented polymer are driving differences in the activity of PAs extracted from grape skins. Each experiment was conducted by looking at the previous experiment and trying to remove variables to arrive at an answer, or partial answer, for the variability seen in 2015. Although the idea of changes in PA activity in the vineyard may

seem like an obscure or esoteric idea, understanding these changes and the factors affecting them can help viticulturists understand how to manipulate properties related to wine quality in the vineyard. The ability to measure and track a parameter that has been shown to be related to the dryness or astringency of PAs throughout the vineyard and winemaking process can provide viticulturists and enologists with a tool to better meet stylistic goals. Furthermore, it can provide data to wineries to understand the cause-effect paradigm and how these decisions, whether in the vineyard or winery, have consequences relating to overall wine quality.

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