PROTEOMICS AND CONDENSED TANNIN: AN OPTIMIZED METHOD TO CHARACTERIZE GRAPE PROTEIN AND THE RELATIONSHIP TO WINE CONDENSED TANNIN CONTENT

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

PROTEOMICS AND CONDENSED TANNIN: AN OPTIMIZED METHOD TO CHARACTERIZE GRAPE PROTEIN AND THE RELATIONSHIP TO WINE CONDENSED TANNIN CONTENT

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and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

I would like to thank my friends and family for all their support during my Ph.D. endeavors. My parents, Mary and Jim Fredrickson for always supporting me and encouraging me to continue to learn and ask questions. My siblings, Sara Chalkley and Eric Fredrickson for keeping me grounded and showing me how to raise a family and still be successful in their careers.

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Last but not least I would like to dedicate this to my family Megan and Magnolia. Megan, you always push me to better, stronger, and never settle, but also support me and my crazy hobbies and interests. I can't wait to see where life takes us next, but I know it will be great! Magnolia, I know my "book" has taken forever to write, but throughout my Ph.D. research and writing, you have always made me smile and laugh. I hope someday you will read this and be proud of what your dad has accomplished. Love you both more than you can imagine!

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PROTEOMICS AND CONDENSED TANNIN: AN OPTIMIZED METHOD TO CHARACTERIZE GRAPE PROTEIN AND THE RELATIONSHIP TO WINE CONDENSED TANNIN CONTENT

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ABSTRACT

Condensed tannin extraction and retention have long been a problem for cool climate grape growing and winemaking regions where *Vitis* interspecific hybrid cultivars are grown. Recently, the link has been made between pathogenesis-related (PR) proteins and low condensed tannin retention in red wine cultivars. At the time of this research, no agreed-upon method for grape juice and wine protein precipitation, quantification, and proteome characterization had been determined. After method optimization, we determine with a coefficient of variation below 15% that the precipitation with acetone/trichloroacetic acid (TCA), solubility buffer of 6M Urea/100 mM ammonium bicarbonate, and bicinchoninic acid (BCA) protein assay for quantification to be most appropriate. For characterization, the use of shotgun-based proteomics with UPLC-ES-TIMS-TOF was used for the characterization of the proteome. Using the optimized method, seven different cultivar's juice and wine were analyzed for protein and condensed tannin. Also, condensed tannin content and proteome from *Vitis* interspecific hybrid cv. Chambourcin wine from 2018 and 2019 was studied more extensively with traditional and novel winemaking methods (exogenous condensed tannin additions early and late, accentuated cut edges), along with the wine and lees of micro fermentations (50 mL) with increasing levels of exogenous condensed tannin additions during fermentation (800-6400 mg/L). The addition of exogenous condensed tannin at 1-month post-press

resulted in condensed tannin retention above 100% and the use of ACE (accentuated cut edges), a practice involving using a blender after crush to increase the skin to juice ratio, increased native condensed tannin content 2.5-fold higher than the control. Total protein does not correlate to condensed tannin concentration, but proteomic data and individual spectrum count do indicate that specific proteins may have more impact on the condensed tannin content in wines. There are many proteins (PR and other groups) that impact condensed tannin retention and is not a select handful that was previously hypothesized.

CHAPTER ONE:
Background

1. Introduction

In 2017, it is estimated that the United States wine industry had an economic impact of $219.9 billion (2017) (Dunham & Associates, 2017). While grapes and wine have both been extensively studied, due to the complex processes and matrices from vine to bottle to the consumer there are still many unknowns relating to all aspects of production (Moreno-Arribas & Polo, 2009). Two areas of research that have gained significant attention in the last 20 years are how condensed tannin and protein are measured and what roles they play in wine quality.

Wine is a complex matrix, composed of phenolics, volatile/aroma compounds, carbohydrates, proteins, acid, alcohol, and many other less abundant components (Moreno-Arribas & Polo, 2009). Condensed tannins, part of the phenolics group play a crucial role in this matrix, providing mouthfeel and the ability to form a new covalently bond compound with anthocyanins that is a more stable color in wine like conditions (Harbertson et al., 2008). With the continued growth of the wine industry worldwide, condensed and hydrolysable tannins have remained an important aspect of wine research, and more recently, become the focus for researchers attempting to understand why the presence and absence of condensed tannins appear to fluctuate depending on cultivar, climate, and region. To better understand and, by extension, manipulate condensed tannin content of wines, it is critical to understand how condensed tannins interact with wine proteins, a poorly understood relationship.

Condensed tannins are made up of flavan-3-ols, which are three-ring structures, two benzene rings bonded to a 2H-pyran (Oxygen) ring in the middle (C-ring). The variation in flavan-3-ols comes from the two R-groups off the 3rd carbon of the C-ring and a third R-group off the B-ring (Figure 1). These flavan-3-ols can combine and polymerize to form proanthocyanidins (condensed tannins). When condensed tannins are heated under acidic conditions they release anthocyanidin pigments (red color like anthocyanins, but without the sugar) (Ribéreau-Gayon et al., 2006). The primary grape-derived proanthocyanidins are B-type which are flavan-3-ols that are linked as C4-C8 and C4-C6 bonds (on the A and C rings). The less common A-type proanthocyanidins (more often in cranberries and cinnamon) have an extra linkage at C2-O-C7 or C2-O-C5. The release of the anthocyanidin can also be split into two groups (for grapes), procyanidins which release the anthocyanidin cyanidin, and prodelphinidins which release delphinidins (Moreno-Arribas & Polo, 2009).

Condensed tannins can vary in size (2-80 units) and are diverse and complex. For example, if tannins were only composed of catechin and epicatechin and there were only oligomers up to 10 units with two interflavan bond types, there could be 524,288 different isomers (Moreno-Arribas and Polo 2009; Naumann et al. 2017). With three to five additional flavan-3-ols and tannins as large as 80 units, the number of unique tannin structures quickly becomes very diverse. The diversity causes issues for analysis as it is very difficult to separate all these structures via chromatography and there is not a representative standard for tannins. In the enology field catechin is used as a standard and numbers are reported in catechin equivalents, yet catechin is not representative of diverse and complex wine tannin structures.

Condensed tannins are highly reactive and there are frequent changes in the structures of tannins once they are extracted into wine. Depolymerization can occur with a weak acid (pH similar to wine) which creates an "active catechin" that has an electrophilic center and can bind with thiols and other nucleophilic compounds. The new compound is highly reactive with other monomers which can polymerize again (Ribéreau-Gayon et al., 2006). Several other reactions occur during the winemaking process (oxidation, co-pigmentation, procyanidin polymerization, etc.) which result in a more stable color suitable for the aging of red wine and a more desirable mouthfeel.

The impact that proteins have on red wine quality has often been left untouched as most researchers believed that proteins were precipitated out quickly by fermentation (temperature) and wine conditions (mostly phenolics, but also alcohol and acid). Most researchers assumed that proteins in grapes were only a problem in white wines because they did not extract phenolic compounds from the skins or seed. Smith et al., (2011) found that Pinot noir wines between 6-32 years old, contained protein content between 50-102 mg/L, indicating that protein is still present in wines even if tannin is present. More recently, Springer et al., (2016) hypothesized that low tannin red wines, mainly hybrid cultivars from cool climates, could be due to a few pathogenesis-related proteins that had been recovered after adding pure seed tannin to recently fermented red wines. Proteins in wine range in size from 9-62 kDa with an isoelectric point from 3-9 (Brissonnet & Maujean, 1991; Hsu & Heatherbell, 1987; Lamikanra & Inyang, 1988). Most proteins are thought to be pathogenesis-related (PR) proteins as they can survive the heat produced during fermentation and acidic conditions, plus they are resistant to proteolysis.

The most abundant PR protein is chitinases (class IV chitinase) which have been reported to make up 50% of the protein in grape berries (Robinson & Davies, 2000). Chitinases were originally found in cell walls of yeast with the ability to catalyze the degradation of chitin, but are present in plants to resist fungal pathogen infections, yet do not inhibit wine yeast growth (higher concentrations were found in grapes with wounds or disease pressure) (Vincenzi et al., 2014). There is no mention of proline and/or histidine-rich residues. Glucosidase is another group of proteins (sometimes classified with chitinases) common in fermenting must/wines which can hydrolyze β-1,3-glucans (Vincenzi et al., 2014). This protein is also found in fungi and is a response to disease pressure in grapes (Ferreira et al., 2001).

Thaumatin-like proteins are 24 kDa in size and are found in the berries once sugar begins to accumulate and some associate them with thaumatin, which is a sweet-tasting protein from an African shrub (Ferreira et al., 2001). However, there are no indications that grape thaumatin-like proteins are sweet. They have been associated with antifungal properties, which could be related to the salicylic and jasmonic signaling pathways (Yan et al., 2017).

The mechanism in which wine protein interacts with tannin is unknown. There is a correlation between soluble cell wall material (often a pathogenesis-related protein) limiting the retention of added exogenous tannin (Bindon et al., 2016; Springer, Sherwood, et al., 2016). More research is needed to determine what proteins are present in juice and wine as there is a wide range of methods to quantify and identify proteins (Smith et al., 2011). In four different studies total protein in grape juice as: Hsu & Heatherbell, (1987) 58-77 mg/L, Ough & Anelli, (1979) 457-786 mg/L, Fombin, (1982)

100-160 mg/L, and Pocock et al., (2000) 31-251 mg/L. Ranges in total wine protein have been reported between 12.2-328 mg/L (Smith et al., 2011; Springer, Chen, et al., 2016; Vincenzi et al., 2005). When characterizing proteins, methods utilize sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins and then identify bands/spots of gel. Although effective, this is time-consuming and cannot characterize an entire juice or wine proteome. For researchers to communicate effectively, method optimization needs to be completed.

The word tannin derives from the interaction and precipitation of protein for the tanning of leather. An important feature of tannins is they are amphipathic molecules which is where the aromatic rings are hydrophobic while a large number of hydroxyl groups are hydrophilic. This ability allows tannins to bind to multiple molecule sites. The main mechanism for tannin protein interactions is hydrogen bonding and hydrophobic interactions. When tannins polymerize, they have more hydroxyl groups (and hydrophobic areas), which results in more active sites for binding. The amount of gallic acid (epigallocatechin and epicatechin gallate) on a condensed tannin also influences the number of active sites as it adds extra hydroxyl groups (Hanlin et al., 2010).

Stereochemistry can also influence tannin reactivity to protein as the straighter the tannin structure is, the quicker the hydroxyl groups can interact with proteins and more hydrophobic regions are accessible. This can be seen with interflavan linkages where with B-type proanthocyanidins the C4-C8 linkage forms a more open configuration (in theory) compared to the C4-C6 formation. One study showed that dimers of C4-C8 had greater protein-tannin interaction than C4-C6 dimers (so they concluded this would be extrapolated in larger tannin molecules) (Hanlin et al., 2010).

The hydrophobic interactions of tannin and protein include Van der Waals interactions and/or π-π stacking (with the electron-rich B-ring or galloyl ester). The hydrogen bonding is a reaction with the hydroxyl groups. Self-association (aggregation) occurs by crosslinking of protein-tannin complexes (thought to be more hydrogen bonding). These aggregates can continue to grow until they coalesce and produce colloidal particles resulting in precipitation (McRae & Kennedy, 2011).

Baxter et al., (1997) titrated different polyphenols (including epicatechin and an epicatechin dimer) into a synthesized proline-rich peptide and characterized the interactions by NMR. They found that the major interaction between the polyphenols and peptide was hydrophobic stacking. They also found that the first proline-proline sequence of the peptide was more favored.

The ability to increase tannin content in red wine is a need and want for winemakers in the industry, especially in low tannin wines made from hybrid cultivars. The ability to apply winemaking techniques (traditional and novel) to improve tannin retention was performed. With increased tannin concentration proteomics was performed on low and high tannin wines to explore differences and shifts in the proteome.

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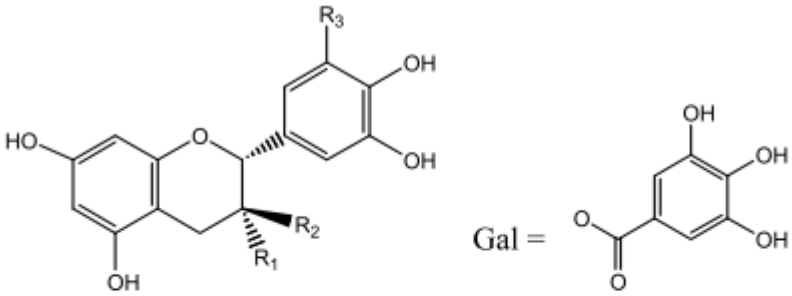
Figure 1: Flavan-3-ols found in wine (McRae & Kennedy, 2011)



Flavan-3-ol Monomer	R_1	R_2	R_3
Epigallocatechin	OH	H	OH
Gallocatechin	H	OH	OH
Epigallocatechin 3-*O*-gallate	O-Gal	H	OH
Catechin	H	OH	H
Epicatechin	OH	H	H

CHAPTER TWO:
An Optimized Protein Precipitation Method for Quantification and Characterization of Grape Juice and Wine Proteins

1. Introduction

Grape and wine proteins play an important role in grape development, winemaking, and wine quality, but methods for measuring and identifying proteins in these systems vary widely. This leads to difficulties in the scientific community and grape and wine industry when comparing results of grape juice and wine protein that has been extracted, precipitated, and quantified using different methods.

After fruit set and throughout the growing season, proteins in grapes are upregulated and downregulated correlating with many biological and physiological changes during fruit development including production and storage of sugars and malate, plus the synthesis of polyphenolics (Martínez-Esteso et al., 2013). Several proline-and glutamate-rich proteins have been associated with grape ripening associated cDNA which play a role in cell wall structure (Davies & Robinson, 2000). Many studies have shown that after vérasion (color change), there are a great increase in the production of pathogenesis-related (PR) proteins, including (but not limited to) chitinases, thaumatin-like, and glucanase (Ferreira et al., 2001). These PR proteins have shown to be an effective defense system against biotic stresses and can contribute to resistance to *U. necator* (causal agent of Powdery mildew), *Botrytis cinerea* (causal agent of Botrytis Bunch Rot), and *P. viticola* (causal agent of Downy mildew) (Girbau et al., 2004a; Moreno-Arribas & Polo, 2009; Toffolatti et al., 2018), all of which are common fungal

diseases of grapevines, especially in cool climate regions. Ultimately the final protein content of fruit is influence by a vast array of factors including cultivar, growing conditions, biotic and abiotic stress. (Gerós et al., 2012).

Grape protein content and makeup influence many factors, even after harvest. During primary fermentation yeasts utilize sugars and anaerobically convert them into ethanol, carbon dioxide, and other byproducts, but for the yeast to thrive many factors go into a healthy fermentation (Fugelsang & Edwards, 2007). One major nutrient for yeast is nitrogen, coming from ammonia and amino acids. Yeasts have been shown to have large amounts of protease activity and can reduce the juice protein by one-third during seven days of fermentation suggesting that these products are likely available for yeast during fermentation (Dizy & Bisson, 2000).

Proteins play a crucial role in flavor development with many enzymes regulating the accumulation of terpene or terpene precursors during grape maturation which includes many desirable compounds like linalool, geraniol, nerol, and rotundone (Emanuelli et al., 2010; Takase et al., 2016). Many esterases have been shown to contribute to the synthesis of esters and are responsible for flavor development in the grape berry (Kambiranda et al., 2016). During fermentation, yeast, in particular, yeast cell walls (partially composed of protein) have been shown to bind volatile compounds leading to a less aromatic wine (Lubbers ~ et al., 1994).

In the production of sparkling wine, bubble characteristics (e.g., size and foam retention) play an important role in the quality perception of the product. Proteins, in particular hydrophobic proteins, are the driving force for foam formation and stability (Brissonnet & Maujean, 1993). Without the ability of sparkling wine to foam, bubbles

quickly rise to the surface and dissipate and do not form a ring around the glass known as a collar, a highly requested attribute for consumer panels on sparkling wine (Cilindre et al., 2010). Mannoproteins from yeast have been shown to inhibit the formation of tartaric acid salts, an undesirable characteristic of bottled wines (Moine-Ledoux et al., 1997). On the negative end, some PR proteins are very stable during the winemaking process and if they are not removed, can end up in bottled wine. If exposed to heat, a haze can form, leading to a cloudy wine which is almost always considered a negative sensory characteristic (Van Sluyter et al., 2015).

In cooler climate regions, as the number of vineyard acres and wine production increases, so does the use of interspecific hybrid cultivars. Hybrid cultivars and cool climate *Vitis vinifera* reds often have lower phenolic content, and in particular, lower condensed tannin concentration in the finished wine. While the majority of *Vitis vinifera* based wines grown in warmer climates have condensed tannin concentrations ranging from 52 to 1895 mg/L, hybrid wines generally have much lower condensed tannin concentrations ranging from 25 to 125 mg/L (Harbertson et al., 2008; Springer & Sacks, 2014). The lower concentration of condensed tannin is not explained by total condensed tannin, as the majority of hybrid cultivars have condensed tannin content in their fruit comparable to cultivars that produce high condensed tannin wines (Springer, Chen, et al., 2016). To improve low phenolic problems, winemakers consistently used exogenous tannin (often condensed and sometimes hydrolysable) additions, but when added at crush, very little tannin is retained in the finished product (Alex J. Fredrickson et al., 2020).

Recent research has identified select PR proteins, many of which bind with tannin and are recovered following centrifugation in a tannin-protein pellet (Springer,

Sherwood, et al., 2016a). In particular, soluble mesocarp proteins may play a role in protein-tannin interactions, and even at low concentration can still bind available tannins at high rates (Bindon et al., 2016a). The use of pectolytic enzymes and removal of insoluble material before maceration can increase condensed tannin extraction and retention (Osete-Alcaraz et al., 2019a). A separate study where must was fermented without pomace saw only limited retention of exogenous condensed tannin in wine from hybrid cultivars (Nicolle et al., 2019).

Currently, there is a wide range of methods that grape and wine researchers use to purify, quantify, and then identify proteins (Table 1). The mechanism(s) in which protein precipitation and solubilization occur can vary between methods which can result in different sizes, types, and functionality of proteins being precipitated, possibly leading to different quantification even with the same assay. For example, sodium dodecyl sulfate (SDS) may precipitate more membrane proteins which may result in different quantities of total protein or individual proteins when comparing with an acetone/trichloroacetic acid (TCA) or ammonium sulfate precipitation method (Zhou et al., 2012). The assay in which protein is quantified and the protein standard used to give results can vary greatly from method to method (Thermo Fisher Scientific, 2017). The wide range of methods used (Table 1) makes cross-comparison of results difficult. While broad qualitative comparisons are possible, quantitative results should not be compared when methods vary. With the wide range of roles proteins play in grapes, juice, and wine, there must be a standard method that the scientific community and industry can utilize and agree upon for protein analysis in a juice and wine matrix.

Although SDS-PAGE has been effective at separating and identifying an individual or small numbers of proteins in juice and wine, there is no information on how the whole proteome could be impacting condensed tannin retention. Another complication is that the protein precipitation/purification method before SDS-PAGE can vary (Table 1), which may impact the concentration and diversity of proteins. Also, SDS-PAGE is labor-intensive and time-consuming and is not recommended for high-throughput protein identification. The previous optimization of precipitation methods in other food and beverage products was necessary for overcoming matrix specific issues to improve repeatability and reproducibility, even when methods had been published for similar, yet different products (Mansor et al., 2020; Vilhena et al., 2015 Chen et al., 2009; Zhu et al., 2009).

Despite the importance proteins play in wine quality, proteome characterization has been limited in grape and wine research. In this study, we aim to optimize a method for protein precipitation, buffer solubilization of pellet, and protein quantification for LC-MS/MS peptide analysis. With an optimized method, we then analyzed the proteome of juice and wine of various cultivars, hybrid, and *Vitis* vinifera, to validate that the method is reproducible and effective. This enabled us to correlate proteome data in juice and wine to condensed tannin retention.

2. Materials and Methods

2.1. Chemical Reagents

Acetone (99.9% purity), acetonitrile (ACN) (99.8% purity), ethanol (95% purity), glacial acetic acid (99.7% purity), Trichloroacetic acid (TCA) (99% purity), albumin from bovine serum (BSA) (98% purity), ammonium bicarbonate (99% purity),

ammonium sulfate (99% purity), (+)-catechin hydrate (96% purity), formic acid (98% purity), iodoacetamide (IAM) (99% purity) sodium chloride (99% purity), sodium dodecyl sulfate (SDS) (98% purity), sodium hydroxide (NaOH) (98% purity), potassium chloride (KCl) (99% purity), triethanolamine (98% purity), Tris-HCl (99% purity), LC-MS grade water (MQ 100 purity), urea (99% purity), and thiourea (99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) (99% purity), iron(III) chloride (98% purity), Coomassie Brilliant Blue G-250 (>95% purity), Hydrochloric acid (37% purity), and phosphoric acid (85% purity) were sourced from Fisher Scientific (Waltham, MA, USA). Sequencing grade modified trypsin was obtained from Promega Corp. (Madison, WI, USA).

2.2. Experimental Design

All winemaking experiments were completely randomized design with three fermentation replications for each treatment. The dependent variables were condensed tannin concentration while the independent variables were exogenous tannin additions (to must or at 1-month post-press and ACE.

Protein was extracted in triplicate for each treatment and quantified with three replications for each extraction. Protein digestion and characterization were done in triplicate for each treatment. Protein concentration, protein spectrum counts, and proteins identified were the dependent variables.

2.3. Comparison of protein precipitation methods

All method optimization experiments were performed on *Vitis* interspecific hybrid cv. Chambourcin harvested from a University of Missouri research vineyard in Mt. Vernon, MO in 2018. The precipitations were run on both juice and wine (post-

fermentation) and samples were collected in 2018 and frozen at -80°C for further analysis.

2.3.1. Trichloroacetic acid (TCA) and acetone precipitation

Following methods from M. R. Smith et al., 2011; Vincenzi et al., 2005, the protein was precipitated in juice/wine using 4 volumes of ice-cold 5% TCA in acetone to one volume of juice/wine and incubated at -20 °C overnight. Protein is recovered by centrifugation at 12000 x g for 30 minutes at 4 °C, followed by carefully removing and discarding the supernatant. Protein was washed with 80% acetone and vortexed vigorously or sonicated until the pellet is broken up, then incubated at -20°C for 10 minutes. Protein was then centrifuged at 20,800 x g for 10 minutes at 4 °C. Repeat rinse for a total of (3) acetone washes. Remove supernatant and dry pellet. The final pellet was dissolved in water or buffer.

2.3.2. SDS with potassium chloride (KDS) protein precipitation:

This method was originally developed by Carraro et al., (1994) for molecular biology as an alternative to acetone precipitation which can sometimes partially lose hydrophobic or low-ionic-strength-insoluble proteins. This was then adopted for grape juice and wine by Vincenzi et al., (2005) and used in wine protein quantification studies (Gazzola et al., 2015; M. R. Smith et al., 2011). Briefly, KDS precipitation was done by adding 10% SDS to juice/wine for a final concentration of 0.125% SDS and incubating in 100°C water bath for 5 minutes, cooled on ice or to room temperature, then 1M KCl was added to reach a concentration of 250 mM KCl and mixed gently for 30 minutes, then centrifuge samples at 12000 x g at 4°C for 30 minutes, remove and discard supernatant, the pellet was washed with 1M KCl and dissolving the pellet with vertexing or sonicating

then centrifuged at 20,800 x g for 10 minutes, and the supernatant was removed and discarded. Then the wash was repeated three times. The final pellet was dissolved in water or buffer.

2.3.3. Ammonium sulfate protein precipitation

This method uses salting in/out to extract protein and is often used for fractionating protein but is less frequently used in juice/wine protein extraction (Springer, Sherwood, et al., 2016b). Protein was precipitated in juice/wine by slowly adding 4 volumes of saturated ammonium sulfate to one volume of juice/wine at 2°C and left at 2°C overnight. The following day samples were centrifuged at 12000 x g at 4°C for 30 minutes. The supernatant was decanted, and the protein pellets were resuspended in water or buffer. To remove salt, samples were dialyzed using tubing with a 3.5 kDa molecular weight cutoff for 20 hours, changing the water twice, once at 6 hours and again at 14 hours. Samples were then lyophilized, and the final pellet was dissolved in water or buffer.

2.3.4. SDS-PAGE Gel

Before analyzing the proteome by LCMS, samples from the three precipitation methods were loaded on a gel to help identify which precipitation methods precipitated the most protein from the juice. Before dissolving the pellet in water or buffer, samples were dissolved in 120 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, and 3.1% (v/w). Due to interferences from the buffer, protein concentrations were considered estimates. Samples were heated at 65°C for 25 minutes, then loaded (5µl for juice and 10µl for wine for an estimated 50µg of protein per lane) on to a 12% acrylamide mini-gel (14 x 16 cm) and a 2 cm 4% acrylamide stacking layer. Samples were run on the gel for 3 hours at 50

mAmp (constant current), then washed with water 3x for ten minutes, to remove any residual SDS. The washed gel was stained overnight with Colloidal Coomassie Brilliant Blue (20% ethanol, 1.6% phosphoric acid, 8% ammonium sulfate, 0.08% Coomassie Brilliant Blue G-250). The gel was incubated at 23°C for six hours under agitation. The excess gel was removed, and the gel was rinsed twice with water for 1 minute per wash. The gel was destained with water for approximately 4 hours, or until the background was low. The gel was imaged using a UMAX PowerLook flatbed scanner (UMAX Technologies, Inc., Dallas, TX).

2.3.5. Digestion and cleanup

For all precipitation methods, pellets were dissolved in the buffer by sonicating and vortexing until the pellet was broken up and the protein resuspended. Protein was diluted with water to achieve a urea concentration below 3 M and protein concentration within the standard curve (25-2000 µg/mL) and then quantified with BCA assay. Once quantified, the protein was normalized for all samples by adding 30 µg of protein to a new test tube and adding making volume up to 100 µL with ELGA PURELAB flex 6.2 megaohm water (ELGA LabWater, High Wycombe, United Kingdom). The normalized protein was digestion and individual proteins were identified using a method from Cui et al., (2020), with slight modifications. Briefly, 5 µL of 100 mM ammonium bicarbonate and 200 mM DTT (reducing agent) was added and mixed by gentle vortex and reduced for 1 hour at 21°C. Then 20 µL of 100 mM ammonium bicarbonate and 200 mM iodoacetamide (alkylating reagent) was added and mixed by gentle vortex and alkylated for 45 minutes at 21°C. Then 20 µL of the reducing agent was added to consume any unreacted iodoacetamide and mixed by gentle vortex and allowed the reaction to occur at

21°C for 20 minutes. Urea concentration was diluted below 0.6 M, for trypsin to retain its activity. Trypsin was added at a protease-to-substrate ratio of 1-to-50. Samples were mixed by gentle vortex and the digestion occurred overnight at 37 °C. The reaction was stopped by adding concentrated formic acid (FA) to 1% v/v. Purification of trypsin digest was needed before analysis. Pierce C18-100 µL Tips (Thermo Scientific, Rockford, lL) were prepared by wetting with 100% acetonitrile, washing with 70% acetonitrile/1% formic acid, then equilibrating with 1% formic acid. To bind peptides, the peptide sample was pipetted into and out of the C18 tip at least 20 times and expelled on the last cycle without introducing air into the tip. Peptides were washed with 1% formic acid and the peptides were expelled by cycling 50 µL of 70% acetonitrile/1% formic acid into and out of the tip at least 20 times and diluted with 50 µL of water. Samples were then lyophilized before analysis.

2.3.6. Chromatography and Mass Spectrometry

For UPLC analysis, samples were resuspended in 30 µL of solvent (5% acetonitrile, 0.1% formic acid) to approximately 1 µg/µL of the initial protein concentration. Peptides were transferred to vials and placed in a refrigerated autosampler (7 °C). Samples, 2 µL, were injected directly onto a 20cm long x 75µm inner diameter pulled-needle analytical column packed with Waters BEH-C18, 1.7 µm reversed phase resin. Peptides were separated via nano ultra-performance liquid chromatography coupled with electrospray ionization-trapped ion mobility spectrometry-time of flight (UPLC-ES-TIMS-TOF) and eluted from the analytical column with a gradient of acetonitrile at 300nL/min. The Bruker nanoElute system (Bruker Daltonics, Billerica, Massachusetts) is attached to a Bruker timsTOF-PRO mass spectrometer via a Bruker

CaptiveSpray source (Beck et al., 2015). LC gradient conditions: Initial conditions were 2%B (A: 0.1% formic acid in water, B: 99.9% acetonitrile, 0.1% formic acid), followed by a 10 min ramp to 17%B. Then 17-25%B over 25 min, 25-37%B over 25 min, 37-80%B over 10 min, hold at 80%B for 15 min, ramp back (2min) and hold (3min) at initial conditions. The total run time was 90min.

MS data were collected in positive-ion data-dependent parallel accumulation serial fragmentation (PASEF) mode over an m/z range of 100 to 1700. PASEF and TIMS One MS and ten PASEF frames were acquired per cycle of 1.27sec (~1MS and 120 MS/MS) (Meier et al., 2015). Target MS intensity for MS was set at 20,000 counts/sec with a minimum threshold of 250 counts/s. The intensity repetition table (default values) was set to On. A charge-state-based rolling collision energy table was used from 76-123% of 42.0 eV. An active exclusion/reconsider precursor method with release after 0.4min was used. If the precursor (within mass width error of 0.015 m/z) was >4X signal intensity in subsequent scans, a second MSMS spectrum was collected. Isolation width was set to 2 m/z (<700m/z) or 3 (800-1500 m/z).

2.3.7. Protein Identification

The acquired data were submitted to the PEAKS X (Bioinformatics Solutions Inc., Waterloo, ON, Canada) search engine for protein identifications. An NCBI-*Vitis vinifera* database (182,373 entries; last update 3/5/2019) and/or UniProt *Vitis vinifera*/yeast protein was searched. Data were searched with trypsin as the enzyme, 2 missed cleavages allowed; carbamidomethyl cysteine as a fixed modification; oxidized methionine and deamidation of N/Q as variable mods; 20ppm mass tolerance on precursor ions, 0.1Da on fragment ions. FDR estimation enabled (a reversed decoy

database is created and searched simultaneously). Search results files were first filtered for 0.1% FDR (peptide false discovery rate) and >1 unique peptide per protein and export from PEAKS.

2.4. Comparison of quantification methods

2.4.1. Fluorescence-based protein quantification

All method optimization before comparing precipitation methods was performed with acetone/TCA. To increase the solubility of membrane proteins after precipitation, a buffer consisting of 6M Urea, 2M thiourea, and 100mM ammonium bicarbonate was used to dissolve protein pellets. Since thiourea interferes with the majority of protein quantification assays, we began with a fluorescence-based protein quantification method mainly used for electrophoresis (Agnew et al., 2004). 1 μL of the sample containing up to 5 μg/μL protein is spotted on paper and an external standard curve (ovalbumin). Once samples are dried, 100% methanol is used to rinse the paper for 5 minutes, then a fluorescent protein stain provided in Thermo Fisher (Waltham, Massachusetts, US) the EZQ protein quantification kit was added with gentle shaking for 30 minutes, then washed three times with 10% methanol, 7% glacial acetic acid for two minutes and then dried. Sample fluorescence intensity was then read on a 96 well plate fluorescence laser scanner. Using the external standard curve, unknown protein samples can be quantified.

2.4.2. Bradford Dye protein quantification

The Bradford method is one of the most commonly used assays to quantify protein, especially in food, and has been reported use in juice/wine protein quantification (Bindon et al., 2016b; Jaeckels et al., 2017; Salazar et al., 2017; E. Waters et al., 1991). Coomassie Blue G-250 dye is added to the protein and the compounds bind together

which shifts the maximum absorbance from 465nm to 595nm where absorbance can be read with a simple spectrometer. Using an external standard curve, the unknown protein can be quantified. The reproducibility is good and maximum color change is achieved after 2 minutes and can be stable for up to an hour making this a very user-friendly assay (Bradford, 1976). Due to the interferences from Urea and Thiourea, this assay used a Tris-HCl, pH 8.6 buffer for solubilizing the protein. To determine the effectiveness of the assay standard additions of BSA were made to cv. Chambourcin bottled wine at rates of 100, 250, and 500 µg/ml and then duplicate acetone/TCA protein precipitation for each rate was done.

2.4.3. Pierce 660 nm protein quantification

Similar to the Bradford Dye assay, the Pierce system uses a dye-based compound that binds protein resulting in a color shift. Rather than using just a dye, this assay uses a dye-metal complex which in this case is a polyhydroxybenzenesulfonephthalein-type dye and a transition metal. The complex is a brown color and when combined with protein shifts to a green color (Antharavally et al., 2009). To our knowledge, this assay has not been used to quantify protein in juice/wine. One advantage is that it is subject to fewer interferences than the Bradford Dye assay. The Pierce 660nm kit was used for this assay (Thermo Fisher, Waltham, Massachusetts, US). As for the Bradford Dye assay, effectiveness was tested by making standard additions of BSA to cv. Chambourcin bottled wine at rates of 100, 150, and 200 µg/mL. Duplicate acetone/TCA protein precipitations were done at each concentration.

2.4.4. Bicinchoninic acid (BCA) protein quantification

This assay uses bicinchoninic acid, a weak acid that forms a purple color upon complexation with cuprous ion (Cu^{1+}). This can be used to monitor the reaction of peptide bond with cupric ions (Cu^{2+}) under alkaline conditions, known as the biuret reaction which forms cuprous ions when peptide bonds are present (Antharavally et al., 2009; P. K. Smith et al., 1985). This assay can be used in a 96-well microplate format to reduce sample size and assay time and has a working range of 20-2000 µg/ml. This assay has been used in wine, with particular success after precipitation (Dzedze, 2018; Vincenzi et al., 2014). After proteins are solubilized in the buffer, the working reagent is added to the sample (BCA and copper (II) sulfate), mixed on a shaker plate for 30 seconds, then incubated in a water bath at 37°C for 30 minutes. Protein is then quantified using absorbance reading at 562 nm and an external standard curve of bovine serum albumin (BSA) (M. R. Smith et al., 2011; Vincenzi et al., 2005). A BCA kit from Thermo Fisher (Waltham, Massachusetts, US) was used for this assay. To test the accuracy of this assay a bottled 2013 Chambourcin wine (University of Missouri, Columbia, MO) and 2018 Chambourcin juice with additions of BSA at rates of 5, 10, 15, 25, and 50 µg/ml. After promising initial results, acetone/TCA protein precipitation was done at each concentration.

2.5. Comparison of buffers

2.5.1. Water vs 6M Urea/100 mM ammonium bicarbonate

After juice protein precipitations, buffer effectiveness was determined by resuspending protein in ELGA PURELAB flex (High Wycombe, United Kingdom) 6.2 megaohm water (pH 7), Protein pellets were resuspended in 1500 µL of water, then sonicated and vortexed until the pellet was broken up and particulates were not visible.

Suspended, yet not dissolved protein, was quantified with BCA protein assay (Thermo Fisher, Waltham, Massachusetts, US) to obtain a total protein estimation. The water samples were then centrifuged at 16,000 x g 10 min at 4°C. The supernatant was carefully transferred into a new 1.5 ml tube (water samples). Leftover pellets from the water samples were then dissolved in 1500 µL of 6M Urea and 100mM ammonium bicarbonate buffer by sonication and vortexing until the pellet was dispersed and particulates were not visible. Samples were centrifuged at 16,000 x g 10min at 4°C and the supernatant was carefully transferred into a new 1.5 ml tube (urea/ammonium bicarbonate samples). Using the quantification estimation, 30 µg of protein for each sample was added to a new 1.5 ml tube and made up to 100 µL with the respective buffer. Samples were then digested and analyzed.

2.5.2. *6M Urea/100 mM ammonium bicarbonate* vs *6M Urea/2M thiourea/100 mM ammonium bicarbonate*

After protein precipitation, all samples were resuspended in 6M Urea in 100 mM of ammonium bicarbonate buffer to start. Juice sample protein pellets were resuspended in 300 µL of buffer and a 1:5 dilution was made for BCA quantification (50 µL of the sample, 200 µL of ELGA PURELAB flex 6.2 megaohm water). Wine sample protein pellets were resuspended in 200 µL of buffer and a 1:3 dilution was made for BCA protein quantification (50 µL of the sample, 100 µL of ELGA PURELAB flex 6.2 megaohm water). Pellets were dissolved by sonication and vortexing. After quantification by BCA, thiourea was added to the thiourea treated samples by adding 3.998 M thiourea, 6M Urea, 100 mM ammonium bicarbonate buffer to reach a final concentration of 6M Urea, 2M thiourea, and 100 mM ammonium bicarbonate). After the addition of thiourea,

samples were vortexed and rested for 30 min. Using the BCA quantification and accounting for dilutions of the thiourea, 28 µg of protein was added to a new 1.5ml tube and made up to 100µL with ELGA PURELAB flex 6.2 megaohm water. Samples were then digested and identified via UPLC-ES-TIMS-TOF.

2.6. Analysis with optimized method

After optimization was complete, quintuple precipitations of juice and wine were performed to verify reproducibility and repeatability. One ton of Chambourcin fruit was machine-harvested into 0.5-ton picking bins from Rocheport, MO on September 25, 2019. Berries were then crushed, and juice samples were collected and placed into 50 ml tubes and frozen at -80° C for later analysis. Wine samples were from the same 0.5-ton picking bins as the juice, but 3,050 g samples of berries were crushed and placed into 3.79 L plastic fermentation vessels. One day later, musts were inoculated with 0.16 g/l of Renaissance Yeast Muse (Gusmer Enterprises, Inc. Waupaca, WI) and 5 mg/l of Lallemand Beta Co-Inoc Malolactic Bacteria (Scott Laboratories Inc., Petaluma, CA), and 0.45 g/l of diammonium phosphate (DAP) (Scott Laboratories Inc., Petaluma, CA). Fermentation proceeded in a 20°C cooler with caps punched down twice per day. On 9/29/2019, 0.225 g/l of DAP was added to fermentations and on October 3, 2019, wines were pressed with a #20 fruit press (9.5 L, 20cm basket press) (MoreFlavor, Pittsburg, CA). 1.89 L of wine was collected. From that, 50 ml of wine was stored in polypropylene tubes and frozen at -80° C for later analysis.

2.7. Final precipitation method

Samples were prepared by centrifuging samples at 12000 x g for 10 minutes at 4°C and then filtered with vacuum filter and #2 filter paper. Samples consisting of 5ml of

juice and 10ml of wine were then transferred into 50 ml centrifuge tubes (Celltreat scientific products, Pepperell, MA). 4x the volume of 5% TCA in acetone was added to each centrifuge tube (20 ml for juice and 40 ml for wine). Tubes were carefully inverted 10 times to mix and avoid sample loss, then incubated at -20°C overnight (max 12 hours). The next day, samples were centrifuged at 12,000 x g for 30 minutes at 4°C. The supernatant was carefully poured off and 0.5 ml of 80% acetone was added to the tube. The pellet was sonicated in ice water until it was broken up. The suspended pellet was then transferred by pipet to a new 2.2 mL centrifuge tube. The centrifuge tube was then rinsed 2x with 0.5 mL 80% acetone and the rinsates were transferred to the 2.2 mL tube. If needed, residual protein pellets were vortexed and sonicated until suspended in solution, then incubated at -20°C for ten minutes. Samples were then centrifuged at 20,800 x g for 10 minutes and the supernatant was removed and discarded. Protein pellets were washed 2x with 1 mL of 80% acetone and vortexed/sonicated to disperse the pellet, then held at -20°C for ten minutes. Samples were then centrifuged at 20,800 x g for 10 minutes and the resulting supernatant removed and discarded. This resulted in a total of 3-80% acetone washes; more washes can be used as necessary to ensure all TCA is removed. Caution must be used to not to lose the pellet as it can become loose after a few 80% acetone washes. After the last supernatant is removed, the pellet was allowed to dry for 15-30 minutes to remove any excess acetone. Drying the pellet completely was avoided as this makes it difficult to redissolve. 300 µL of 6M Urea and 100 mM ammonium bicarbonate buffer was then added to the pellets. The 2.2 mL tubes were repeatedly vortexed, sonicated, and rested for 30 minutes to resuspend the pellet, warming (<37°C) helped dissolve pellets. After pellets were dissolved, samples were

centrifuged at 16,000 x g for 10min at 4°C to remove and nonprotein insoluble residue. Before quantification, samples were diluted in LC-MS grade water (Sigma-Aldrich, Inc., St. Louis, MO) to adjust concentration to within the assay's standard curve range and to avoid interference with Urea (max 3M). A 1:4 dilution (50µL of the sample, 150 µL water) was made for juice samples and a 1:3 dilution (30 µL of the sample, 60 µL of water) for wine samples. Diluted protein samples were then quantified using the BCA protein assay, as stated above. Protein digestion and LCMS analysis were done as stated above.

2.8. Cultivar variation

The optimized method was used on a range of *Vitis vinifera* and Interspecific hybrid cultivars to investigate identified proteins from juice and wine in cultivars with a wide range of parentage. We also aimed to explore whether certain proteins in juice or wine (or changes between them) show any trends that can be related to changes in phenolic content.

2.8.1. Winemaking

Approximately three clusters from the following cultivars were hand-harvested and frozen at -20°C: 2018 Chambourcin (Mt. Vernon, MO) at 28 °Brix, 2019 Chambourcin (Ste Genevieve, MO) 21 °Brix, 2019 Syrah (CA) 25 °Brix, 2019 Riesling (Fredonia, NY) 19 °Brix, 2019 Tannat (Fredonia, NY) 17 °Brix, 2019 Petit Verdot (Fredonia, NY) 22 °Brix, 2019 Concord (Fredonia, NY) 15 °Brix, and 2019 Vignoles (Ste Genevieve, MO) 21 °Brix. On June 26, 2020, 65g of randomly selected berries were sampled from each variety in quadruplicate. The berries were macerated by hand in a cup and 8 ml of juice was removed from the samples with 5 mL transferred into a new 50mL

centrifuge tube and frozen at -80°C. 40 mL of the remaining crushed must was added to a 50mL (for 20% headspace) centrifuge tube (Celltreat scientific products, Pepperell, MA). Musts were inoculated with 1 g/l of Lalvin ICV Yeast GRE (Scott Laboratories Inc., Petaluma, CA) and 0.3 g/l of DAP was added (Scott Laboratories Inc., Petaluma, CA). Caps were placed on tubes just less than tight (allowing for CO_2 to escape as pressure increased) with fermentation taking place in a 28°C water bath. Simulated "punch-downs" occurred twice per day, by inverting the tubes five times to mix the solids with the liquid. After seven days, the wine was separated from the skins by centrifugation at 10000 x g for 10 minutes at 4°C and directly decanted and filtered with Whatman #2 filter paper. A 10 mL aliquot of the filtered wine was placed in a new 50mL centrifuge tube for protein precipitation and a 2 mL aliquot of wine was collected in a 2.2 mL tube for condensed tannin analysis. Wine samples were taken and frozen at -80°C for later analysis. Protein precipitation was performed according to the final protein precipitation method above.

2.8.2. Tannin analysis

Protein precipitable tannin analysis was performed via Adams-Harbertson tannin assay (Harbertson et al., 2002a). Briefly, 0.5 mL of wine (diluted up to 4-fold with DI water) was added to a 2.2 mL tube with 1 mL of 200 mM glacial acetic acid, 170 mM sodium chloride, and 1 mg/mL bovine serum albumin at pH 4.9. Tubes were inverted 10 times to mix wine and protein solution and incubated at room temperature with gentle agitation for 15 minutes. Samples were centrifuged for 5 minutes at 20800 x g. The supernatant was removed and carefully 0.875 mL of 5% triethanolamine and 5% SDS at pH 9.4 buffer was added to the pellet and incubated at room temperature for 10 minutes.

Samples were then vortexed vigorously until the pellet was completely dissolved. Samples were incubated at room temperature for an additional ten minutes. The entire 0.875 mL sample was transferred to a 1.5 mL microcuvette and the background absorbance was read at 510 nanometers. Then 0.125 mL of 10 mM of iron(III) chloride in 0.01 N HCl was added to the sample and inverted to mix and then incubated at room temperature for 10 minutes. The sample was then incubated for a final ten minutes and the second absorbance was read at 510 nm. Using an external standard curve of catechin, the condensed tannin concentration is calculated and reported in mg/L catechin equivalents (mg/L CE).

2.9. *Data Processing and Statistical analysis*

Data were collected and stored using Microsoft Excel (Redmond, WA). Mean comparisons of protein and condensed tannin concentrations were performed using Tukey-Kramer HSD (multiple mean comparison) or one-tailed t-test ($p < 0.05$) (one mean comparison) on JMP statistical software (SAS Institute, Inc.). The principal component analysis and partial least squares discriminant analysis was performed using the MetaboAnalyst statistical analysis website which uses R (Xia & Wishart, 2011). Before analysis, data were filtered to remove variables unlikely to be used in the model using the interquartile range to remove 25% of the variables. Data were normalized by log transformation and auto-scaling (mean-centered divided by the standard deviation of each variable).

3. Results and Discussion

3.1. Comparison of protein precipitation methods

Protein samples from the three precipitation methods being evaluated were first screened using SDS-PAGE gel to determine visually if and how much protein was precipitated. For all precipitation methods the juice protein precipitated and separated on the SDS-PAGE (Figure 1) revealed distinctive bands from 20-30 kDa and another around 60 kDa. This separation pattern is similar to what has been observed in other studies using SDS-PAGE on grape juice (Jaeckels et al., 2015, 2017; Vincenzi et al., 2005). Protein recovered in the acetone/TCA and SDS/KCl, precipitates were visually higher SDS/KCl were also visually darker than those recovered by acetone/TCA. This is similar to what (Vincenzi et al., 2005) observed suggesting the strong denaturation effect of TCA can inhibit the protein solubility and migration into the SDS-PAGE gel. In effect, this reduces the amount of protein run on the gel. The ammonium sulfate method precipitated less protein when compared to the other methods as estimated by SDS-PAGE. Although previous reports from Springer, Sherwood, et al., (2016a) indicated success with the ammonium sulfate protein precipitation, there was never a direct SDS-PAGE comparison with other precipitation methods. Other grape and wine research using ammonium sulfate precipitation used acetone to precipitate the protein a second time (Yokotsuka & Singleton, 1997). This was likely one way to eliminated interferences in SDS-PAGE when residual ammonium sulfate is present (Aguilar et al., 1999). With the goal of this study to precipitate, quantify, and identify the whole (or close to) proteome of juice and wine, plus do it in a quick and efficient method, it was determined that we discontinued using the ammonium sulfate precipitation method.

3.1.1. Quantification via BCA protein assay

The remaining precipitation methods were quantified for total protein in juice and wine using the BCA protein assay. Acetone/TCA juice precipitations had an average of 92.8 μg/mL BSAeq of protein in 2019 Chambourcin juice samples with a mean coefficient of variation (CV) of 8%. The SDS/KCl precipitations had an average of 128.6 μg/mL BSAeq of protein in 2019 Chambourcin juice samples with a CV of 4%. Recently fermented wines of 2019 Chambourcin had protein concentrations of 30.5 μg/mL BSAeq (5% mean CV) and 54.5 μg/mL BSAeq (6% mean CV), respectively. Consistent with SDS-PAGE analysis, the SDS-KCl visually precipitated the most protein with 28% more protein in the juice and 44% more protein in the wine.

3.1.2. Chromatography and Mass Spectrometry

Spectrum counting is a method that uses MS/MS data and after database searching to identify proteins, individual proteins can be compared between treatments that have the same spectrum-to-peptide matches (Bantscheff et al., 2007). Due to variation in signal strength for peptides, the differences in total spectrum count do not indicate more or less protein, just a change in what proteins are present. There was a large difference in the diversity of proteins from Acetone/TCA vs SDS/KCl precipitated juice samples (Figure 2) as represented by total spectrum counts of 12,956 and 7,789, respectively. The large variability in the Acetone/TCA wine samples (Figure 2) was due to a sample that came back with zero proteins identified, but even with that, the total spectrum count in the acetone/TCA samples was almost 4 times higher than the SDS/KCl at 4,172 vs 1,050, indicating a large change in the proteome that was identified. It was clear that there was significant interference from a detergent (Figure 3), likely residual SDS that was not completely removed by KCl precipitation or C18 cleanup making it

impossible to distinguish individual peaks. Residual SDS in a sample that is run an HPLC can have a significant reduction in peptide identification when above 0.3% (Aguilar et al., 1999). This interference greatly reduced the number of peptides that could be distinguished, plus the detergent was difficult to wash from the column between runs, so there was a bleed of SDS to some of the acetone/TCA wine samples (that came after the SDS/KCl samples). The acetone/TCA juice samples were run first, so SDS interference was not an issue. This level of interference by SDS was unexpected as previous studies have shown that SDS/KCl precipitation removed over 99.9% of SDS while retaining 80% of the peptides for LCMS analysis (Zhou et al., 2012). One big difference from previous studies is this precipitation method followed methods from Vincenzi et al., (2005) where KCl precipitation was used with intact juice/wine proteins, while other studies used KCl on tryptic peptides which may allow for easier KCl precipitation of SDS. Precipitating SDS after digestion may be difficult as there can be interference from SDS in protein quantification assays that are necessary to achieve a protein: trypsin digestion ratio of 50:1 and to standardize the protein in all of the samples. Since acetone/TCA precipitation was effective for LCMS protein characterization we used acetone/TCA as the sole precipitation method for all subsequent analyses.

3.2. Comparison of quantification methods

3.2.1. Fluorescence-based protein quantification

When using the fluorescence-based protein quantification method the BSA standard curve had a R^2 of 0.9718 which was determined to be acceptable for this quantification method. When using cv. Chambourcin juice, the mean CV was 41% and a range of 8.8-66.2 μg/mL ovalbumin equivalent while post-fermentation wine had a mean

CV of 29% and a range of 0.1-5.1 μg/mL ovalbumin eq. This high coefficient of variation was consistent with previous reports of protein quantification in wine. Vincenzi et al., (2005) found that wines before bentonite fining (after fermentation, but before bottling) had protein levels between 12.2-328.0 μg/mL BSAeq which indicates that the fluorescence-based protein quantification method is not properly quantifying protein in grapes or wine. The use of ovalbumin as a standard could account for some of the lower values as the protein fluorescence of BSA is 1.25 times higher than ovalbumin, but the low protein juice and wine samples are still far below the BCA quantification assay (Thermo Fisher Scientific, 2017). To our knowledge, this method has not previously been tested on grape juice or wine. The fluorescence measures cystine (Agnew et al., 2004), and grapes are not known as high cystine foods, but Gutiérrez-Gamboa et al., (2018) quantified amino acids in cv. Carignan juice and found levels of cystine between 4.27-7.75 mg/L which should be sufficient for protein quantification. One explanation for low quantification in wine could be because cystine is used in yeast metabolism's production of volatile sulfur compounds (Moreira et al., 2002).

3.2.2. Bradford Dye protein quantification

The BSA standard curve for the Bradford Dye assay had an R^2 of 0.946. After quantifying the protein, the linear relationship for BSA standard addition had an R^2 of 0.23. This indicates that the use of Acetone/TCA precipitation is problematic for estimating protein concentration in wine. This was a similar trend to what (Vincenzi et al., 2005) found when quantifying standard additions of BSA. Although (M. R. Smith et al., 2011) had success quantifying protein with Bradford Dye when using invertase as the protein standard, our preliminary work with invertase resulted in a large sample to sample

variation. Although yeast invertase may be a better representation of glycoproteins in wine, it only accounts for a small portion of juice/wine proteins. Our thought was that a more consistent assay with low variance would be better than having an assay with a higher variance that is closer to the actual protein concentration. The inconsistent measurement of protein could be due to possible interferences of bound phenolic compounds that end up in the precipitated protein after extraction and cleanup. Ethanol and phenolic compounds have demonstrated the ability to reduce the protein measured in juice and wine to 30-90% of the initial values (Marchal et al., 1997).

3.2.3. Pierce 660nm protein quantification

The BSA standard curve had an R^2 of 0.999. After quantifying the protein, the linear relationship for BSA standard addition had an R^2 of 0.985. Although the linear relationship was excellent, the control wine with no BSA addition was continually measuring zero protein. This indicated that the polyhydroxybenzenesulfonephthalein-type dye and a transition metal may not interact with grape and wine proteins. Similar to other dye-binding assays, there are limitations due to types of proteins (amino acid structure), isoelectric point, secondary structures, and prosthetic groups (Antharavally et al., 2009). It is also possible that phenolics can interact with proteins and end up in the precipitates and can ultimately contribute to assay interferences. BSA is also one protein tested that had one of the highest color responses possibly explaining why the grape protein analyses are zero or close to zero when using BSA for the standard curve. With a more representative grape protein standard, we may be able to obtain more accurate quantification. Regardless, the absorbance values were very low indicating that there may be issues with the dye-binding with grape proteins.

3.2.4. bicinchoninic acid (BCA) protein quantification

The BSA standard curve had an R^2 of 0.998. The mean CV for each addition to wine was 14, 10, 6, 3, 2, and 5% for 0-50 µg/mL BSA additions, respectively (Figure 4). While the mean CV for each juice addition was 5, 2, 3, 2, 4, 2, and 6% for 0-100 BSA additions, respectively (Figure 4). The wine protein content on average was 14.7 µg/mL BSAeq. This may seem low, but Vincenzi et al., 2005 found commercially bottled wines (white and red) to have 12.2-30.5 µg/mL BSAeq, and Jaeckels et al., 2017 found pre bentonite treated white wines to have a protein concentration between 10.7-67.4 µg/mL BSAeq (measured with Bradford assay). The juice protein concentration was determined to be 78.5 µg/mL BSAeq (Figure 4). This seems to be with previous studies that measured 112 µg/mL BSAeq of protein in Sauvignon blanc juice (Salazar et al., 2017) while (Bayly & Berg, 1967) found protein concentration in a wide range of whites to be between 51-260 µg/mL. The use of the BCA assay to quantify juice and wine protein is not very common, but the previous work has been encouraging with Gazzola et al., (2015) showing that there was less protein to protein variation between commonly used standards (BSA, lysozyme, ovalbumin, and *Thaumatococcus daniellii* (thaumatin)) when compared to the Bradford assay and the protein concentration slope was similar to that of BSA and lysozyme.

3.3. Comparison of buffers

3.3.1. Water vs 6M Urea/100 mM ammonium bicarbonate

After extraction and quantification, there was on average 160 µg/mL BSAeq of protein in the cv. Chambourcin juice. This was higher than the previous juices analyzed in this study but was in range with other juice protein analysis studies (Bayly & Berg,

1967; Salazar et al., 2017). The mean CV for the extractions was 13%, indicating good replicability of the precipitation method and quantification assay. After centrifugation, the majority of protein diversity is lost in the water resuspension treatment as total spectrum counts are 2.2-fold higher with 1.7-fold more proteins identified in the 6 M Urea/100 mM ammonium bicarbonate buffer (Figure 5). It's possible that without centrifugation of the proteins suspended in water and adding trypsin directly the resulting peptides would dissolve during digestion and MS analysis would work fine (Sung et al., 2006). The issue with unknown juice amounts is that quantification needs to occur to standardize protein concentrations for digestion. Also, centrifugation of proteins suspended in the buffer can help remove non-protein impurities that may have been precipitated along with the protein from the juice. The use of water as a resuspension solution is a more practical method when analyzing cellular proteins as a known concentration of cells can be used avoiding the need to quantify proteins in advance. With grapes, many environmental factors play a role in protein content. Under disease pressure like powdery mildew pathogenesis-related proteins have been observed in white free run *V. vinifera* juice and in low condensed tannin red wine that was recently fermented (Girbau et al., 2004b; Springer, Sherwood, et al., 2016b). A wide range of protein concentrations between different cultivars (red and white) has also been observed where total juice protein ranged from 31-251 µg/mL BSAeq (Pocock et al., 2000). This makes it quite difficult to match the unknown amount of protein in a juice sample which makes using water as a protein dispersion method difficult.

3.3.2. 6 M Urea/100 mM ammonium bicarbonate vs. 6 M Urea/2 M thiourea/100 mM ammonium bicarbonate

Before adding the thiourea, total protein was quantified and on average there was 104.3 µg/mL BSAeq in cv. Chambourcin juice and 17.2 µg/mL BSAeq in Chambourcin wine. Although the juice protein from this study is 55.7 µg/mL BSAeq lower than the same extraction and buffer from the urea/ammonium bicarbonate vs water study, the variation could be since the Chambourcin juice from this experiment was stored at -20°C which could lead to degradation of the protein. Since the protein was not being compared between experiments this was not a huge concern as the total juice protein was still within the 31-251 µg/mL range of the BSAeq standard curve (Pocock et al., 2000).

After dividing the samples and adding the thiourea treatment, samples were analyzed by MS. There is no difference in the number of unique identified proteins between the buffers (Figure 6), while the average total spectrum counts were 18,508 for 6 M Urea/ 100 mM ammonium bicarbonate and 18,233 for 6 M Urea/2 M thiourea/100 mM ammonium bicarbonate. In wine, there was also a reduction in the number of proteins identified (Figure 6) and spectrum count (6,271 and 2,500, for with and without thiourea, respectively). This is surprising since thiourea is known to increase the solubility of membrane proteins (Natarajan et al., 2005). Without a grape protein solubility study with thiourea, it is difficult to explain these differences. One factor could be that the protein concentration was not properly standardized following thiourea addition, leading to a reduction in signal. However, with 40% of the total spectrum intensity, it seems unlikely that this is the explanation for this difference. The reduction in spectrum count could be because iodoacetamide can be scavenged by thiourea which leads to poorly alkylated proteins, especially free -SH groups on proteins, like cysteine (Galvani et al., 2001). Whether or not thiourea is interfering with protein

solubility/identification, the use of 6 M Urea/100 mM ammonium bicarbonate is effective at solubilizing grape proteins after centrifuging to remove non-protein compounds and does not interfere with BCA protein quantification, so this buffer was used for the optimized method.

3.4. Analysis with optimized method

There is strong repeatability of this method as the number of proteins identified and total spectrum count both had a mean CV at 15% or below (Table 3) for the optimized method analysis and previous experiments that had proteomics data. Total protein via BCA q was on average 81.1 µg/mL BSAeq in juice (Table 4) and 24.0 µg/mL BSAeq in wine (Table 5). Both averages were in the range of previous studies for juice and wine (Salazar et al., 2017; Vincenzi et al., 2005). The use of shotgun proteomics in grape juice and wine has not been widely reported as the majority of previous studies used SDS-PAGE gel for purification and identification (D'Amato et al., 2011; Deytieux et al., 2007; Springer, Sherwood, et al., 2016b). An attempt to identify the whole proteome with the use of SDS-PAGE gel was done by stopping the gel once the protein entered the separating gel as one band, which was then cut out and digested in-gel and identified 1411 unique proteins using a nonspecific database (Kambiranda et al., 2016). Our method identified 1240 unique proteins (average 425 per replicate) in juice using UniProt *Vitis vinifera* and yeast protein database search. The number of unique proteins identified with our method is on par with Kambiranda et al., (2016), but the average number of proteins identified is lower. We used a database specific to grape and yeast protein which could be the reason for the reduced number of proteins identified in the sample, also the other study precipitated protein from the whole grape berry as opposed

to the pressed juice (Kambiranda et al., 2016). Other studies using 2-dimension gel separation reported approximately 300 unique spots (George et al., 2015) which are in range with what we have observed in our method. The observation that our results in terms of numbers of proteins identified are within the range of previous studies with a low CV is a good indication that the method developed herein is effective at quantifying and identifying grape and wine protein.

3.5. Cultivar variation

With an optimized method, the seven different cultivars were collected to analyze the proteome of juice and wine plus condensed tannin content of wines. Low condensed tannin cultivars Chambourcin and Concord had the highest total juice protein while cv. Petit Verdot, a higher condensed tannin *Vitis vinifera* cultivar had the lowest amount of total juice protein (Table 6). The most surprising total juice protein number was from Tannat which is known to have high concentrations of condensed tannin in the wine (George et al., 2015), but had high levels of juice protein comparable to 2019 Chambourcin and Concord. The protein in Tannat was over twice as concentrated in wine protein when compared to the juice after maceration indicating that more protein was extracted from grape solids (skin, flesh, and seeds). Except for the 2018 Chambourcin, there was an increase in protein for all samples from juice to wine. With the increased concentration of ethanol during fermentation and aging, wine protein is often lower. It is possible the fermentations were not finished in the 50 mL centrifuge tubes, leading to lower alcohol levels and less protein precipitating from the wine. Extraction of condensed tannin did not seem to be a problem in relation to protein content and fermentation size as cultivars Tannat, Petit Verdot, and Vignoles all had condensed tannin s levels above 800

mg/L CE (Table 6). These condensed tannin concentrations are on par with high condensed tannin red wines from warm climates (Harbertson et al., 2008). The high condensed tannin concentration in Tannat is even more interesting as it also had high juice and wine protein concentrations indicating that not all proteins are precipitating condensed tannin. This may explain why hybrid cultivars are lower in condensed tannin concentration when compared to *Vitis vinifera* cultivars. Total protein does not seem to be a good predictor of wine condensed tannin concentration.

After identification, the protein analyses from juice and wines were used to run a PCA to determine protein differences between the cultivars. PCA analysis indicates differences among cultivars explains 52.3% of the variance with every other individual component below 10%. Except for a 2019 Chambourcin, all replicates are grouped nicely together with 2018/2019 Chambourcin, and 2019 Syrah in one group. Riesling and Tannat were grouped while Vignoles and Petit Verdot formed another group. Concord could be considered grouped with Vignoles and Petit Verdot but is also a bit distant and could be considered a separate group. This is interesting as one would expect hybrid cultivars bred for cold hardiness and disease resistances (pathogenesis-related proteins linked to diseases resistance) would have more similar proteomes and the *Vitis vinifera* would form a separate group. With condensed tannin concentration, there is not an obvious grouping, but with three of the four low condensed tannin cultivars grouping together (2018/2019 Chambourcin and Syrah), there may be some features (i.e., individual proteins) that are influencing low condensed tannin cultivars. Previous studies using PCA, observed genome differences in gape berries with downy mildew and found three *Vitis vinifera* cultivars to separate to different regions in a PCA that explained 71%

of the variation, but the same cultivar inoculated with and without the pathogen did not have obvious PCA differences (Toffolatti et al., 2018). Alternatively, a comparison between *V. rupestris* and *V. vinifera* stilbene profiles had clear differences (Stempien et al., 2018). Total proteome (of juice and wine) comparisons of different cultivars have not been previously reported. The finding that hybrid and *V. vinifera* cultivars are grouped which could indicate that protein diversity may not be the only (or primary) factor for differences in phenolic composition and content.

There are few groups of proteins that are important features in the predictive model, (endo)chitinase, Beta-fructofuranosidase (invertase), Oryzain alpha chain, Thaumatin-like, and Non-specific lipid-transfer proteins (Table 7). Chitinases can degrade chitin which is believed to be an important plant defense system against pathogens that contain chitin (Punja & Zhang, 1993). Although not the same as the mature protein, Springer et al., 2016 recovered a class IV chitinase precursor in a condensed tannin pellet that was precipitated when added to recently fermented low condensed tannin red wine, so chitinases ability to precipitate protein seems probable. Except for Endochitinase EP3 (accession ID: A0A438DFT5) which was not detected in the wine, there was a reduction from 60-92% in spectrum counts from juice to wine. This suggests that these chitinases may not be fully precipitating during the winemaking process, but are present in the wine matrix.

Beta-fructofuranosidase which helps with sucrose degradation is significantly reduced in abundance/activity when berries ripen from veraison to harvest (Degu et al., 2014). This particular protein was not found in wine samples which could indicate that it is highly reactive, or it precipitates easily in the wine matrix. On the other end, the only

protein coming from wine in the top ten important feature variables was the Oryzain alpha chain (Table 7) which does not have much information on its function other than it is similar to a cysteine-type peptidase (Roach et al., 2018). There were mixed trends with this protein with half of the cultivars exhibiting increasing concentrations from juice to wine while the concentration decreased in the other half. Except for Concord, it seems most low condensed tannin wines increase in Oryzain alpha chain from juice to wine.

Both Thaumatin-like and Non-specific lipid transfer proteins are two other plant defense proteins involved in abiotic and biotic stress response with both proteins seeming to have the ability to permeate the cell membrane of pathogens (Scheurer & Schülke, 2018; Vigers et al., 1992). Both these proteins had a 69-98% reduction from juice to wine across all cultivars (Table 7), but it still may not be efficiently precipitated during the winemaking process.

It seems that these proteins may play a role in condensed tannin retention as there was a trend with similar biotic stress-related proteins showing up in the predictive model. To what degree these proteins play in condensed tannin retained in the final product is still unknown.

4. Conclusion

This study was able to optimize a method that is reproducible with a low variation for determining total protein then characterizing protein via LC-MS in grape juice and wine. This method was optimized in grape juice and wine as opposed to relying on methods based on other matrixes, like tomatoes which may not account for matrix specific issues. With a coefficient of variation below 15% for protein quantification and MS data, this method is effective and reproducible. The method could be used as a

baseline for comparative quantification and identification of grape and wine proteins. This method was tested on a wide range of cultivars including *Vitis vinifera* and hybrid reds and white varieties from two different wine growing regions, suggesting it may be a powerful tool for analyzing different cultivars, regions, or winemaking conditions.

With the complexity of the wine matrix, including phenolics, alcohols, organic acids, and proteins as major components there is no surprise that we still do not fully understand predictors or reasons for varying levels of condensed tannin in wine when grapes have comparable condensed tannin amounts. Although using a predictive PLS model does not indicate that there is not a single or group of protein(s) related to condensed tannin variability in total protein concentration or proteome, there did seem to be a continued trend that pathogenesis-related proteins may play a role in condensed tannin retention that warrants further study. Determining if and how pathogenesis-related proteins interact with condensed tannin will go aways into determining if proteins are a driving force in reduced condensed tannin content.

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Table 1: Previous grape and wine protein methods. A summary of methods from previously published research on *Vitis* juice and wine for precipitation/purification of proteins, buffer used to resuspend protein, the total protein quantification, and identification of proteins.

Publication	Juice, Wine, or Both	Precipitation/purification method	Resuspension buffer used	Protein Quantification method used	Protein identification method used
(M. R. Smith et al., 2011)	Wine (red)	Acetone/TCA	Distilled water	Bradford assay with yeast invertase standard	N/A
(Vincenzi et al., 2005)	Wine (red and white)	SDS/KCl	Distilled water	BCA Protein assay with BSA standard	N/A
(Springer, Sherwood, et al., 2016a)	Juice and wine (red)	Ammonium Sulfate	Distilled water	SDS-PAGE/densitometry	Single protein band in gel digest, then LC/MS/MS
(Girbau et al., 2004a)	Juice and wine	Size exclusion (eluted >6,000 Da)	N/A, but eluted with water	HPLC and 220 nm peak area against a standard (horse heart cytochrome c)	Retention times of previously ID proteins and MS
(Van Sluyter et al., 2009)	Juice	Cation exchange, ammonium sulfate, hydrophobic interaction chromatography (HIC)	0.1 M malic acid adjusted to pH 3.5 (KOH)	N/A	X-ray Crystallography
(Springer, Chen, et al., 2016)	Juice (red)	25 µL of a 1 M Tris, 100g/L SDS buffer, and 100 µL of a 500 g/L TCA solution, syringe filtration	N/A	Amido Black staining	N/A
(Cilindre et al., 2008)	Wine (white)	10 kDa cutoff filtration, then ethanol/TCA	7 M urea, 2 M thiourea, 4% (w/v) (CHAPS), 0.5% (v/v) (IPG) buffer 3-10, 60 mM 1,4- (DTT), and traces of bromophenol blue	Two-Dimensional Electrophoresis (2DE)	Individual protein spots in-gel digest, then LC/MS/MS
(Salazar et al., 2017)	Juice and wine (white)	Ultrafiltration and Two-Dimensional Electrophoresis (2DE)	N/A	Juice and Wine directly with Bradford assay and BSA standard	Individual protein band in gel digest, then MALDI-MS/MS
(Carpentieri et al., 2019)	berries	Liquid nitrogen, powder, extracted with (Urea 8 M, Thiourea 2 M, CHAPS 1%, Tris-HCl 100 mM), Chloroform/Methanol precipitation	N/A	N/A	Digest, then LC/MS/MS

(Deytieux et al., 2007)	berries	Phenol extract, then ammonium acetate precipitation	7 M urea, 2 M thiourea, 25 mM DTT, 4% CHAPS, 1% IPG buffer	Two-Dimensional Electrophoresis (2DE)	Individual protein spots in-gel digest, then LC/MS/MS
(Fusi et al., 2010)	Wine (white)	SDS/KCl	Water	BCA Protein assay with BSA standard	N/A
(Nicolle et al., 2019)	Wine (Red)	TCA	0.1 M NaOH	BCA Protein assay with BSA standard	N/A

Table 2: Method optimization experimental summary. A summary of experiments and treatments executed to determine the optimal method for sample preparation to be used in shotgun proteomics

Experiment	Treatments	How Optimal Method was Determined?	The optimal method for LC-MS/MS
Protein Precipitation	1. Acetone/TCA 2. SDS/KCl 3. Ammonium sulfate	1. SDS-PAGE Gel 2. LC-MS/MS	Acetone/TCA
Total Protein Quantification	1. Fluorescence-based (EZQ) 2. Bradford Dye 3. Pierce 660 nm 4. Bicinchoninic acid (BCA)	1. Quantification results of juice and wine only 2. Standard addition of BSA to juice and wine 3. LC-MS/MS	Bicinchoninic acid (BCA)
Protein Resuspension Buffer	1. Water 2. 6M Urea/ 100 mM ammonium bicarbonate 3. 6M Urea/100 mM ammonium bicarbonate vs 6M Urea/2M thiourea/100 mM ammonium bicarbonate	1. BCA quantification 2. LC-MS/MS	6M Urea/ 100 mM ammonium bicarbonate

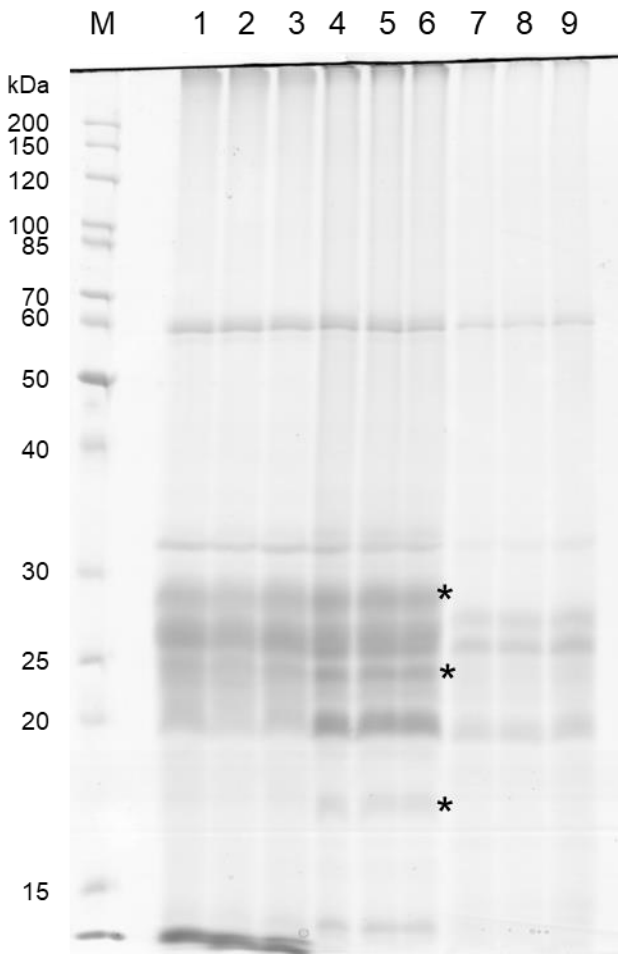

Figure 1: SDS-PAGE of juice samples. Samples were heated, centrifuged, and the supernatant transferred to fresh tubes. An equal volume (5µL) of each sample was loaded on the gel to achieve approximately 50 µg/lane. The 12% acrylamide gel was run at 50 mA for 3 hours and then stained with Colloidal Coomassie Brilliant and destained. The gel was analyzed using a UMAX PowerLook flatbed scanner (UMAX Technologies, Inc., Dallas, TX). Molecular weight marker, sizes are shown in kiloDaltons (M). Asterisks indicate clear differential bands present/increased in the SDS/KCl method. Samples 1-3 are acetone/TCA precipitation; samples 4-6 are SDS/KCl; and 7-9 are ammonium sulfate precipitation.

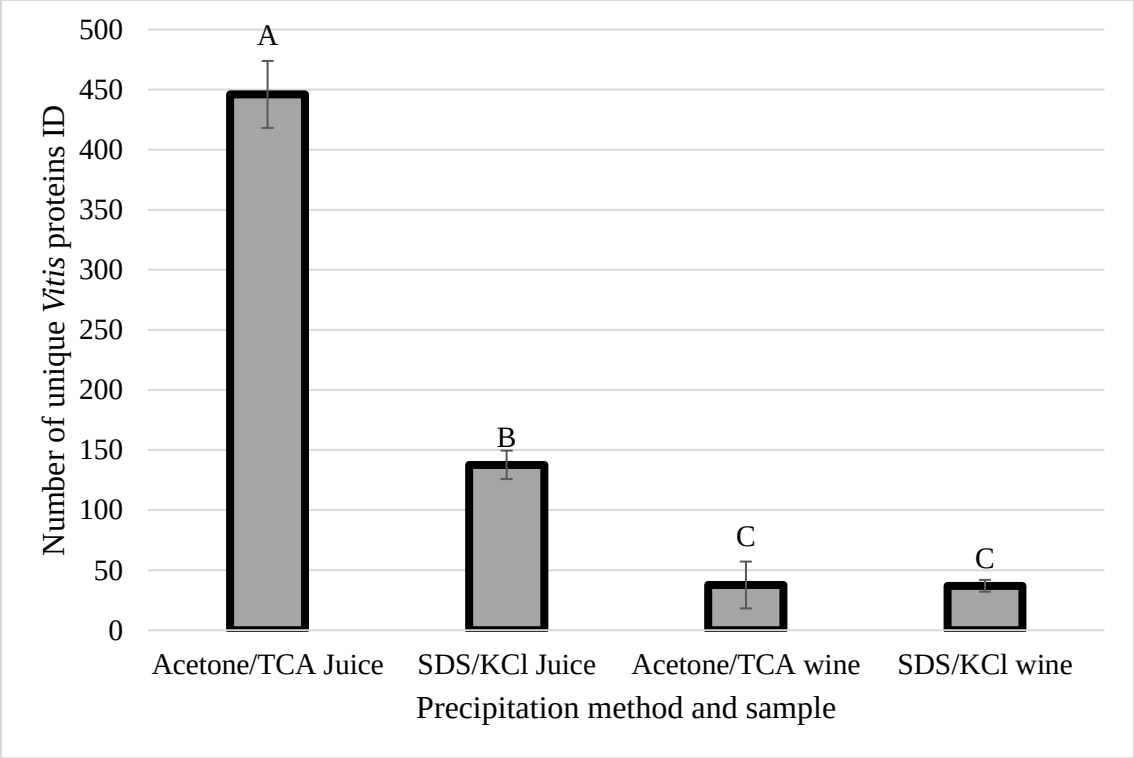

Figure 2: Identified proteins in precipitation methods. Number of identified proteins in juice and wine from two precipitation methods, Acetone/TCA and SDS/KCl after triplicate precipitations of 2019 cv. Chambourcin (M. R. Smith et al., 2011; Vincenzi et al., 2005). Before LC-MS/MS, total protein was quantified using the BCA assay using BSA as the standard (P. K. Smith et al., 1985). 30 µg of protein was used to trypsin digest and identify proteins via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020). Means not followed by a common letter are significantly different according to Tukey-Kramer HSD.

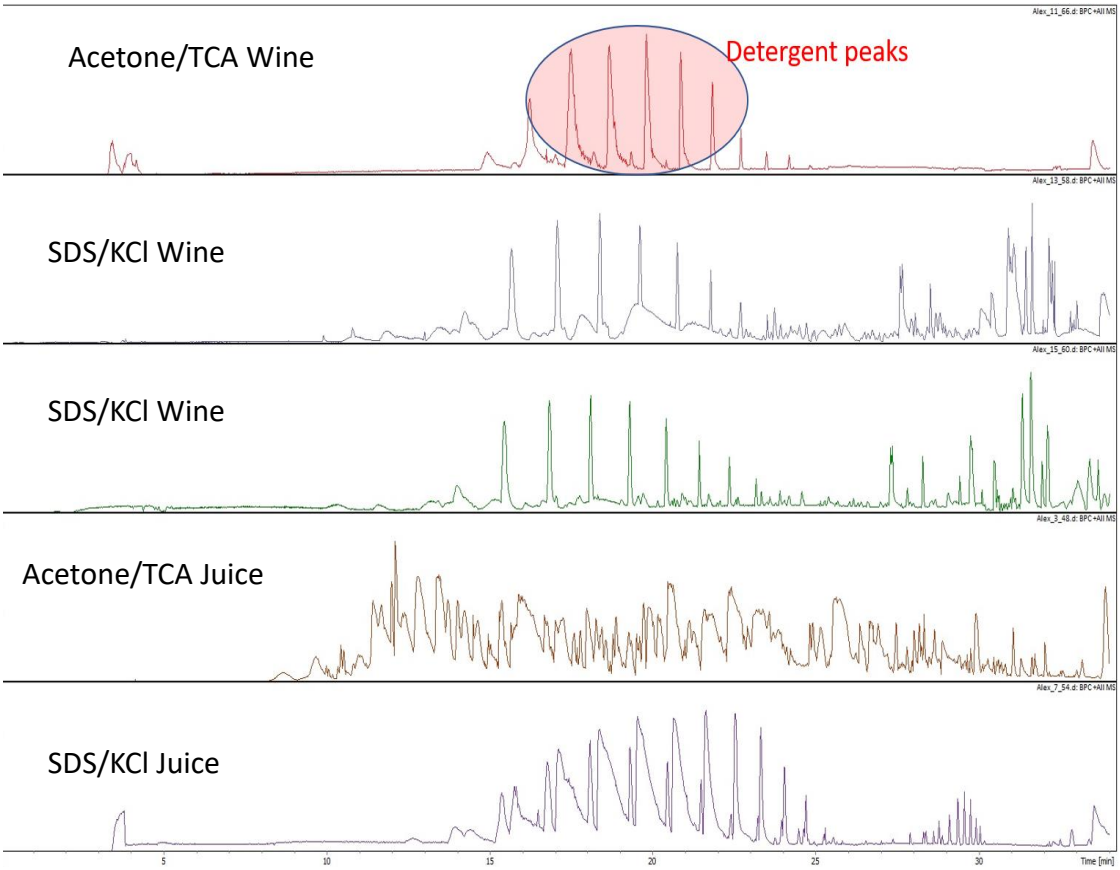

Figure 3 Chromatogram of samples with interference. Base peak chromatogram of samples on LCMS after 2019 cv. Chambourcin juice and recently fermented wine with acetone/TCA and SDS/KCl protein precipitation, both with total BCA protein quantification (M. R. Smith et al., 2011; P. K. Smith et al., 1985; Vincenzi et al., 2005). Acetone/TCA Juice is of a typical run while the rest of the samples have interference, due to residual detergent

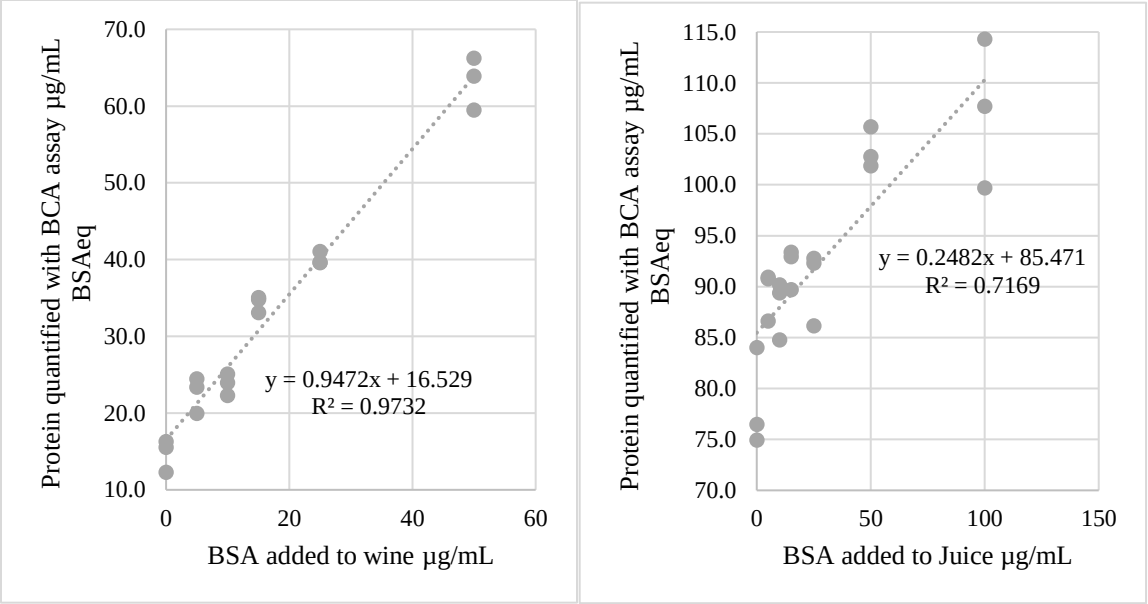

Figure 4: BCA protein quantification after standard additions. A. BSA additions of 5, 10, 15, 25, and 50 µg/mL to bottled 2013 cv. Chambourcin wine and B. BSA additions of 5, 10, 15, 25, 50, and 100 µg/mL to bottled 2018 cv. Chambourcin juice. All protein was precipitated via acetone/TCA precipitation in triplicate (M. R. Smith et al., 2011; P. K. Smith et al., 1985; Vincenzi et al., 2005). Protein pellets were suspended in a Tris-HCl, pH 8.6 buffer and then quantified via BCA protein quantification assay (M. R. Smith et al., 2011).

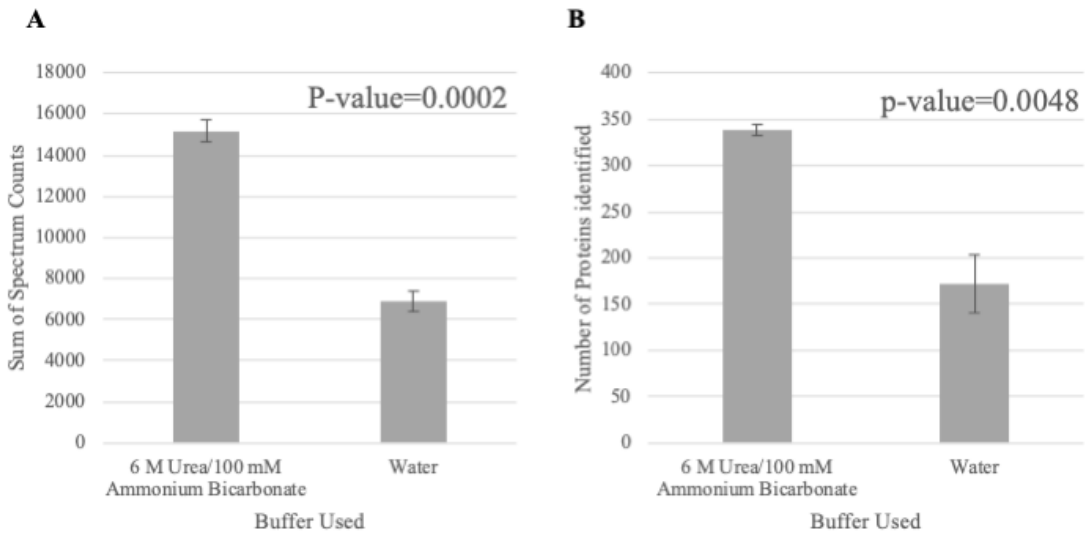

Figure 5: Spectrum count and proteins identified in buffer vs water. A. Spectrum counts, and B. number of identified proteins from the sequential addition of buffers water and Urea/ammonium bicarbonate tested after 2019 cv. Chambourcin juice acetone/TCA extraction and total BCA protein quantification before centrifugation (M. R. Smith et al., 2011; P. K. Smith et al., 1985). From quantification, the same volume from each buffer was used to estimate 30 µg of protein for trypsin digestion and identify proteins via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020). Differences in means were determined by a one-tailed t-test ($p < 0.05$).

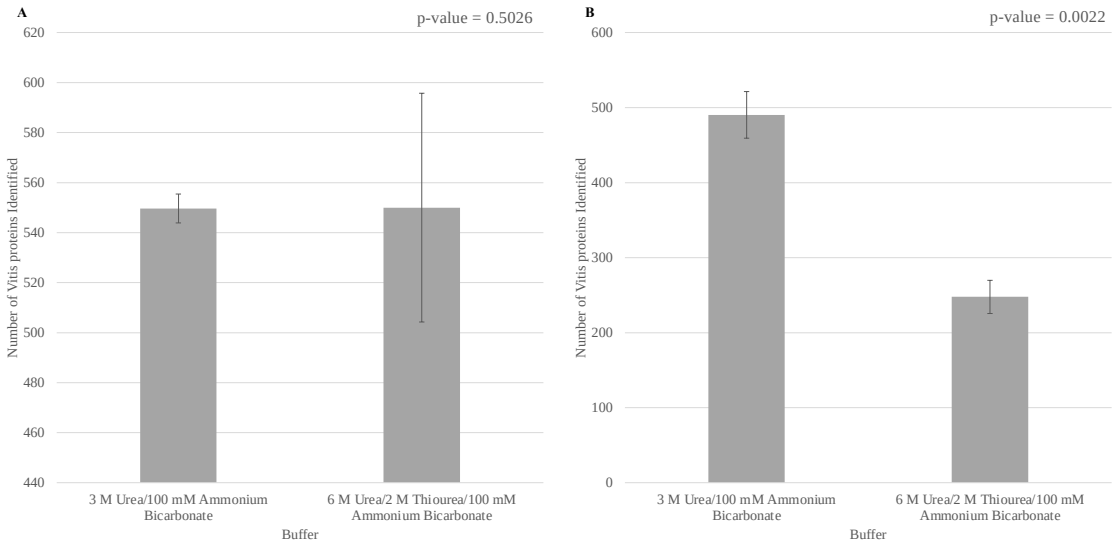

Figure 6: Number of proteins identified in comparison of buffers. The number of identified proteins from two suspension buffers Urea/ammonium bicarbonate and Urea/thiourea/ammonium bicarbonate tested after triplicate precipitations of 2019 cv. Chambourcin acetone/TCA extraction and total BCA protein (BSAeq) quantification before the addition of thiourea (M. R. Smith et al., 2011; P. K. Smith et al., 1985). 30 µg of protein was used to trypsin digest and identify proteins via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020). A. Juice and B. Wine. Differences in means were determined by a one-tailed t-test ($p < 0.05$).

Table 3: Optimized method coefficient of variation. 2019 cv. Chambourcin juice acetone/TCA extraction and total BCA protein quantification (M. R. Smith et al., 2011; P. K. Smith et al., 1985). From quantification, the same volume from each buffer was used to estimate 30 µg of protein for trypsin digestion and identify proteins via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020). The coefficient of variation (CV) is between 3 replicates for method optimization studies (precipitation comparison and buffer optimization) and 5 replicates for optimized method analysis. The number of proteins identified is the number of unique proteins that were identified in Peaks X data search. Total spectrum count (coverage) is the spectrum sum for every protein identified. The average CV for all individual proteins is the CV for every identified protein and then averaging all the individual proteins.

Experiment	**Number proteins Identified CV**	**Total Spectrum Count (Sum) CV**	**Average CV for all individual proteins**
Precipitation Comparison Juice	5%	1%	14%
Water vs 6 M Urea/100 mM Ammonium Bicarbonate Juice	2%	6%	11%
6 M Urea/100 mM Ammonium Bicarbonate vs “”+2 M Thiourea Buffer juice	2%	3%	11%
Analysis with optimized method Juice	**8%**	**13%**	**25%**
6M Urea/100mM Ammonium bicarbonate vs “”+ 2M thiourea buffer wine	11%	15%	13%
Analysis with optimized method wine	**3%**	**5%**	**15%**

Table 4: Final optimized method run in juice. Five replications of 2019 cv. Chambourcin juice acetone/TCA extraction and total BCA protein quantification (M. R. Smith et al., 2011; P. K. Smith et al., 1985). From quantification, the same volume from each buffer was used to estimate 30 µg of protein for trypsin digestion and identify proteins via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020)

Replicate	Juice [protein] ug/mL BSAeq	Total proteins ID	Sum of spectrum count
Precipitation 1	82.8	421	13832
Precipitation 2	75.7	464	15882
Precipitation 3	80.0	451	15411
Precipitation 4	80.6	376	11663
Precipitation 5	86.5	413	12275
Total average	81.1	425.0	13812.6
Total standard deviation	4.0	34.5	1858.9
coefficient of variation	5%	8%	13%

Table 5: Final optimized method run in wine. Five replications of 2019 cv. Chambourcin juice acetone/TCA extraction and total BCA protein quantification (M. R. Smith et al., 2011; P. K. Smith et al., 1985). From quantification, the same volume from each buffer was used to estimate 30 µg of protein for trypsin digestion and identify proteins via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020)

Replicate	Wine [protein] ug/mL BSAeq	Total proteins ID	sum spectrum count
Precipitation 1	25.1	309	6591
Precipitation 2	24.7	313	6449
Precipitation 3	23.3	286	6731
Precipitation 4	22.7	305	7236
Precipitation 5	24.0	328	6943
Total average	24.0	311.8	6790.0
Total standard deviation	0.9	9.7	311.4
coefficient of variation	4%	4%	5%

Table 6: Protein and condensed tannin in various cultivars. Three replications of each cultivar juice were pressed by hand and wine samples were taken after 7 days of maceration and separated from skins. Protein analysis was done by acetone/TCA precipitation and total BCA protein quantification (M. R. Smith et al., 2011; P. K. Smith et al., 1985). Tannin analysis was performed via Adams-Harbertson tannin assay (Harbertson et al., 2002a). Reported numbers are the mean of replicates with standard error mean. Letters within in each analysis (column) designate significant differences between cultivars determined via Tukey-Kramer HSD.

Cultivar	**Juice Protein µg/mL BSAeq**	**Wine Protein µg/mL BSAeq**	**[Condensed tannin] mg/L CE**
2018 Chambourcin (Mt. Vernon, MO)	86.8 ± 3.2^{A}	55.7 ± 1.1^{C}	69.2 ± 4.9^{DE}
2019 Chambourcin (Ste Genevieve, MO)	75.0 ± 2.6^{AB}	98.9 ± 6.2^{B}	58.8 ± 8.5^{E}
2019 Concord (Fredonia, NY)	67.7 ± 4.9^{B}	99.0 ± 6.6^{B}	34.1 ± 13.9^{E}
2019 Vignoles (Genevieve, MO)	42.7 ± 1.0^{C}	46.2 ± 1.7^{C}	847.4 ± 62.3^{C}
2019 Petit Verdot (Fredonia, NY)	10.9 ± 0.8^{D}	26.7 ± 0.5^{D}	1028.9 ± 67.0^{B}
2019 Riesling (Fredonia, NY)	44.6 ± 1.9^{C}	47.5 ± 1.7^{C}	176.5 ± 32.7^{D}
2019 Tannat (Fredonia, NY)	69.7 ± 1.9^{B}	241.1 ± 6.0^{A}	1189.2 ± 35.0^{A}
2019 Syrah (CA)	32.0 ± 5.4^{C}	95.5 ± 2.7^{B}	34.8 ± 3.8^{E}

Figure 7: PCA scores plot. The first and second components in a 2D score plot of PCA with all identified proteins, protein concentration (Juice and wine), and condensed tannin concentration (wine). Three replications of each cultivar juice were pressed by hand and wine samples were taken after 7 days of maceration and separated from skins. Protein analysis was done by acetone/TCA precipitation and total BCA protein quantification (M. R. Smith et al., 2011; P. K. Smith et al., 1985). Tannin analysis was performed via Adams-Harbertson tannin assay (Harbertson et al., 2002a). 30 µg of protein for trypsin digestion and identify proteins via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020). Before analysis, data were filtered to remove variables unlikely to be used in the model using the interquartile range to remove 25% of the variables. Data were normalized by log transformation and auto-scaling (mean-centered divided by the standard deviation of each variable).

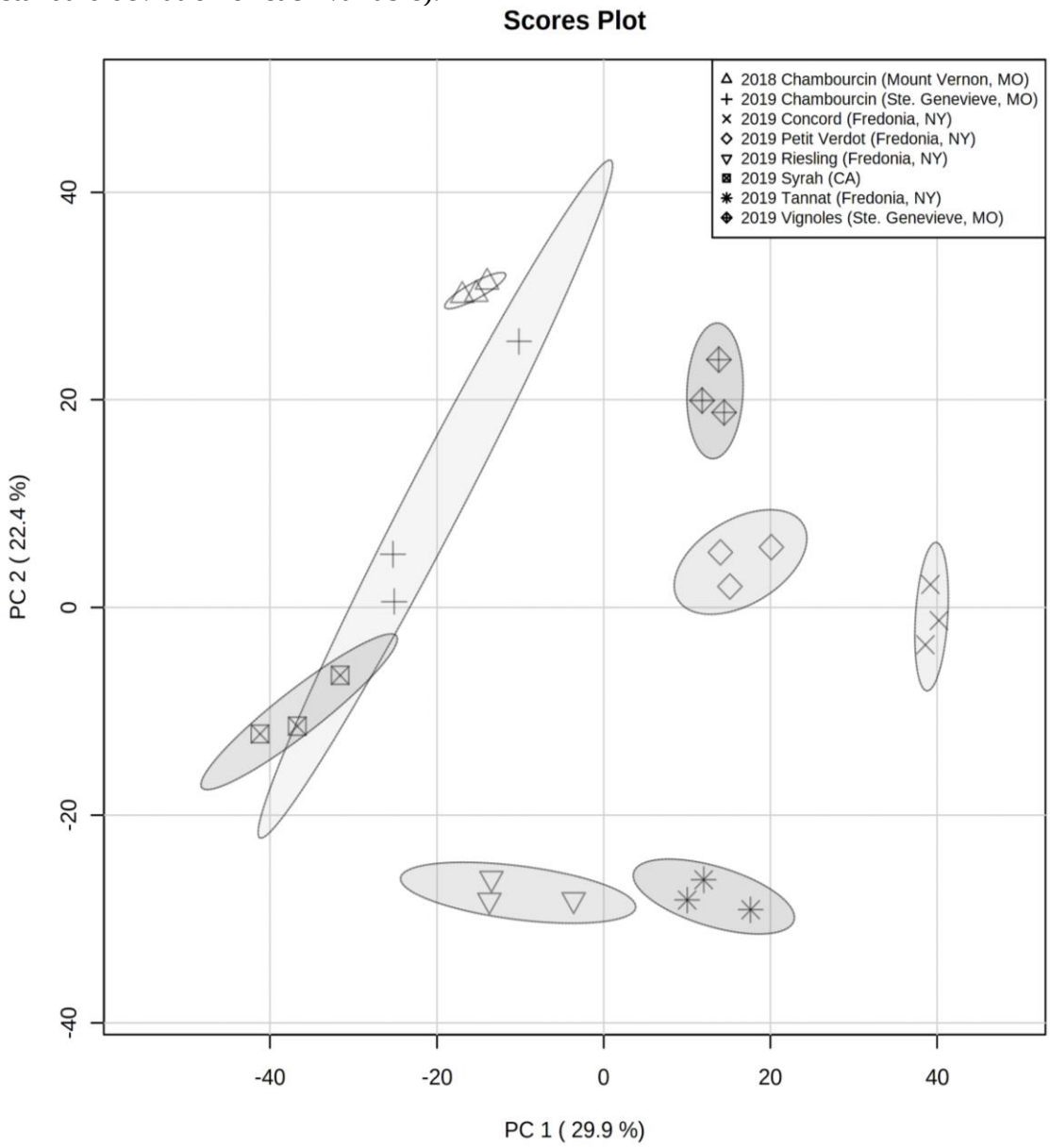

Table 7: PLS prediction model for condensed tannin. Partial least squares model to predict condensed tannin concentration with all identified proteins and protein concentration (Juice and wine), with a total of 3,187 independent variables. The model was trained and run with Leave-One-Out Cross-Validation (LOOCV). Important features were determined with variable importance for the predictive model with the top 15 variables listed. Three replications of each cultivar juice were pressed by hand and wine samples were taken after 7 days of maceration and separated from skins. Protein analysis was done by acetone/TCA precipitation and total BCA protein quantification (M. R. Smith et al., 2011; P. K. Smith et al., 1985). Tannin analysis was performed via Adams-Harbertson tannin assay (Harbertson et al., 2002a). 30 µg of protein for trypsin digestion and identify proteins via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020).

(Sample) accession ID	Description of protein	Cultivar							
		2018 Chambourcin	2019 Chambourcin	2019 Syrah	2019 Riesling	2019 Tannat	2019 Petit Verdot	2019 Concord	2019 Vignoles
Juice Q7XAU6	Class IV chitinase OS	1068	746	502	779	180	315	563	143
Juice O24531	Class IV endochitinase (Fragment) OS	867	641	455	756	168	305	563	142
Juice A0A438DFT5	Endochitinase EP3 OS	857	636	452	752	167	304	555	140
Juice O24530	Class IV endochitinase OS	788	562	448	667	163	293	483	124
Juice F6HAU0	Uncharacterized protein OS	1271	1172	987	435	340	882	348	774
Juice A0A438IRV9	Beta-fructofuranosidase soluble isoenzyme I OS	1144	1045	900	389	313	811	309	704
Wine A0A438D8U6	Oryzain alpha chain	786	738	474	689	79	275	98	237
Juice F6HUG9	Uncharacterized protein OS	714	392	387	412	39	415	97	78
Juice A0A438JJ53	Thaumatin-like protein	611	307	366	417	31	535	71	73
Juice A0A438DZR8	Non-specific lipid-transfer protein	26	87	570	187	14	28	35	27
Juice A3QRB4	Thaumatin-like protein	658	347	314	329	27	394	70	64
Juice Q850K5	Non-specific lipid-transfer protein	28	88	501	148	15	26	45	27
Juice A3QRB5	Thaumatin-like protein	660	357	410	366	35	335	68	79
Juice F6GXX3	Non-specific lipid-transfer protein	76	195	548	68	6	123	17	25
Juice A0A438DX78	Beta-fructofuranosidase	867	641	455	756	168	305	563	142

CHAPTER THREE:
Changes to Wine Proteome when Winemaking Methods are Used to Increase Condensed tannin Content

1. Introduction

Astringency, often described in terms of sensory attributes as 'drying', 'roughing' and 'puckering' is one of the most important mouthfeel characteristics in red wine (Gawel, 1998). Phenolic compounds and in particular condensed tannin s are secondary metabolites in grapes that are the primary compounds in wine sensory astringency. Although there are contradicting reports, tannins may be more bitter and less astringent as molecular weight (degree of polymerization) decreases (McRae & Kennedy, 2011b). Seed and skin condensed tannin fractions in wine-like solutions both have similar bitterness and astringency sensory intensities (even with differences in molecular weight and galloylation percentage) (Brossaud et al., 2001). Condensed tannin s are also important in wine color stability and overall appearance. For example, they can condense with anthocyanins with the help of an aldehyde to form a stable purple pigment (Liu et al., 2013).

Hybrid cultivars have been increasing in production acreage in cool climate regions of the United States that also are associated with high disease pressure (Alex J. Fredrickson et al., 2020). Often these hybrid red cultivars have lower amounts of condensed tannin present in the skins (comparable in the seeds). When vinified they also have very low levels of condensed tannin when compared to *Vitis vinifera* cultivars (Narduzzi et al., 2015). Even when the condensed tannin content in hybrid fruit is comparable to *Vitis vinifera* cultivars, the hybrid wines that are made have lower

concentrations of condensed tannin. This is likely due to poor extraction or retention of condensed tannin possibly due to cell wall material (soluble and insoluble) (Springer & Sacks, 2014). To increase condensed tannin content in hybrid wines, winemakers often use exogenous tannin (often condensed and sometimes hydrolysable) products before and after fermentation (Alex James Fredrickson, 2015; Parker et al., 2007a). Increasing extraction surface area through accentuated cut edges (ACE) which reduces skin particle size with mechanical skin fragmentation has also been used for increasing condensed tannin content in wines, in particular in *Vitis vinifera* cultivars (Sparrow, Holt, et al., 2016).

In recent years protein, in particular, pathogenesis-related (PR) protein in hybrid cultivars juice and recently fermented wine, have been linked to lower levels of condensed tannin in wine (Bindon et al., 2016a; Springer, Sherwood, et al., 2016a). While a good correlation between the presence of these soluble proteins and a lower level of condensed tannin has been demonstrated there is much related to the mechanism and kinetics yet to be understood. PR proteins have been extensively studied for their ability to improve disease resistance and their concentration increases as disease pressure increases (Monteiro et al., 2007). They have mainly been studied in white wines as they are highly soluble and can make it into the final wine product and form a haze when exposed to heat (Girbau et al., 2004a; Van Sluyter et al., 2015). A few of these proteins were recovered when condensed seed tannin was added to recently fermented wines (Springer, Sherwood, et al., 2016a) and identified via SDS-PAGE and LCMS/MS. The recent use of shotgun proteomics to analyze the whole wine proteome has allowed for a more comprehensive analysis of proteins that may be impacting condensed tannin

extraction and/or retention as opposed to analyzing <20 proteins from an SDS-PAGE gel (Fredrickson and Kwasniewski, Chapter One).

In this study, we aimed to assess the role of native grape proteins in condensed tannin retention (native and exogenous) in wine from hybrid grapes. To this end, we made wine from one hybrid cultivar and characterized the proteomes of wines (and lees) with different levels of protein precipitable condensed tannin to help understand if and what proteins or protein groups play a role in condensed tannin retention.

2. Materials and Methods

2.1. Chemical Reagents.

Acetone (99.9% purity), acetonitrile (ACN) (99.8% purity), ethanol (95% purity), glacial acetic acid (99.7% purity), Trichloroacetic acid (TCA) (99% purity), albumin from bovine serum (BSA) (98% purity), ammonium bicarbonate (99% purity), ammonium sulfate (99% purity), (+)-catechin hydrate (96% purity), formic acid (98% purity), iodoacetamide (IAM) (99% purity) sodium chloride (99% purity), sodium dodecyl sulfate (SDS) (98% purity), sodium hydroxide (NaOH) (98% purity), potassium chloride (KCl) (99% purity), triethanolamine (98% purity), Tris-HCl (99% purity), LC-MS grade water (MQ 100 purity), urea (99% purity), and thiourea (99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) (99% purity), iron(III) chloride (98% purity), Coomassie Brilliant Blue G-250 (>95% purity), Hydrochloric acid (37% purity), and phosphoric acid (85% purity) were sourced from Fisher Scientific (Waltham, MA, USA). Sequencing grade modified trypsin was obtained from Promega Corp. (Madison, WI, USA). Potassium metabisulfite (KMBS) was

obtained from MoreFlavor (Pittsburg, CA). Grape derived exogenous condensed tannin was obtained from Enartis USA Inc. (Windsor, CA).

2.2. Experimental design

All winemaking experiments were completely randomized design with three fermentation replications for each treatment. Wines from 2018 and 2019 had the following treatments: standard winemaking procedure (Control); Exogenous condensed tannin addition at crush (Crush +); Exogenous condensed tannin addition at 1-month post-press (1 Month Post Press +); and ACE to must (ACE). The dependent variable was condensed tannin concentration while the independent variables were exogenous condensed tannin additions (to must or at 1-month post-press) and ACE.

Protein was extracted in triplicate for each treatment and quantified with three replications for each extraction. Protein digestion and characterization were done in triplicate for each treatment. Protein concentration, protein spectrum counts, and proteins identified were the dependent variables.

2.3. Relationship of wines with different condensed tannin concentration and protein content and diversity

2.3.1. Winemaking

In 2018, cv. Chambourcin was hand-harvested from Mt. Vernon, MO (37◦4 027.1700N, 93◦52046.7000W, altitude 376 m) on 9/17-18/2018 (Maimaitiyiming et al., 2017). Approximately 45 kg of fruit were stored in a -20°C freezer for later use. On 9/19/2018 fruit was destemmed and crushed into a 450 kg macro bin and mixed by hand. For each treatment, 7.7 kg of fruit was added to 9.5 L PET fermenters. Juice samples were taken from each fermenter but were considered identical. 50 mg/L of sulfur dioxide

(SO_2) in the form of KMBS was added to the juice. On 9/20/2018 1400 mg/L of grape-derived exogenous condensed tannin was added to musts at crush condensed tannin addition treatment (Crush +). Also, using an immersion blender, musts were completely blended as an ACE treatment (ACE) (Sparrow et al., 2016). All musts were inoculated with *Saccharomyces cerevisiae*-GRE (Scott Laboratories Inc., Petaluma, CA) at a rate of 0.25 g/L and hydrated in GoFerm Protect (Scott Laboratories Inc., Petaluma, CA) at a rate of 0.3 g/L. Ferments were punched down twice a day and kept in a temperature-controlled cooler at 18°C. On 10/2/2018, all wines were pressed with a #20 fruit press (9.5 L, 20cm basket press) (MoreFlavor, Pittsburg, CA) and 3.79 L of wine was collected. On 10/9/2018 wines were tested for residual sugar with Clinitest reducing sugar tablets (Bayer Corporation Wippany, NJ) and all had a 0.25-0.5% residual sugar which was considered dry (completely fermented). On 10/10/2018 50 mg/L SO_2 in the form of KMS was added to all wines. On 10/31/2018 all wines were racked off settled lees and on 11/2/2019, 1400 mg/L of exogenous condensed tannin was added to 1-month post-press condensed tannin addition treatment lots (1 Month Post Press +). On 12/18/2018, wines were filtered with Buon Vino Super Jet Wine Filter and a number 1 course (7 microns) filter pad (Buon Vino Manufacturing, Cambridge, ON) to remove residual sediment. Filtration was followed by the addition of 50 mg/L of sulfur dioxide (SO_2) as KMBS. Wines were bottled in 187/375 mL crown cap bottles on 12/19/2018 and stored at 7°C and 55% relative humidity. On 4/4/2019 one bottle of each wine was opened for a 6-month post-press sampling collected into one 50 mL centrifuge tube that was frozen at -80°C for later analysis and one 2.2 ml centrifuge tube for condensed tannin analysis.

In 2019, on 10/4/2019 approximately 7200 kg of cv. Chambourcin from Etlah, Missouri was machine harvested into ½ ton macro bins. Approximately 10 L from each bin was collected and placed into three 60 L totes. A homogenous juice sample from each of the three totes was collected and frozen at -80 °C for later analysis. The 12 ferments (four treatments x three replicates) were randomized with each consisting of 3,056 g of fruit added to a 3.79 L PET container. 50 mg/L of sulfur dioxide (SO_2) as KMBS was added to the juice. Wines were vinified as in 2018 with the following exceptions: upon pressing only 1.89 L of wine was collected. Wines were checked for residual sugar weekly and mixed until 11/1/2019 when samples were all below 0.75% RS by Clinitest (Bayer Corporation Wippany, NJ). We considered the wine "dry" at this stage to reduce the risk of oxidation in small fermentation vessels and then added 50 mg/L of SO_2 in the form of KMS. On 11/2/2019, 1400 mg/L of exogenous condensed tannin was added to wines for the 1-month post-press condensed tannin addition treatment. On 11/18/2019 all wines were racked from settled lees into 950 mL containers until full. On 12/19/2019 50 mg/L SO_2 in the form of KMBS was added to all wines that were then bottled into three, 187 mL crown cap bottles and stored at 18°C in a temperature-controlled room. On 6/9/2020 one bottle of each wine was opened as an 8.5-month post-press sampling that was collected into one 50 mL centrifuge tube, frozen at -80°C for later analysis, and into one 2.2 mL centrifuge tube for condensed tannin analysis.

2.3.2. Tannin analysis

Condensed tannin content was quantified via protein precipitable tannin analysis, known as Adams-Harbertson tannin assay (Harbertson et al., 2002a). Briefly, 0.5 mL of wine was added to a 2.2 mL centrifuge tube with 1 mL of 200 mM glacial acetic acid,

170 mM sodium chloride, and 1 mg/mL bovine serum albumin at pH 4.9. Tubes were inverted 10 times to mix wine and protein solution and incubated at room temperature with gentle agitation for 15 minutes. Samples were then centrifuged for 5 minutes at 20,800 x g. The supernatant was removed and 0.875 mL of a 5% triethanolamine and 5% SDS pH 9.4 buffer was carefully added to the pellet and incubated at room temperature for 10 minutes. Samples were then vortexed vigorously until the pellet was completely dissolved, then incubated at room temperature for an additional ten minutes. The entire 0.875 mL sample was quantitatively transferred to a 1.5 mL microcuvette and the background absorbance was read at 510 nanometers. 0.125 mL of 10 mM of iron(III) chloride in 0.01 N HCl was added to the sample and inverted to mix and then incubated at room temperature for 10 minutes and the final absorbance was read at 510 nm. Using an external standard curve of catechin, the condensed tannin concentration was calculated and reported in mg/L catechin equivalents (mg/L CE).

2.3.3. SDS-PAGE Gel

To determine if the two exogenous condensed tannin products used in this study contain protein, one condensed tannin product (seed-derived and one hydrolysable tannin product (oak derived) were analyzed by SDS-PAGE. Samples were resuspended at 50mg/mL in SDS buffer (1X Laemmli buffer: 60 mM Tris pH 6.8, 10% glycerol, 100 mM DTT, 2% SDS). Following heating at 65 °C for 25 min, samples were loaded (25 µL) onto a 12% T Acrylamide mini-gel (7 x 7 cm) with a 2 cm 4% acrylamide stacking layer, run for 3 hours at 25 mAmp (constant current), and then washed 3 times with water. The gel was stained overnight with Colloidal Coomassie Brilliant Blue (20% ethanol, 1.6% phosphoric acid, 8% ammonium sulfate, 0.08% Coomassie Brilliant Blue

G-250). The gel was then destained with water and imaged using a UMAX PowerLook flatbed scanner (UMAX Technologies, Inc., Dallas, TX).

2.3.4. Protein precipitation and quantification

Protein content was quantified via acetone/TCA precipitation and BCA quantification (Fredrickson and Kwasniewski, Chapter one). Samples were processed by centrifuging at 12000 x g for 10 minutes at 4 °C and then vacuum-filtered using #2 filter paper. Samples (5 mL of juice or 10 mL) were then transferred into 50 mL centrifuge tubes. Four times the volume of 5% TCA in acetone pre-chilled in ice, was added to each centrifuge tube (20 mL for juice and 40 mL for wine). Tubes were carefully inverted 10 times to mix then incubated at -20°C overnight (max 12 hours). The next day samples were centrifuged at 12000 x g for 30 minutes at 4 °C. The supernatant was removed, and 0.5 mL of 80% acetone was added to the tube. Tubes with pellets were sonicated in ice water until dispersed, then the pellet was transferred to a new 2.2 mL centrifuge tube. The 50 mL centrifuge tube was rinsed with 0.5 mL 80% acetone two more times to transfer any remaining protein to the 2.2 mL tube. The new tube with the pellet was vortexed and sonicated and then incubated at -20°C for ten minutes. The 2.2 mL tube was centrifuged at 20,800 x g for 10 minutes at 4°C then the supernatant was removed and discarded. Protein pellets were then washed 2x with 1 mL of 80% acetone. After the last supernatant was removed, the pellet was air-dried for 15-30 minutes to remove any excess acetone. 300 µL of 6M Urea and 100 mM ammonium bicarbonate was added to the juice pellets and 200 µL to the wine pellets. The 2.2 mL tubes were vortexed and sonicated to resuspend the pellet. After pellets were dissolved, samples were centrifuged at 16,000 x g for 10 min at 4 °C to remove any nonprotein insoluble residue. Before quantification,

samples were diluted in LC-MS grade water to achieve concentrations that were within the protein assay standard curve and to avoid interference with Urea (max 3 M). A 1:4 dilution (50 µL of the sample, 150 µL water) was made for juice samples and 1:3 dilution (30 µL of the sample, 60 µL of water) was made for wine samples. Diluted protein samples were then quantified using the BCA protein assay and reported in µg/mL BSA equivalents (BSAeq) (P. K. Smith et al., 1985).

2.3.5. Digestion and cleanup

Protein concentration was normalized based on the above quantification for all samples by adding 30 µg of protein to a new test tube and diluting with LC-MS grade water to 100 µL. The normalized protein was digested and individual proteins were identified using a method from Cui et al., (2020), with slight modifications. Briefly, 5 µL of 100 mM ammonium bicarbonate and 200 mM DTT (reducing agent) was added and mixed by gentle vortex and reduced for 1 hour at 21°C. Then 20 µL of 100 mM ammonium bicarbonate and 200 mM iodoacetamide (alkylating reagent) was added and mixed by gentle vortex and alkylated for 45 minutes at 21°C. 20 µL of the reducing agent was added to consume any unreacted iodoacetamide and mixed by gentle vortex and allowed the reaction to occur at 21°C for 20 minutes. Urea concentration was diluted below 0.6 M, for trypsin to retain its activity. Trypsin was added at a protease-to-substrate ratio of 1-to-50. Samples were mixed by gentle vortex and the digestion occurred overnight at 37 °C. The reaction was stopped by adding concentrated formic acid (FA) to 1% v/v. Purification of trypsin digest was needed before analysis. Pierce C18-100 µL Tips (Thermo Scientific, Rockford, lL) were prepared by wetting with 100% acetonitrile, washing with 70% acetonitrile/1% formic acid, then equilibrating with 1%

formic acid. To bind peptides, a peptide sample was pipetted into and out of the C18 tip at least 20 times and expelled on the last cycle without introducing air into the tip. Peptides were washed with 1% formic acid and the peptides were expelled by cycling 50 µL of 70% acetonitrile/1% formic acid into and out of the tip at least 20 times and diluted with 50 µL of water. Samples were then lyophilized before analysis.

2.3.6. Chromatography and Mass Spectrometry

For UPLC analysis, samples were resuspended in 30 µL of solvent (5% acetonitrile, 0.1% formic acid) to approximately 1 µg/µL of the initial protein concentration. Peptides were transferred to vials and placed in a refrigerated autosampler (7 °C). Samples, 2 µL, were injected directly onto a 20cm long x 75µm inner diameter pulled-needle analytical column packed with Waters BEH-C18, 1.7 µm reversed phase resin. Peptides were separated via nano ultra-performance liquid chromatography coupled with electrospray ionization-trapped ion mobility spectrometry-time of flight (UPLC-ES-TIMS-TOF) and eluted from the analytical column with a gradient of acetonitrile at 300nL/min. A Bruker nanoElute system (Bruker Daltonics, Billerica, Massachusetts) was attached to a Bruker timsTOF-PRO mass spectrometer via a Bruker CaptiveSpray source (Beck et al., 2015). LC gradient conditions: Initial conditions were 2% B (A: 0.1% formic acid in water, B: 99.9% acetonitrile, 0.1% formic acid), followed by 10 min ramp to 17% B. Then 17-25% B over 25 min, 25-37% B over 25 min, 37-80% B over 10 min, hold at 80% B for 15 min, ramp back (2min) and hold (3min) at initial conditions. The total run time was 90min.

MS data were collected in positive-ion data-dependent parallel accumulation serial fragmentation (PASEF) mode over an m/z range of 100 to 1700. PASEF and TIMS

One MS and ten PASEF frames were acquired per cycle of 1.27sec (~1MS and 120 MS/MS) (Meier et al., 2015). Target MS intensity for MS was set at 20,000 counts/sec with a minimum threshold of 250 counts/s. The intensity repetition table (default values) was set to On. A charge-state-based rolling collision energy table was used from 76-123% of 42.0 eV. An active exclusion/reconsider precursor method with release after 0.4min was used. If the precursor (within mass width error of 0.015 m/z) was >4X signal intensity in subsequent scans, a second MSMS spectrum was collected. Isolation width was set to 2 m/z (<700m/z) or 3 (800-1500 m/z).

2.3.7. Protein Identification

The acquired data were submitted to the PEAKS X (Bioinformatics Solutions Inc., Waterloo, ON, Canada) search engine for protein identifications. An NCBI-*Vitis vinifera* database (182,373 entries; last update 3/5/2019) and/or UniProt *Vitis vinifera*/yeast protein was searched. Data were searched with trypsin as the enzyme, 2 missed cleavages allowed; carbamidomethyl cysteine as a fixed modification; oxidized methionine and deamidation of N/Q as variable mods; 20ppm mass tolerance on precursor ions, 0.1Da on fragment ions. FDR estimation enabled (a reversed decoy database is created and searched simultaneously). Search results files were first filtered for 0.1% FDR (peptide false discovery rate) and >1 unique peptide per protein and export from PEAKS.

2.4. Impact of exogenous condensed tannin rate on protein content and proteomics

2.4.1. Winemaking

Previously frozen 2018 Chambourcin (Mt. Vernon, MO) whole berries (3 L) were thawed overnight in a 4 °C fridge. A total of fifteen ferments were made by weighing 50

g ± 1g of fruit out and homogenizing in a zippered plastic bag and homogenizer (Stomacher Lab-blender 400, Seward, London, UK). Samples were quantitively transferred to 50 mL centrifuge tubes which was 45 mL of crushed fruit (10% headspace for fermentation). Ferments were allowed to warm up to room temperature, then musts were inoculated with 1 g/L of Lalvin ICV Yeast GRE (Scott Laboratories Inc., Petaluma, CA) and 0.3 g/L of DAP (Scott Laboratories Inc., Petaluma, CA). Caps were placed on tubes just less than tight (allowing for CO_2 to escape as pressure increased) with fermentation taking place in a 28°C water bath. Simulated "punch-downs" occurred twice per day, by inverting the tubes 5 times to mix the solids with the liquid. After three days of maceration, wines were pressed by hand with two layers of cheesecloth until at least 25 mL of juice/wine was collected. Once ferments had 25 mL, exogenous condensed tannin addition treatments were completed. The five treatments (with three fermentation replicates) were, control (no condensed tannin add), 800 mg/L, 1600 mg/L, 3200 mg/L, and 6400 mg/L exogenous condensed tannin additions. Treatments were allowed to continue fermenting in a 28 °C water bath for four days, a total of 7 days of fermentation. The wine was separated from gross lees by centrifugation at 10,000 x G for 10 minutes at 4 °C. The wine was carefully decanted and filtered with a Whatman #2 filter paper. 10 mL sample was transferred to a new 50 mL centrifuge tube for protein analysis and 2 mL sample was transferred to a new 2.2 mL centrifuge tube for condensed tannin analysis and froze at -80 °C. The sediment (lees) from the 25mL after fermentation was saved for protein analysis and froze at -80 °C.

For the juice and wines, the same acetone/TCA protein precipitation method was used as before, but wines were resuspended in 300 µL of buffer because they were

recently fermented wines, we expected higher amounts of total protein. Since the lees already had insoluble protein, the method was modified. First, 0.5 mL of 80% acetone was added and vortexed for 30 seconds to suspend the lees and transferring to a new 2.2 mL centrifuge tube. Repeat 80% acetone wash of 50 mL tube two more times for a total of 1.5 mL which came to 2 mL with solids. The pellets were vortexed and sonicated until broken up, then incubated at -20°C for ten minutes. The 80% acetone wash was repeated three additional times (for a total of four 80% acetone washes). After the last supernatant was removed, the pellet was dried for 30 minutes to remove any excess acetone. 300 µL of 6M Urea and 100 mM ammonium bicarbonate was added to pellets. The pellets were suspended/dissolved by sitting/vortexing/sonicating the 2.2 mL tubes. After pellets were dissolved, samples were centrifuged at 16,000 x g for 10 minutes at 4 °C to remove and nonprotein insoluble residue. Before quantification, samples were diluted in LC-MS grade water (Sigma-Aldrich, Inc., St. Louis, MO) to fit in protein assay's standard curve and to avoid interference with urea (max 3M), 1:20 dilution (50 µL of the sample, 950 µL water) was made for samples through 3200 mg/L of exogenous condensed tannin added and 1:40 dilution (50µL of the sample, 1950 µL water) for 6400 mg/L of exogenous condensed tannin added. Quantification, digestion, and identification follow the sampling procedure as before.

Condensed tannin analysis was done via Adams-Harbertson tannin assay (Harbertson et al., 2002a) as stated above. Since the exogenous condensed tannin additions were made on material mass and not protein precipitable tannin content, we calculated exogenous condensed tannin retention rates based on the condensed tannin content of the product. The exogenous condensed tannin product used contained 26%

protein precipitable tannin, so when calculated condensed tannin retention we used the formula: (condensed tannin content of treatment)-(condensed tannin content of control)/(exogenous condensed tannin addition * 26%).

2.5. Data Processing and Statistical analysis

Data were collected and stored using Microsoft Excel (Redmond, WA). Mean comparisons of protein and condensed tannin concentrations were performed using Tukey-Kramer HSD (multiple mean comparison) or one-tailed t-test ($p < 0.05$) (one mean comparison) on JMP statistical software (SAS Institute, Inc.). Principal component analysis (discriminate analysis) and partial least squares discriminant analysis was performed using the MetaboAnalyst statistical analysis website which uses R (Xia & Wishart, 2011). Before the analysis above, data were filtered to remove variables unlikely to be used in the model using the interquartile range to remove 25% of the variables. Data were normalized by log transformation and auto-scaling (mean-centered divided by the standard deviation of each variable) to allow for lower abundance proteins to have a meaningful impact in score plots as well as to create more normally distributed data. Prediction model of partial least squares regression (PLC-R) with Leave-One-Out Cross-Validation (LOOCV) used condensed tannin concentration as the dependent variable and spectrum counts of all identified individual proteins from juice and wine along with total protein in wines as the independent variables (2322 predictors). Variable Importance in Projection (VIP) was used to determine which variables had the most influence on the model. The prediction used R studio statistical package (Boston, MA).

3. Results and Discussion

3.1. Relationship between wines with different condensed tannin concentration and protein content and diversity

3.1.1. Winemaking

Except for ACE from 2019, the condensed tannin content for all treatments was higher than the control for both years with the control wines having very little condensed tannin with a concentration of 46.5 mg/L CE in 2018 and 35.8 mg/L CE in 2019 (Figure 1). ACE treatment in 2019 had a fermentation replicate that was an outliner in range of the control, which if removed brings ACE higher than the control. In 2018 condensed tannin content in the ACE treatment was 3-fold higher than the control and was similar to the Crush Condensed tannin + treatment (142 mg/L CE) at 145 mg/L CE. Although ACE increased condensed tannin content in Pinot noir 6-fold compared with traditional winemaking methods, we were uncertain whether similar results could be achieved with hybrid cultivars because of previous reports of cell wall material and pathogenesis-related proteins being linked to poor condensed tannin retention (Bindon et al., 2016a; Sparrow, Smart, et al., 2016; Springer, Sherwood, et al., 2016a). ACE improves extraction, but not to the levels of *Vitis vinifera* cultivars, as there is still a poor retention problem that ACE did not overcome. The Crush Condensed tannin + in 2018 only retained 30% of the condensed tannin that was added. This is probably not an economically viable option if a winemaker is looking to improve condensed tannin content, though other benefits have been noted with early condensed tannin addition (Canuti et al., 2012; Scollary et al., 2012). This retention rate for exogenous condensed tannin additions to must is on par with other studies that observed retention rates below 30% for low-condensed tannin red

hybrids (Alex J. Fredrickson et al., 2020). The 1 Month Post Press Condensed tannin + had the highest final wine condensed tannin content with retention rates above 100%. This indicates polymerization may be increasing the condensed tannin content above what was added and/or facilitating retention of endogenous condensed tannin (Moreno-arribas & Polo, 2009). It could be due to the change in matrix which results in better solubility or decreased interactions. The higher retention rate, during later addition, was similar to previous studies examining the timing of exogenous condensed tannin addition on retention (Alex James Fredrickson, 2015).

However, Jeffries, (2018) found that adding exogenous condensed tannin (oak derived) at crush resulted in condensed tannin content ten-fold higher than the control. Jeffries, (2018) used a spectrophotometric method that measured absorbance in wine directly as opposed to measuring protein precipitable, iron reactive condensed tannin which we did in this study. It is important to take methods into account as even with methods that precipitate condensed tannin can have significant differences between assays (Mercurio & Smith, 2008). Although large increases when exogenous condensed tannin was added at crush were observed in previous studies the differences in methods make it difficult to confirm or refute the results.

In 2019 similar trends were observed with the exogenous condensed tannin addition treatments, with the crush condensed tannin addition 40 mg/L CE higher and the late exogenous condensed tannin addition 80 mg/L CE lower (Figure 1) than in 2018. The ACE treatment was over 50 mg/L CE lower than in 2018, partially due to the outlier and to lower extraction with traditional winemaking methods (control).

3.1.2. Total Protein

In 2018 the control along with ACE treatment had the lowest amount of protein with 20.4 µg/mL BSAeq (21.7 µg/mL BSAeq in ACE) and the 1 Month Post Press Condensed tannin + had the most protein at 26.0 µg/mL BSAeq (Figure 2). The 2019 wines had slightly lower protein concentrations between 15 and 19 µg/mL BSAeq (Figure 2). Although the methods for precipitating and quantifying protein varied widely, our values were in the range of those reported in previous studies (Marchal et al., 1997; M. R. Smith et al., 2011; Vincenzi et al., 2005). Surprisingly, the 2018 wines with higher amounts of condensed tannin also had higher amounts of protein with both exogenous condensed tannin addition treatments having higher protein contents than the control. Indicating that in these wines total final protein content would be a poor predictor of condensed tannin content and that not all proteins present in wine precipitate condensed tannins (even condensed tannin species quantified via a protein precipitation-based assay). In 2019, there were no statistically significant differences in protein content among the treatments, but the exogenous condensed tannin addition treatments still followed trends similar to 2018 with protein in exogenous condensed tannin addition treatments slightly higher than control and ACE.

When examining the linear relationship between total protein and condensed tannin concentration there is an R^2 of 0.2146 and a p-value of 0.0226. This was surprising as one may expect lower amounts of total protein to be an indicator of increased levels of condensed tannin, as condensed tannin is known to precipitate protein (Harborne, 1993). Also, the majority of deposits found in bottled red wine are composed of phenolics (condensed tannin and anthocyanins) and protein (E. J. Waters et al., 1994). Alternatively, there may be significant differences in the types of proteins and it is known

that protein size, amino acid composition, and pI can impact protein precipitation (Harborne, 1993). In another wine-protein area of interest, haze formation, total protein is not an indicator of haze formation (Moreno-arribas & Polo, 2009). This study indicates that in cv. Chambourcin, total protein is not a good indicator of final condensed tannin concentration and for an understating of condensed tannin protein interactions, we must look to understanding individual proteins rather than pooling them.

3.1.3. Proteomics

The only treatment that had a lower number of proteins identified (>1 spectrum count) than the control in 2019, as identified UPLC-ES-TIMS-TOF was 1 Month Post Press Condensed tannin + (Figure 4). It doesn't seem like there are any obvious trends, except that generally, the wines with higher amounts of condensed tannin, seemed to have higher amounts of total protein, but when the protein concentration was normalized for proteomic analysis the diversity of the protein decreased. There is a poor negative linear relationship of proteins identified and condensed tannin concentration with an R^2 of 0.3608 (p-value 0.0019), however, if the outlier from 2018 ACE is removed the R^2 improved to 0.6959, indicating that there could be value in redoing this study with greater replication. Protein identification with the PEAKS database has default mass error reduction and false discovery filters, that allow proteins with a spectrum count of 1 or greater to be considered real. We also evaluated the data using extra filtering of the data that can be done to remove low-abundance proteins which will likely have a lower impact on the proteome (Yang et al., 2020). Increasing the spectrum count threshold to >10 reduces the number of identified proteins on average by 49% (Figure 5) Using this additional stringency improves the correlation between the number of identified proteins

and condensed tannin concentration (Figure 1) with a negative linear relationship and R^2 of 0.7293 (p-value of 0.0001). The variability in proteins with low spectrum counts is high which likely results in a reduced R^2 correlation when including proteins with spectrum counts between 1-9. This additional filtering reduced the 2018 ACE treatment coefficient of variation for the number of proteins identified from 37% to 1%. The 1 Month Post Press Condensed tannin + wines had on average of 50% fewer proteins identified than the control, indicating that a large number of proteins are unidentified with the increase in condensed tannin content.

The total spectrum counts as the treatments in 2018 are the inverse of condensed tannin concentration (Figure 1 and 6) with the control having the highest total spectrum count, then ACE and Crush Condensed tannin + in the middle, and 1 Month Post Press Condensed tannin + having the lowest total spectrum count. The total spectrum count does not indicate that there is more or less protein as each protein gives different ion intensities resulting in a different spectrum (Figure 6). It does, however, indicate that the proteome in the wine is changing as protein precipitable condensed tannin concentration increases (Choi et al., 2008). The differences in proteomes are further evident with the partial least squares-discriminate analysis, which in 2018 had clear separation of the treatments from the components one and three (explaining 62.1% of the variance) except for the ACE which overlapped with the control and Crush Condensed tannin + (Figure 7). In 2019, all four treatments are separated with 47.9% of the variance explained (Figure 7). The 1 Month Post Press Condensed tannin + exhibited the greatest separation from the control in both years. This treatment had over 2-fold more condensed tannin than the next

highest sample and almost 10-fold more than the control indicating that increasing the condensed tannin content in the wine had an impact on proteome (Figure 7).

The optimal PLC-R prediction model for 2018 had three components, an R^2 of 0.8982, and a Mean Absolute Error (MAE) Of 44.5. In 2019 the same predictive model with two components resulted in an R^2 of 0.5300 and an MAE of 83.2. Given the high number of environmental biotic (fungi, bacteria, viruses, fungal cell wall, and oligosaccharides) and abiotic (ethylene, salicylic acid, ozone, UV light) factors that can influence PR protein synthesis it is not surprising that this predictive model exhibits year to year variability (Punja & Zhang, 1993). Several genes related to downy mildew (*Plasmopara viticola*) resistance are activated in response to stress which results in more protein synthesis (Toffolatti et al., 2018). Although abiotic and biotic stresses may not change the total protein content, the diversity, and content of PR proteins changes which may have a larger impact on predicting condensed tannin content. An R^2 of 0.5300 and 0.8982 makes this model a possible method for condensed tannin prediction. Combining the two years into the model resulted in an R^2 of 0.6493 and an MAE of 80.5.

The relationship between the spectrum count of the whole proteome and condensed tannin concentration of wines revealed a negative linear correlation with an R^2 of 0.8031 (p-value <0.0001) and a negative logarithmic relationship with an R^2 of 0.8531 (p-value <0.0001) (Table 1). To ensure that the former relationship wasn't due to dilution when 30 µg of protein was taken from each precipitation for digestion, each sample and individual spectrum counts were standardized to the volume of the buffer that was added. In this case, the negative linear relationship decreased, but the trend stayed the same with

an R-squared of 0.6502. Although total protein did not have a relationship to condensed tannin concentration in wine, proteome composition did.

Examination of specific proteins and their VIP scores for the PLS-R predictive model revealed that the variable with the highest score was a presumed vacuolar invertase (accession number: F6HAU0) (Table 1). This is a very abundant glycoprotein found in grapes that is an important enzyme in ripening as it hydrolyzes sucrose into glucose and fructose (Hovasse et al., 2016a). The spectrum counts for this protein were highest in the control at 329 and lowest in 1 Month Post Press + treatment at 16.8. Although the protein is still detected in the higher condensed tannin wines, there is a relationship between condensed tannin concentration and the spectral counts of this protein with a logarithmic trendline of 0.8531 and a p-value less <0.0001. The second highest (and the 9th highest) VIP score protein is a Beta-fructofuranosidase which also functions at cleaving sugar (Schomburg & Salzmann, 1991). This is a new class of condensed tannin retention related protein as recently, condensed tannin retention issues have been linked to PR proteins and these proteins are associated with metabolism and not PR functions (Springer, Sherwood, et al., 2016a). Of the top ten proteins in the model, five of them are pathogenesis-related proteins, four Thaumatin-like, and one Osmotin-like protein. Neither chitinase nor *Vitis* endo-1,3-β-glucosidase, both known to be PR proteins (Monteiro et al., 2003, 2007; Springer, Sherwood, et al., 2016a) were detected. There were many chitinases with high spectrum counts in the juice samples, so they may have some influence on condensed tannin concentration during the winemaking process, but they don't seem to be a driving force for condensed tannin differences in aged wines. One interesting trend is that all 8 of the *Vitis* proteins detected followed a

strong logarithmic relationship with condensed tannin concentration (greater than 0.8000 R^2) while the two yeast proteins had much lower R^2 values.

One yeast protein, Endo-1 3(4)-beta-glucanase was in the top ten VIP proteins for the PLS-R predictive model for condensed tannin retention and is unrelated to the *Vitis* PR glucosidases (Donzelli et al., 2001). Yeast cells and cell walls can interact and form aggregates with condensed tannin (J Mekoue Nguela et al., 2014, 2016; Julie Mekoue Nguela et al., 2015). Although individual proteins have not been identified for their ability to interact with condensed tannin, it seems likely that yeast protein plays a role in condensed tannin retention.

3.2. Impact of exogenous condensed tannin on protein content and proteome composition

3.2.1. Condensed tannin and Protein

With the treatments, there was a linear correlation (R^2 of 0.9992) condensed tannin content with addition rates (Figure 9). This is consistent with previous studies investigating the rate of exogenous condensed tannin addition on its retention in fermented wine (Alex J. Fredrickson et al., 2020; Harbertson et al., 2012). All the retention rates are above 100% which could be a result of polymerization of monomeric or oligomeric phenolics (Es-Safi et al., 1999; Parker et al., 2007b; Peleg et al., 1999). The lowest retention rate was 800 mg/L addition at 123% while the 6400 mg/L addition had a retention rate of 145% and there was an R^2 of 0.6589 when correlated to dosage (addition rates). Total protein in the wine follows a similar trend in our previous experiment with high exogenous condensed tannin additions having slightly more protein (Figure 8). Total protein in wine has no linear relationship to condensed tannin concentration with an R^2 of

0.1609 (p-value 0.1384). Similar to our previous experiment there seems to be no indication that total protein relates to condensed tannin content.

The lees, on the other hand, give a different story with increasing amounts of protein as condensed tannin concentration increases. In contrast to wine, there is a strong positive linear relationship between lees protein concentration and wine condensed tannin concentration with an R^2 of 0.8543 (p-value <0.0001). It seems that there are a significant number of proteins in the lees that are not accounted for in the Control when compared to the 6400 mg/L addition (Figure 8). Previous studies have shown that there are higher concentrations of protein in cell wall material (100-131.1 mg/g in skins and 73.1-143.8 mg/g in the pulp) than in juice or wine (Ortega-Regules et al., 2008). We also know that a portion of the cell wall material is soluble, including protein that can limit the extraction and/or retention of condensed tannins (Bindon et al., 2010). In this study, we attempted to limit the impact of insoluble cell wall material to understand the impact soluble proteins in juice and wine have on condensed tannin retention by removing skins and pulp before adding condensed tannin. Therefore, the increase in lees protein being due to extra insoluble cell wall material is unlikely. Another possibility is that exogenous condensed tannin products are often grape-derived and since they are not pure condensed tannin or phenolics, there may be protein present in these products that are measurable in lees. This could explain why protein content increases slightly when exogenous condensed tannin is added. Although not measured directly, a previous study used Fourier transform mid-infrared spectroscopy (FTIR) to compare the chemical composition of different exogenous condensed tannin products and found skin-derived products to be chemically more diverse than seed-derived products in a principal component analysis (PCA) of

FTIR spectra of dried exogenous condensed tannin products with significant reference compounds (i.e. purified seed and skin condensed tannin, monomeric, and anthocyanin compounds) (Li et al., 2018). Another study found that some non-grape-derived exogenous condensed tannin products contain up to 10.5 mg/L BSAeq protein when measured with the Bradford Dye method (Marchal et al., 1997). However, when analyzing two condensed tannin products for protein, we found no protein in either condensed tannin product (Figure 3), so it seems unlikely that exogenous condensed tannin additions contribute to protein content in wine or lees. Studies have shown that protein-condensed tannin complexes do no always precipitate and remain soluble (Harborne, 1993; McRae & Kennedy, 2011a). It could also be possible that at lower condensed tannin concentration there are soluble protein-condensed tannin complexes that are not quantified with protein precipitable condensed tannin quantification method used in this study, but as exogenous condensed tannin content increases these complexes become less soluble and eventually become insoluble.

3.2.2. Proteomics

After normalizing proteomics data, then combining all features in a partial least square discriminate analysis (PLS-D), 64.4% of the variance could be explained in the first two components of the wine samples (Figure 10). The five treatments have good separation with some overlap between the four condensed tannin addition treatments. The control is the one treatment that doesn't overlap at all, although this difference seems to mainly be from the first component which only explains 7.1% of the variance. The results indicate that exogenous condensed tannin addition, regardless of rate, has an impact on the control wine proteome and as proteins were not found in extracts from the added

product these changes are not due directly to the addition of exogenous proteins. The majority of variance (57.3%) is in the second component which mainly affected the 6400 mg/L condensed tannin addition and one of the control fermentations. The lees first two components explained 73.6% of the variance and the five treatments are separated by an even greater distance with only a small amount of overlap between the three higher addition rates. It seems that there is an even larger shift in the proteome of the lees or insoluble protein after the addition of exogenous condensed tannin additions are made.

The proteomics data indicate that in wine there is a general decrease in spectrum count and the number of proteins identified as exogenous condensed tannin addition rate increases. This was expected given the results from the previous experiment. The negative linear relationship between condensed tannin concentration and the sum of spectrum count had an R^2 of 0.4740 (p-value 0.0045) while condensed tannin concentration and the number of proteins identified that had >10 spectrum count resulted in an R^2 of 0.4660 (p-value 0.0050). Although a correlation exists, a large amount of variance is not explained. The unexplained variance could be from specific protein response to condensed tannin (reactivity not predictive) or replicate variability. When replicates were averaged, the negative linear relationship had an R^2 above 0.8 for both spectrum count and proteins identified.

For the lees, we were expecting the inverse of what was observed in the wines, but there was a large decrease in the number of proteins identified (Table 2). The negative relationship between condensed tannin concentration and lees protein spectrum count had an R^2 of 0.6800 (p-value of 0.0002). The R^2 for the negative linear relationship between condensed tannin concentration and the number of proteins identified with a

spectrum count >10 was 0.6763 (p-value 0.0002). The lees samples have a stronger negative correlation between condensed tannin concentration and proteomics data when compared to the wine proteomics data.

Like the previous study, a prediction model with PLS-R was developed using condensed tannin concentration with protein and proteomics data (total protein and individual protein spectrum counts) with wine and/or lees as the predictive variables. This model had 4085 predictors for wine and lees alone and, then 6128 predictors when combined. The prediction model for the wines using two components had an R^2 of 0.4876 and an MAE of 510. In comparison, the lees proteomics prediction of the wine condensed tannin concentration model had a higher R^2 of 0.6010 and an MAE of 425 when compared to the wine proteomics. When combining the wine and lees in the prediction model, the R^2 increased to 0.7304 and MAE 338. The proteomics data from both wine and lees seems to be a more powerful prediction model than with either dataset alone.

Using the VIP scores to determine the highest leverage variables in the PLS-R prediction model for wine condensed tannin concentrations we found that there is a mix of wine (four) and lees (six) proteins that impacting the model the most (Table 3). For the wine, the protein with the highest score was once again the protein with the accession number F6HAU0 which is predicted to be a vacuolar invertase (Carpentieri et al., 2019). In the lees, protein F6HAU0 is present but is at a higher concentration in the control (26 in control and 10 in 6400 mg/L addition). This could indicate that this protein is not fully accounted for in the lees. The low level of F6HAU0 in the lees could be that there was more total protein in the lees when compared to the wine (6-fold more in the control and 14-fold more in 6400 mg/L condensed tannin addition treatment). When standardized to

30 µg less, fewer of the low concentration, wine-soluble proteins may have been present. One of the other wine proteins with a high VIP score was A0A438DX78, another invertase. Invertases are highly *N*-glycosylated proteins, where the glycan is attached to a nitrogen, often associated with the amide group of an amino acid). These glycoproteins in juice and wine have been shown to impact many sensory properties including organoleptic and foaming properties and can also increase the solubility of other proteins (Hovasse et al., 2016b). If they interact to form complexes or precipitate with phenolics/condensed tannin this could be an important protein and group of proteins that are highly soluble and can negatively impact condensed tannin concentration. Invertases are also considered PR proteins, with a cascading effect, where invertase is induced by sugars (from the plant) which can further increase sugar concentrations and which then can, in turn, induces defense genes (including PR genes) (Kulshrestha et al., 2013).

The other two wine proteins detected were type IV (endo)chitinases, which have been extensively studied as PR proteins. They can function as a defense mechanism against insects and fungi (cell walls) for plants with the ability to hydrolyze chitin and oligomers of *N*-acetylglucosamine (Vincenzi et al., 2014). These PR proteins had the highest linear R-squared value in relation to condensed tannin concentration. Chitinases had the highest VIP scores (for PR proteins) in this experiment while Thaumatin-like PR proteins were more prevalent in the relationship between wines with different condensed tannin concentrations and protein content and diversity study (Table 1). This could be an indication that chitinases and thaumatin-like PR proteins both have an impact on condensed tannin extraction/retention. The former may be less stable in wine-like conditions as this experiment investigated wine immediately after fermentation while the

relationship between wines with different condensed tannin concentrations and protein content and diversity study examined wines 6-8.5 months after pressing.

In the lees all but one of the proteins with the highest VIP scores originated from yeast. The function of the one *Vitis* protein detected is unknown and there is currently insufficient information to infer if it is related to any PR proteins. Four of the five lees proteins detected were all part of glycolysis (Roach et al., 2018). These proteins were predictive of condensed tannin in the wine with negative relationships to condensed tannin concentration (table 3) as well as *Vitis* related proteins. This indicates that proteins internally and externally from the grape may play a role in condensed tannin retention. This confirms and expands on results from Springer et al., (2016) as yeast related proteins were not found in their study, but if the proteins do not end up in the finished wine, then these observations need to be made during fermentation or from the lees.

4. Conclusion

This study demonstrates the power of shotgun proteomics to analyze the entire proteome of wine and lees in understating how proteins relate to condensed tannin concentration. The proteomics results from this study showed that there were changes in the wine proteome as condensed tannin concentration changed at post-press and as well as 8.5 months after pressing. This was often illustrated by a negative linear relationship between increasing condensed tannin concentration and decreasing proteome spectrum intensity. In this study, we did not observe a significant relationship between the total protein concentration of wines and condensed tannin concentration. In contrast, the total protein of the lees has a positive linear relationship to condensed tannin concentration while the proteomics spectrum had a negative linear relationship.

We used partial least squares regression analysis and Leave-One-Out Cross-Validation to create a predictive model for condensed tannin concentration in a wine based on proteomics data. The Variable Importance in Projection for all wine models mostly pointed towards *Vitis* proteins related to pathogen resistance as being the most comment although they were less common in the lees. For the lees, most of the proteins from the Variable Importance in Projection were related to glycolysis (yeast) and there were no pathogenesis-related proteins (PR).

As condensed tannin concentration increased there was evidence the proteome analysis revealed a lower diversity of *Vitis and* yeast proteins. For soluble proteins in wine, we conclude that PR proteins have a negative impact on condensed tannin concentration in finished wines. It is unclear from our results how large or how specific of a factor PR proteins may play as the whole proteome decreased as condensed tannin concentration increased. It doesn't seem that there is a smoking gun of a few proteins that could be eliminated to increase condensed tannin content in these wines, but rather a whole proteome that impacts condensed tannin. In general, the majority of *Vitis* proteins have a negative linear relationship and an even stronger negative logarithmic relationship to wine condensed tannin content. We found little if any evidence that one can use a "sacrificial" condensed tannin at crush to improve extraction of native condensed tannin and phenolics. A bigger factor is proteome characteristics and diversity.

Further work at understanding how these proteins interact with condensed tannin (physical, chemical) and if they remain soluble needs to be done. When an interaction occurs, does condensed tannin content, protein content, or both change? Understanding if any cofactors in the wine matrix contribute to interaction/precipitation/solubility of

protein and condensed tannin would be important as well. Also, observing proteomes in other regions and cultivars may give better insight into which proteins are groups of proteins are more impactful than others.

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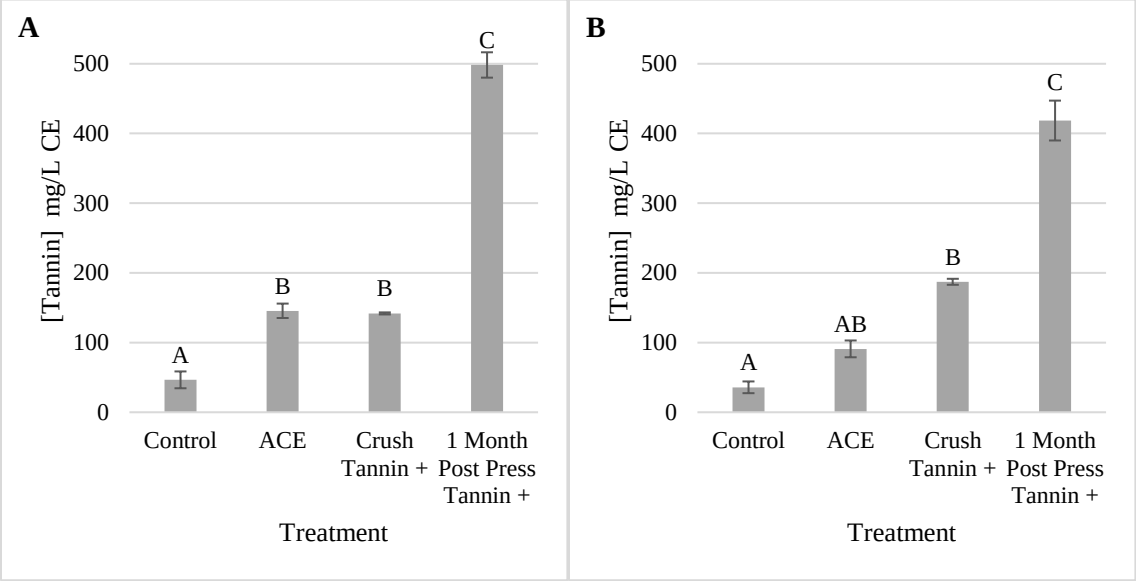

Figure 1: Condensed tannin content of cv. Chambourcin. Three replications of each treatment were completed with control (traditional winemaking), accentuated cut edges to reduce skin surface area (ACE), exogenous condensed tannin additions of 1400 mg/L made at crush (Crush Condensed tannin +), and 1-month after pressing from pomace (1 Month Post Press Condensed tannin +). Wines from 2018 (**A)** and 2019 (**B**). Condensed tannin analysis was performed via Adams-Harbertson condensed tannin assay and reported in mg/L catechin equivalents (CE) (Harbertson et al., 2002a). Reported numbers are the mean of replicates with standard error mean. Letters above each treatment (column) designate significant differences between treatments within a year determined via Tukey-Kramer HSD.

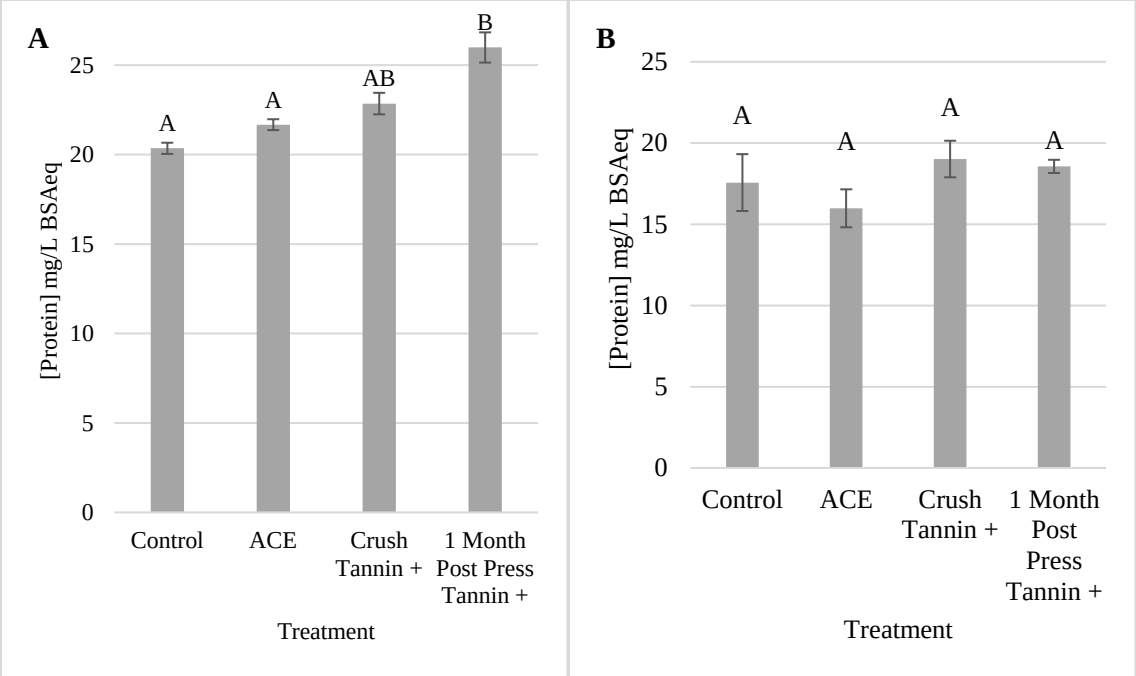

Figure 2: Protein content cv. Chambourcin. Total protein content mg/L BSAeq of wines from 2018 (**A**) and 2019 (**B**). Winemaking treatments include traditional winemaking (Control), accentuated cut edges to reduce skin surface area (ACE), exogenous condensed tannin additions of 1400 mg/L made at crush (Crush Condensed tannin +), and 1-month after pressing from pomace (1 Month Post Press Condensed tannin +). Protein analysis was done by acetone/TCA precipitation and total BCA protein quantification (Fredrickson and Kwasniewski, unpublished; P. K. Smith et al., 1985). Reported numbers are the mean of replicates with standard error mean. Letters above each treatment (column) designate significant differences between treatments within a year determined via Tukey-Kramer HSD.

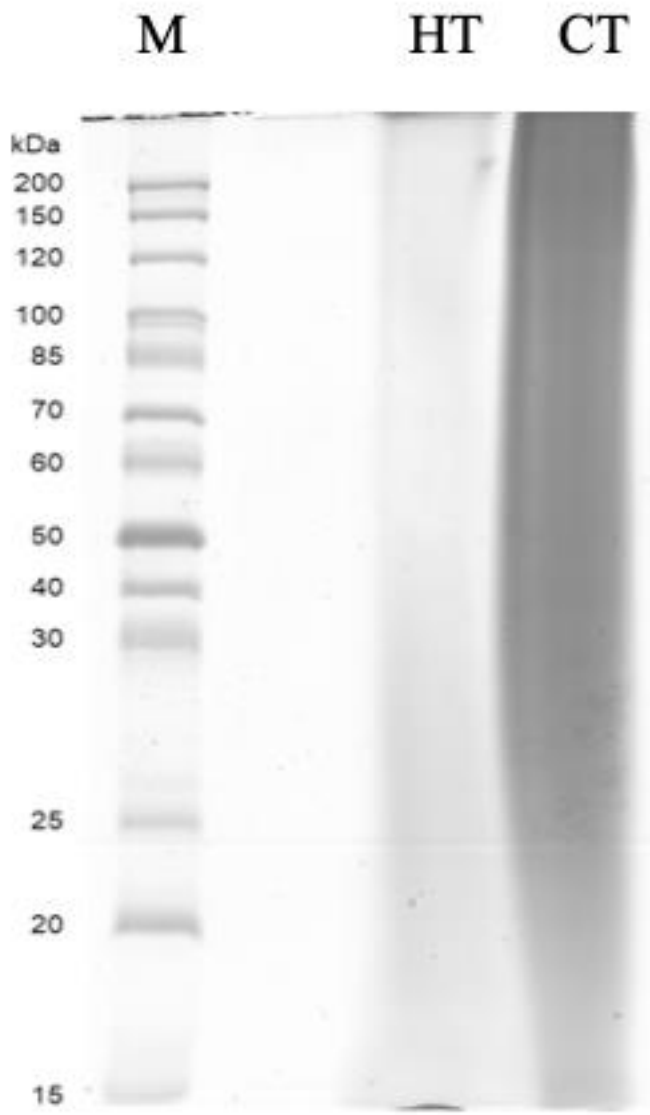

Figure 3: SDS-PAGE of exogenous tannin samples. One seed-derived condensed tannin product (CT) and one oak-derived hydrolysable tannin product (HT) were analyzed. The tannin samples were heated in SDS buffer and 25uL loaded per lane. A 12% acrylamide gel was run at 25 mA for 3 hours and then stained with colloidal coomassie blue. The gel was destained with water and imaged using a UMAX PowerLook flatbed scanner (UMAX Technologies, Inc., Dallas, TX). molecular weight marker (M), sizes are shown in kiloDaltons.

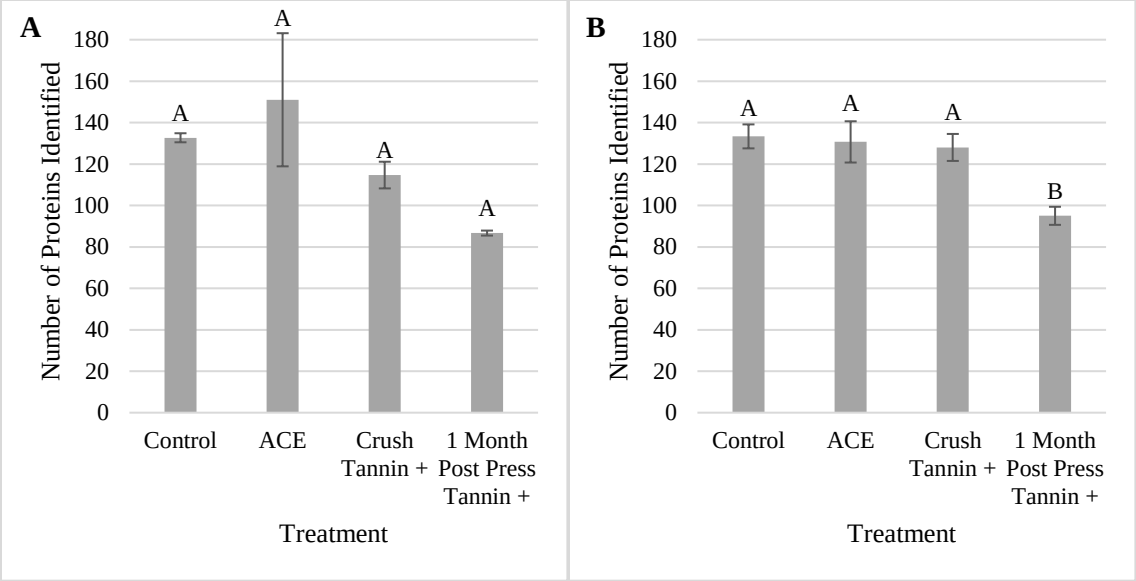

Figure 4: Identified proteins in cv. Chambourcin > 1 count. The number of proteins identified with >1 spectrum count after 30 µg of protein was digested from winemaking treatments of traditional winemaking (Control), accentuated cut edges to reduce skin surface area (ACE), exogenous condensed tannin additions of 1400 mg/L made at crush (Crush Condensed tannin +), and 1-month after pressing from pomace (1 Month Post Press Condensed tannin +). Digestion occurred with trypsin and proteins were identified via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020). Wines from 2018 (**A**) and 2019 (**B**). Reported numbers are the mean of replicates with standard error mean. Letters above each treatment (column) designate significant differences between treatments within a year determined via Tukey-Kramer HSD.

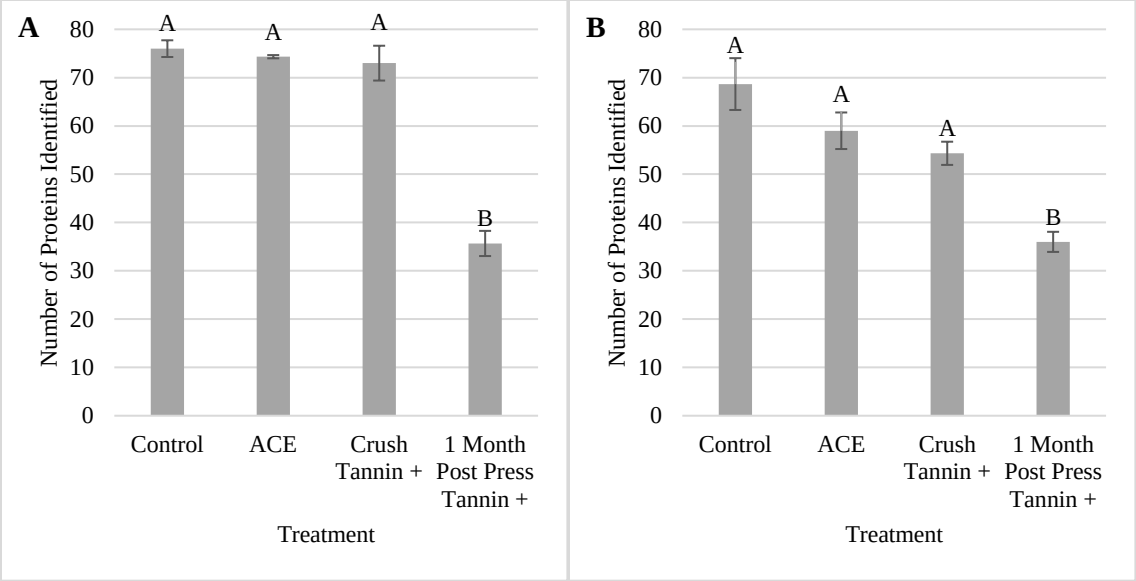

Figure 5: Identified proteins in cv. Chambourcin > 10 count. The number of proteins identified with >10 spectrum count after 30 µg of protein was digested from winemaking treatments of traditional winemaking (Control), accentuated cut edges to reduce skin surface area (ACE), exogenous condensed tannin additions of 1400 mg/L made at crush (Crush Condensed tannin +), and 1-month after pressing from pomace (1 Month Post Press Condensed tannin +). Digestion occurred with trypsin and proteins were identified via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020) in 2018 (**A**) and 2019 (**B**) wines. Wines from 2018 are on the left and 2019 wines are on the right. Reported numbers are the mean of replicates with standard error mean. Letters above each treatment (column) designate significant differences between treatments within a year determined via Tukey-Kramer HSD.

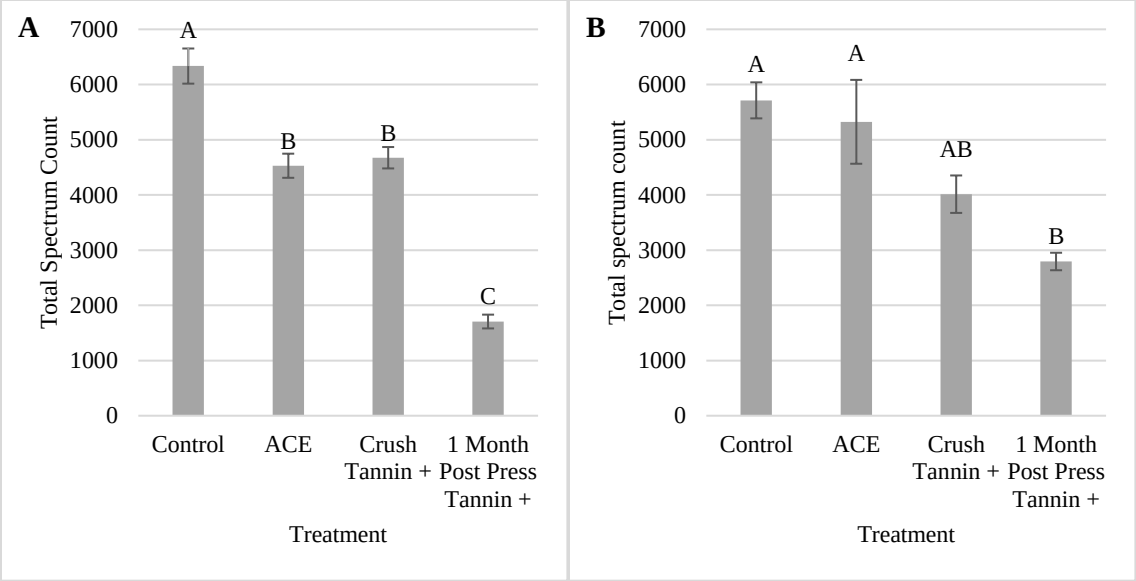

Figure 6: Total spectrum count of cv Chambourcin**.** Total spectrum count of proteome after 30 µg of protein was digested from winemaking treatments of traditional winemaking (Control), accentuated cut edges to reduce skin surface area (ACE), exogenous condensed tannin additions of 1400 mg/L made at crush (Crush Condensed tannin +), and 1-month after pressing from pomace (1 Month Post Press Condensed tannin +). Digestion occurred with trypsin and proteins were identified via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020) in 2018 (left) and 2019 (right) wines. Wines from 2018 (**A**) and 2019 (**B)**. Reported numbers are the mean of replicates with standard error mean. Letters above each treatment (column) designate significant differences between treatments within a year determined via Tukey-Kramer HSD.

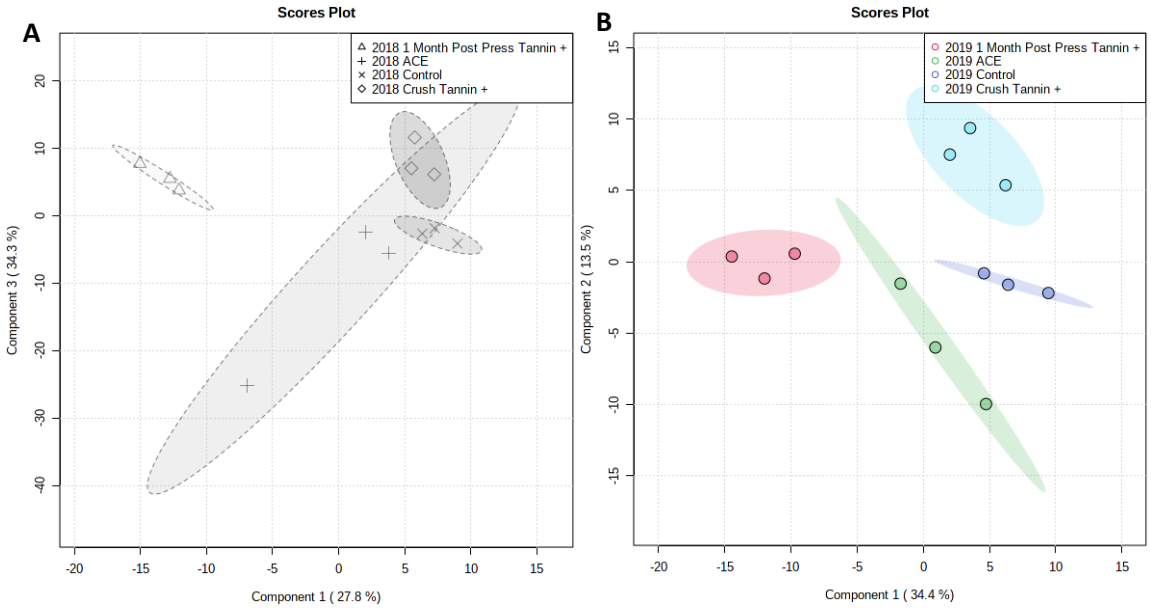

Figure 7: PLS-DS scores plot. Partial least squares discriminate analysis (PLS) score plots from winemaking treatments of traditional winemaking (Control), accentuated cut edges to reduce skin surface area (ACE), exogenous condensed tannin additions of 1400 mg/L made at crush (Crush Condensed tannin +), and 1-month after pressing from pomace (1 Month Post Press Condensed tannin +) in 2018 (**A**) and 2019 (**B**) wines. For each treatment and year, three fermentation replicates were prepared. Shown are the two most important components (Component 1, Component 3 for 2018, and Component 1, Component 2 for 2019) of variation. The X-axis (Component 1) and y-axis (component 2 or component 3) represent the variance weights of Component 1 and Component 2 (or 3), percentages represent the proportions of the component variances on total variance. PLS were performed using Metaboanalyst software based on the intensity values of protein abundance in the proteomes of cv. Chambourcin wines with different winemaking treatments that increased condensed tannin concentration.

Table 1: Top proteins in PLS-R prediction model. Spectrum Counts of total and top ten proteins from Variable Importance in Projection (VIP) in Partial Least Squares-Regression prediction model for condensed tannin concentration with proteomics data. Winemaking methods from 2018 and 2019 include traditional winemaking (Control), accentuated cut edges to reduce skin surface area (ACE), exogenous condensed tannin additions of 1400 mg/L made at crush (Crush Condensed tannin +), and 1-month after pressing from pomace (1 Month Post Press Condensed tannin +). Condensed tannin content against spectrum counts of proteins with each linear and logarithmic regression r-squared values and p-values.

(Sample) accession ID	description of protein	Spectrum count of Treatment				Condensed tannin vs spectrum count regression			
		Control	Crush Condensed tannin +	1-Month Post Press Condensed tannin +	ACE	Linear Regression R^2	P-Value	logarithmic regression R^2	P-value
N/A	Sum of spectrum count	6023.8	4343.8	2250.5	4926.5	0.8031	<0.0001	0.8531	<0.0001
F6HAU0	*Vitis:* vacuolar invertase	328.7	74.3	16.8	126.2	0.4787	0.0002	0.8557	<0.0001
A0A438IRV9	*Vitis:* Beta-fructofuranosidase	278	56.7	13.0	108.5	0.4658	0.0002	0.8738	<0.0001
A0A438JJ53	*Vitis:* Thaumatin-like protein	195.0	135.5	38	171.2	0.7050	<0.0001	0.8346	<0.0001
F6HUG9	*Vitis:* Uncharacterized, (Thaumatin-like)	191.5	63.5	24.8	109.3	0.7159	<0.0001	0.8388	<0.0001
Q7XAU7	*Vitis:* Thaumatin-like protein	217.7	144.8	43.2	190.7	0.6772	<0.0001	0.802	<0.0001
P93621	*Vitis:* Osmotin-like protein	165.7	109.3	21.2	126.3	0.6361	<0.0001	0.8005	<0.0001
A3QRB4	*Vitis*: Thaumatin-like protein	176.7	124	30.8	153	0.5698	<0.0001	0.8310	<0.0001
P38288	Yeast: Protein TOS1 OS=Saccharomyces cerevisiae	232.2	164.3	81.8	224	0.5451	<0.0001	0.6010	<0.0001
A0A438DX78	*Vitis:* Beta-fructofuranosidase	104.2	67.5	11.5	71.0	0.4447	0.0004	0.8453	<0.0001
P53753	Yeast: Endo-1 3(4)-beta-glucanase	200.3	159.5	73.3	163.5	0.3132	0.0045	0.4567	0.0003

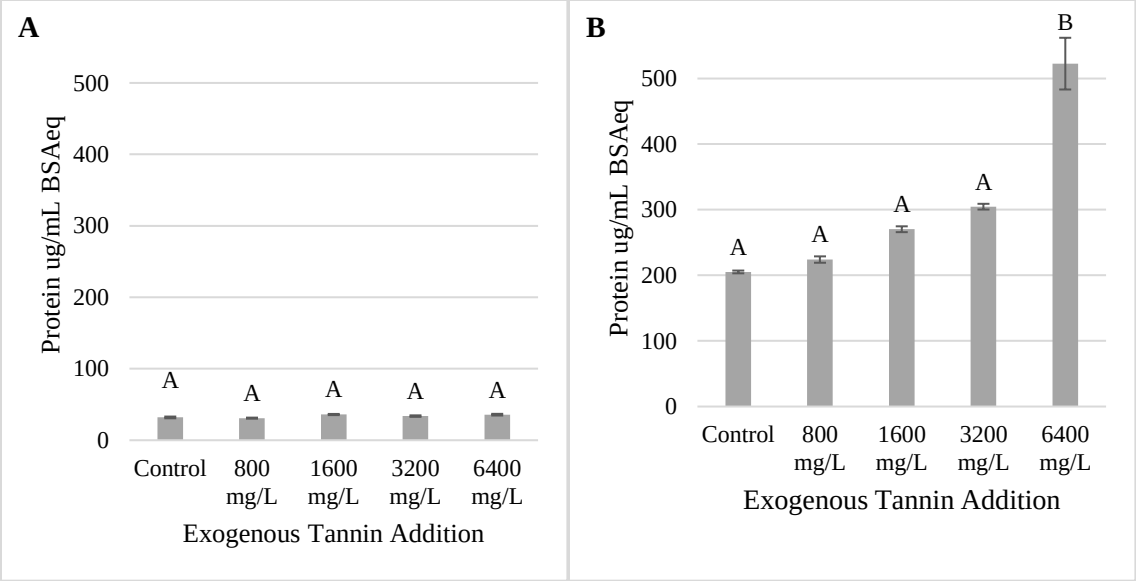

Figure 8: Total protein content of wine and lees with exogenous condensed tannin addition. Total protein content mg/L BSAeq of wine (**A**) and lees (**B**). Ferments of 2018 cv. Chambourcin were pressed after 4 days of maceration and exogenous condensed tannin additions of 0, 800, 1600, 3200, and 6400 mg/L were made. After 7 days of fermentation wine and gross lees were collected. Protein analysis was done by acetone/TCA precipitation and total BCA protein quantification (Fredrickson and Kwasniewski, unpublished; P. K. Smith et al., 1985). Reported numbers are the mean of replicates with standard error mean. Letters above each treatment (column) designate significant differences between treatments within a matrix determined via Tukey-Kramer HSD.

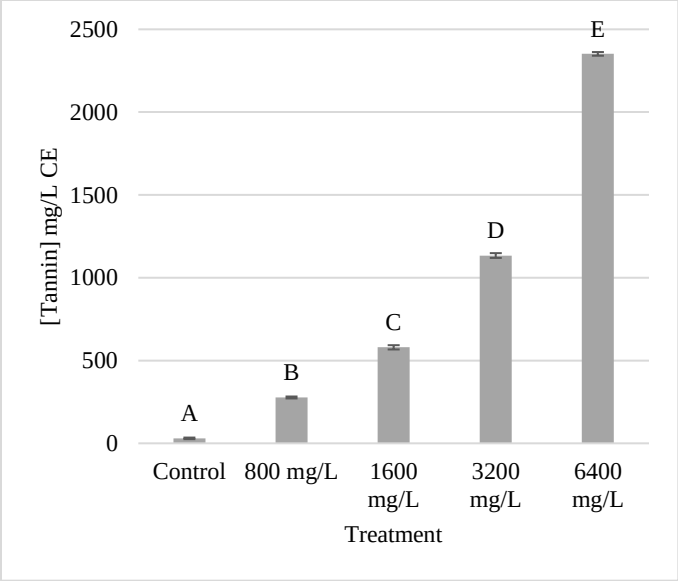

Figure 9: Condensed tannin Content mg/L CE of Wines at post-press with exogenous condensed tannin additions. Ferments of 2018 cv. Chambourcin were pressed after 4 days of maceration and exogenous condensed tannin additions of 0, 800, 1600, 3200, and 6400 mg/L were made. After 7 days of fermentation, the wine was collected for analysis. Condensed tannin analysis was performed via Adams-Harbertson condensed tannin assay and reported in mg/L catechin equivalents (CE) (Harbertson et al., 2002a). Reported numbers are the mean of replicates with standard error mean. Letters above each treatment (column) designate significant differences between treatments within a matrix determined via Tukey-Kramer HSD.

Table 2: Proteomics of wine and lees**.** Ferments of 2018 cv. Chambourcin were pressed after 4 days of maceration and exogenous condensed tannin additions of 0, 800, 1600, 3200, and 6400 mg/L were made. After 7 days of fermentation wine and gross lees were collected. 30 µg of protein was digested with trypsin and proteins were identified via UPLC-ES-TIMS-TOF and PEAKS X data analysis (Cui et al., 2020) Total spectrum count and the number of proteins ID with (>1 spectrum count) and >10 spectrum count are mean of replicates with standard error mean. Letters above each treatment (column) designate significant differences between treatments within a column determined via Tukey-Kramer HSD.

	Wine		Lees	
Treatment	**Spectrum Count**	**Protein ID**	**Spectrum Count**	**Protein ID**
Control	3807±1020^{A}	(516)103±37^{A}	10952±1432^{A}	(890)262±37^{A}
800 mg/L	4277±1280^{A}	(601)103±24^{A}	10129±1113^{AB}	(904)249±31^{A}
1600 mg/L	4097±899^{A}	(596)94±28^{A}	5444±783^{C}	(625)125±23^{BC}
3200 mg/L	3330±519^{A}	(488)71±6^{A}	7177±557^{BC}	(727)167±15^{AB}
6400 mg/L	1139±895^{A}	(193)18±9^{A}	2068±675^{D}	(310)36±17^{C}

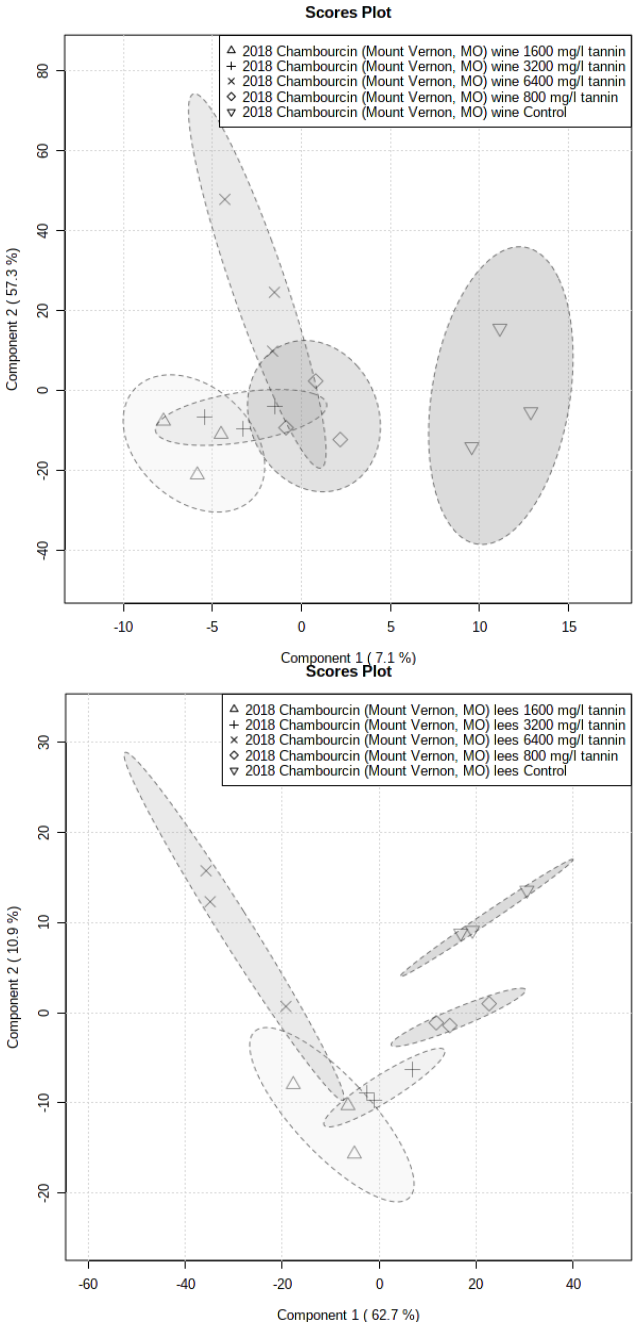

Figure 10: PLC-DS score plots with exogenous condensed tannin additions. Exogenous condensed tannin additions of 0, 800, 1600, 3200, and 6400 mg/L were made to pressed wines after 4 days of maceration and continued to ferment for a total of 7 days. For each treatment, three fermentation replicates were prepared. Plots from wines (**A**) and lees (**B**) show the two most important components (Component 1, Component 2) of variation. The x-axis (Component 1) and y-axis (component 2) represent the variance weights of Component 1 and Component 2, percentages represent the proportions of the component variances on total variance. The partial least squares-discriminate analysis was performed using Metaboanalyst software based on the intensity values of protein abundance in the proteomes of cv. Chambourcin wines with increasing exogenous condensed tannin additions.

Table 3: PLS-R prediction model for exogenous condensed tannin additions**.** Spectrum Counts of top ten proteins from Variable Importance in Projection (VIP) in Partial Least Squares-Regression prediction model for condensed tannin concentration with proteomics data. Treatments were exogenous condensed tannin additions of 0, 800, 1600, 3200, and 6400 mg/L that were made to pressed wines after 4 days of maceration and continued to ferment for a total of 7 days. The last 4 columns are condensed tannin concentrations against spectrum counts of individual proteins with the linear and logarithmic regression r-squared values and p-values.

Wine or Lees	(Sample) accession ID	description of protein	Spectrum count of Treatment					Condensed tannin vs spectrum count regression			
			Control	800 mg/L	1600 mg/L	3200 mg/L	6400 mg/L	Linear R-Squared	P-Value	logarithmic R-Squared	P-value
Wine	F6HAU0	*Vitis:* presumed vacuolar invertase	931	362	236	264	100	0.3920	0.0126	0.6894	0.0001
Wine	Q7XAU6	*Vitis:* Class IV chitinase	340	364	255	183	57	0.7271	<0.0001	0.5133	0.0027
Lees	P00560	*Yeast:* Phosphoglycerate kinase	417	385	175	268	68	0.6600	0.0002	0.7339	<0.0001
Lees	A0A438IKZ4	*Vitis:* Uncharacterized protein	388	489	418	363	197	0.6619	0.0002	0.7078	<0.0001
Wine	A0A438DX78	*Vitis:* Beta-fructofuranosidase soluble isoenzyme	408	190	125	124	53	0.4187	0.0091	0.6600	0.0002
Wine	O24531	*Vitis:* Class IV endochitinase	283	304	210	156	51	0.7523	<0.0001	0.8041	<0.0001
Lees	P00924	*Yeast*: Enolase 1	396	324	174	281	87	0.5556	0.0014	0.6744	0.0002
Lees	P00360	Yeast: Glyceraldehyde-3-phosphate dehydrogenase 1	285	271	117	176	50	0.6280	0.0004	0.7329	<0.0001
Lees	P06169	*Yeast:* Pyruvate decarboxylase isozyme 1	334	292	125	235	55	0.5205	0.0024	0.6645	0.0002
Lees	P00925	Yeast: Enolase 2	328	266	145	224	76	0.5741	0.0011	0.6968	0.0001

CHAPTER FOUR:
Optimized and Novel Winemaking Techniques to Improve Condensed tannin Concentration in Cv. Chambourcin Wines

1. Introduction

Improving condensed tannin content in low condensed tannin wines is important to wine quality and is desired by many winemakers and enologists. Optimizing current winemaking practices and exploring new practices is important to moving wine quality forward, especially for low condensed tannin cultivars and in regions where condensed tannins are naturally low.

Due to long and cold winters, plus wet and humid summers in the state of Missouri and throughout the Midwest, the majority of vineyards are planted to hybrid cultivars (Church, 1982). Many of these cultivars including cv. Chambourcin, a French-American hybrid has high levels of phenols, but often very low levels of flavan-3-ols and condensed tannin (Auw et al., 1996). This can lead to less desirable sensory attributes and the ability to age wine produced from these cultivars. Although condensed tannin levels in some hybrid cultivar berries are comparable to high condensed tannin *Vitis vinifera* cultivars, the uses of traditional winemaking techniques have been unsuccessful at increasing condensed tannin concentrations in the finished product (Manns et al., 2013; Springer & Sacks, 2014).

More recently, exogenous condensed tannins have been used to supplement condensed tannin content for low condensed tannin wines (Thomas, 2013). The majority

of these condensed tannin products are marketed towards *Vitis vinifera* cultivars and are normally added at crush or low dosage rates at post-fermentation (Canuti et al., 2012). When exogenous condensed tannins are added to hybrid musts/wines at the recommended stage, the majority of condensed tannin from the exogenous product is not retained (Alex J. Fredrickson et al., 2020). The complete reasoning for condensed tannins not being retained is currently unknown. It has been demonstrated that cell wall material, including insoluble and soluble fractions, limits the extraction and/or retention of phenolic material including monomeric and oligomeric flavan-3-ols and protein precipitable condensed tannin (Bindon et al., 2016a). Recently the focus has been on the role of soluble cell wall material, in particular pathogenesis-related (PR) proteins. These proteins are highly soluble and in higher concentrations in grapes from in cool/wet climates and hybrid cultivars due to their function in defending against grapevine diseases and insects (Datta & Muthukrishnan, 1999). PR proteins may have the ability to precipitate with condensed tannin, reducing the amount of condensed tannin retained in wine from these hybrid cultivars (Springer, Sherwood, et al., 2016b).

The use of new processing techniques and adjustment of current practices need to be further explored to determine if the condensed tannin naturally present in low condensed tannin wine cultivars can be better extracted and retained to improve wine quality. The use of accentuated cut edges (ACE) is a new winemaking technique that uses mechanical blending to increase skin surface area of grape skins which improves the extraction of phenolics (condensed tannin and anthocyanins). In *Vitis vinifera* cv. Pinot noir, researchers were able to increase condensed tannin content in wines by 7-fold (Sparrow, Smart, et al., 2016). However, it is unclear how this treatment may impact the

extraction of undesirable grape components such as PR proteins. The use of enzymes to improve wine quality has been employed for some time, the use of pectinase to limit condensed tannin-cell wall interactions has recently been investigated with limited success (Castro-López et al., 2016). Also, limiting protein condensed tannin interactions by removing protein in juice with bentonite has shown success at reducing protein in juice and wine, but limited success in improving condensed tannin extraction/retention in various hybrid wines (Nicolle et al., 2019). This method has yet to be tested on cv. Chambourcin fruit.

The objectives of this study were to improve condensed tannin in cv. Chambourcin wines by performing an extensive exogenous condensed tannin addition timing trial to determine the ideal timing for the addition of exogenous condensed tannin to maximize retention in wine. We also investigated modifications to traditional winemaking methods to determine if condensed tannin extraction and retention can be improved for both native and exogenous condensed tannins. The study of these parameters will better enable winemakers to make informed decisions on ways to improve condensed tannin content and overall quality in wine from low condensed tannin cultivars and climates.

2. Materials and Methods

2.1. Chemical Reagents.

Acetone (99.9% purity), acetonitrile (ACN) (99.8% purity), ethanol (95% purity), glacial acetic acid (99.7% purity), Trichloroacetic acid (TCA) (99% purity), albumin from bovine serum (BSA) (98% purity), ammonium bicarbonate (99% purity), (+)-catechin hydrate (96% purity), formic acid (98% purity), iodoacetamide (IAM) (99%

purity) sodium chloride (99% purity), sodium dodecyl sulfate (SDS) (98% purity), sodium hydroxide (NaOH) (98% purity), triethanolamine (98% purity), and urea (99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Iron (III) chloride (98% purity), and hydrochloric acid (37% purity) were sourced from Fisher Scientific (Waltham, MA, USA). Potassium metabisulfite (KMBS) and Diammonium Phosphate (DAP) were obtained from MoreFlavor (Pittsburg, CA). Grape derived exogenous condensed tannin was obtained from Enartis USA Inc. (Windsor, CA). Oak derived exogenous hydrolysable tannin was obtained from Oak Solutions Group (Napa, CA). The enzyme was obtained from Scott Laboratories (Scott Laboratories Inc., Petaluma, CA).

2.2. Experimental design

All winemaking experiments were completely randomized design with three fermentation replications for each treatment. The dependent variables were condensed tannin concentration while the independent variables were exogenous tannin additions (to must or at 1-month post-press) and ACE.

Protein was extracted in triplicate for each treatment and quantified with three replications for each extraction. Protein concentration was the dependent variable.

2.3. Tannin Analysis

Condensed and hydrolysable tannin content was quantified via protein precipitable tannin analysis, commonly known as the Adams-Harbertson tannin assay (Harbertson et al., 2002a). To measure condensed and hydrolysable tannin, 0.5 mL of wine (diluted if condensed and hydrolysable tannin concentration was above standard curve) was added to a 2.2 mL centrifuge tube with 1 mL of 200 mM glacial acetic acid, 170 mM sodium chloride, and 1 mg/mL bovine serum albumin at pH 4.9. Tubes were

inverted 10 times to mix wine and protein solution and incubated at room temperature with gentle agitation for 15 minutes. Samples were centrifuged for 5 minutes at 20,800 x g Supernatant was carefully decanted and the residual liquid was removed with transfer pipet and 0.875 mL of 5% triethanolamine and 5% SDS at pH 9.4 buffer was carefully added to the pellet and incubated at room temperature for 10 minutes. Samples were then vortexed vigorously until the pellet was completely dissolved, then incubated at room temperature for an additional ten minutes. The entire 0.875 mL sample was quantitatively transferred to a 1.5 mL microcuvette and the background absorbance was read at 510 nanometers. 0.125 mL of 10 mM of iron (III) chloride in 0.01 N HCl was added to the sample and inverted to mix and then incubated at room temperature for 10 minutes followed by a final absorbance reading at 510 nm. Using an external standard curve of catechin, the hydrolysable and condensed tannin concentration is calculated and reported in mg/L catechin equivalents (CE).

2.4. Tannin Product Analysis

To determine how much protein precipitable tannin was in the powered exogenous condensed or hydrolysable tannin products, approximately 17 mg of exogenous tannin was added to 25 mL of model wine (0.5% potassium bitartrate, 12% ethanol, and adjusted to pH 3.3 with HCl) and vortexed and sonicated to dissolve the powder. Once dissolved, the products were analyzed for protein precipitable tannin and reported in mg/L CE and percentage of protein precipitable tannin based on the concentration of the initial solution.

2.5. Grape Native Condensed Tannin Determination

Based on a method from Springer and Sacks (2014), the cv. Chambourcin fruit in this study from 2018 and 2019 was used to determine the total condensed tannin content of fruit. For each sample, fifty berries were collected in duplicate. Skin, seed, and pulp were divided and weighed. All components were extracted in 70% acetone overnight. The following day solids were filtered from the liquid. Acetone was removed from a 1 mL aliquot and then volume was made up to the original 1 mL with water. The extract was then measured via protein precipitable tannin and reported in mg (of condensed tannin)/ g (of grape).

2.6. Winemaking

2.6.1. Exogenous Condensed tannin Addition Timing

In 2018 cv. Chambourcin was hand-harvested from Mt. Vernon, MO on 9/17-18/2018. On 9/19/2018 fruit was destemmed, and transferred into a ½ ton macro bin, and mixed by hand. 7.7 kg of fruit was added to 9.5 L PET fermenters. Juice samples were taken from each fermenter. 50 mg/L of sulfur dioxide (SO_2) in the form of KMBS was added to the juice. On 9/20/2018 1400 mg/L of exogenous condensed tannin were added for a "Crush" condensed tannin addition treatment. Musts were inoculated with yeast strain GRE (Scott Laboratories Inc., Petaluma, CA) at the recommended rate of 0.25 g/L and hydrated in GoFerm Protect (Scott Laboratories Inc., Petaluma, CA) at a rate of 0.3 g/L. Ferments were punched down twice a day and kept in a temperature-controlled cooler at 18 °C. On 9/21/2020, the three ferments for "20 °Brix" exogenous condensed tannin addition treatment had Brix readings of 20.1, 20.1, and 20.5, when 1400 mg/L of exogenous condensed tannin were added. On 9/24/2020, the three ferments for "5 °Brix" exogenous condensed tannin addition treatment had Brix readings of 5.3, 5.4, and 5.5, so

1400 mg/L of exogenous condensed tannin were added. On 10/2/2018, all wines were pressed with a #20 fruit press (9.5 L, 20 cm basket press) (MoreFlavor, Pittsburg, CA) and 3.79 L of wine was collected. The wines for "Post Press" exogenous condensed tannin addition had 1400 mg/L of exogenous condensed tannin added immediately after pressing. On 10/9/2018 wines were tested for residual sugar with Clinitest reducing sugar tablets (Bayer Corporation Wippany, NJ) and all had a 0.25-0.5% residual sugar which was considered dry (completely fermented). On 10/10/2018 all wines had 50 mg/L SO_2 in the form of KMS added. The wines for the "1 Week Post Press" treatment had 1400 mg/L of exogenous condensed tannin added. On 10/31/2018 all wines were racked off settled lees and on 11/2/2019, 1400 mg/L of exogenous condensed tannin were added to the "1 Month Post Press" condensed tannin addition treatment. On 12/2/2019 1400 mg/L of exogenous condensed tannin were added to the "2 Months Post Press" condensed tannin addition treatment. On 12/18/2018, except for the 3-months post-press treatment, all wines were filtered with Buon Vino Super Jet Wine Filter and a number 1 course filter pad (Buon Vino Manufacturing, Cambridge, ON) which is rated to 7 microns to remove any unwanted sediment and then 50 mg/L of sulfur dioxide (SO_2) in the form of KMS was added. Wines were bottled in 187 (3) and 375 (1) mL crown cap bottles on 12/19/2018 and stored in a 7 °C cooler at 55% relative humidity. On 1/10/2019, 1400 mg/L exogenous condensed tannin was added to the "3 Months Post Press" condensed tannin addition treatment. After four days, the wines from 3 Months Post Press were filtered and bottled like the other treatments. Samples for all ferments were taken at post-press, post condensed tannin addition, 1-month post-press, at bottling, and 6-months post pressing.

2.6.2. Accentuated Cut Edges

The same winemaking procedure for exogenous condensed tannin addition timing was used, but no exogenous condensed tannin additions were made. On 9/20/2018, ACE at crush treatments were completely blended with an immersion blender which broke down skins and pulp into a soup-like consistency and kept seeds intact as determined by visual inspection. Punch downs continued twice a day until caps sank which occurred after three days. For 24 hours before pressing, ACE was applied on 10/1/2018 whereby skins and pulp were blended with an immersion blender until all skins were fragmented into a similar consistency as the crush treatment. On 10/2/2018, all wines were pressed by adding a double-layered cheesecloth to the #20 fruit press (9.5 L, 20 cm basket press) (MoreFlavor, Pittsburg, CA) and then adding the blended must slowly to allow for a large amount of free run to drain and then the pomace was pressed until 3.79 L of wine was collected. After pressing, the same winemaking protocol for finishing, bottling, and storage was used from the exogenous condensed tannin addition timing method (2.4.2.).

2.6.3. *Enzyme Treatment of Wine*

The same winemaking procedure from the exogenous condensed tannin addition timing method (2.4.2.) was used to collect wine. Once the wine was pressed and the three treatments were implemented, enzyme (blend of pectinase and hemicellulase) addition of 0.379 g/L was added to one of the treatments. One week after the enzyme was added, 1400 mg/L of exogenous condensed tannin product was added and the treatments labeled ‘Enzyme at Pressing’, and ‘Condensed tannin Add 1 Week Post Pressing’. The lots of one treatment that had no enzyme added also had 1400 mg/L of exogenous condensed tannin added and labeled as ‘1 Week Post Press Condensed tannin Addition'. After

exogenous condensed tannin addition was made, all wines had 50 mg/L of SO_2 in the form of KMS added. Wines were treated similar to exogenous condensed tannin addition treatments after 10/9/2018.

2.6.4. *Bentonite Treatment of Juice*

The same procedure for obtaining and dividing into fermenters was used. Three lots were pressed as a white with a #20 fruit press (9.5 L, 20 cm basket press) (MoreFlavor, Pittsburg, CA). The juice was finned with hydrated Canaton bentonite (MoreFlavor, Pittsburg, CA) at a rate of 0.6 g/L by adding the bentonite slurry and mixing with a stir bar for five minutes, then allowing the bentonite to settle overnight. The following day the finned juice was carefully racked off the lees back onto the skins from which the juice was pressed. Wines were then fermented, pressed, and finished following the same procedure from above. Protein content was determine following method from (Fredrickson and Kwasniewski, unpublished) to confirm protein was removed from bentonite treatments.

2.6.5. Exogenous Condensed tannin Addition and ACE

On 10/4/2019 approximately 8 tons of cv. Chambourcin from Etlah, Missouri was machine harvested with mostly whole berries into ½ ton macro bins. Approximately 5 L of whole berries from each bin was taken and placed into three 60 L totes. A homogenous juice sample from the three totes was collected and frozen at -80 °C for later analysis. The 18 ferments were randomized and had 3,056 g of fruit added to a 3.79 L PET container. 50 mg/L of SO_2 in the form of KMS was added to the juice. 1400 mg/L of exogenous condensed tannin was added to ferments of the “Crush Condensed tannin Addition” treatment. With an immersion blender, the must of (three treatments) nine

ferments were thoroughly blended to a thick soup-like consistency similar to 2018. Of the ACE treatments, one had 1400 mg/L of exogenous condensed tannin added before yeast inoculation for an "ACE and Crush Condensed tannin Addition" treatment. All musts were inoculated with GRE (Scott Laboratories Inc., Petaluma, CA) at a rate of 0.25g/L and hydrated in GoFerm Protect (Scott Laboratories Inc., Petaluma, CA) at a rate of 0.3 g/L. Ferments were punched down twice a day and kept in a temperature-controlled cooler at 18 °C. On 10/14/2019 all wines were pressed with a #20 fruit press (9.5 L, 20 cm basket press) (MoreFlavor, Pittsburg, CA) and 1.89 L of wine was collected. Wines were checked for residual sugar weekly and mixed until 11/1/2019 when samples were all below 0.75% RS (which was considered dry to reduce the risk of oxidation in small fermentation vessels) by Clinitest (Bayer Corporation Wippany, NJ), so 50 mg/L of SO_2 in the form of KMS was added. On 11/2/2019, 1400 mg/L of exogenous condensed tannin was added to traditional winemaking wines for "1 Month Post Press Condensed tannin Addition" treatment and to an ACE maceration for an "ACE and 1 Month Post Press Condensed tannin Addition" treatment. The ACE maceration with no condensed tannin additions was known as the "ACE" treatment. On 11/18/2019 all wines were racked from settled lees into 950 mL containers until full. On 12/19/2019 all wines were bottled into 3, 187ml crown cap bottles with 50 mg/L SO2 in the form of KMS was added and stored in an 18 °C cellar. Samples for all ferments were taken at post-press, at bottling, and 8.5-months post pressing.

2.6.6. *Double Exogenous Hydrolysable Tannin Additions*

Exogenous hydrolysable tannin used for this experiment was oak-derived (as opposed to grape-derived). On 10/4/2019 approximately 8 tons of cv. Chambourcin from

Etlah, Missouri was machine harvested with mostly whole berries from into ½ ton macro bins. In collaboration with Les Bourgeois Vineyards (Rouchport, MO) the fruit was crushed and added evenly to two stainless steel tanks until 3,785 L of the must was added to each. 50 mg/L of SO_2 in the form of KMS was added to the must of each tank. 400 mg/L of exogenous hydrolysable tannin was added to one of the two tanks. On 10/5/2019 musts were inoculated with 0.16 g/L of Muse yeast (Gusmer Enterprises, Inc, Fresno, CA) and 5 mg/L of Lallemand Beta Co-Inoc, *Oenococcus oeni* (Scott Laboratories Inc., Petaluma, CA), and 0.45 g/L DAP. Wines were pumped over twice a day and after 1/3 of sugar was consumed on 10/7/2019 0.225 g/L DAP was added to fermentations. On 10/14/2019 both wines were pressed and stored in stainless steel tanks. Wines were checked for residual sugar until 11/1/2019 when samples were all below 0.75% RS by Clinitest, so 50 mg/L of SO_2 in the form of KMS was added to both wines. Wines were stored in stainless steel tanks until 2/19/2020 when 8 L of each treatment was taken. The two wines were both divided into eight, 1 L lots. Of the eight lots, four received 400 mg/L of exogenous hydrolysable tannin. This resulted in four treatments; Control (no hydrolysable tannin addition); 400 mg/L exogenous hydrolysable tannin added at crush (Crush +); 400 mg/L exogenous hydrolysable tannin added at 4 months post-press (4-Months Post Press +); and 400 mg/L exogenous hydrolysable tannin added at crush and 4 months post-press (Crush + and 4 Months Post Press +). On 2/27/2020 samples were taken for condensed and hydrolysable tannins analysis as a post hydrolysable tannin addition sample. On 3/19/2020 wines were bottled in 187 mL bottles, 50 mg/L of SO_2 in the form of KMS was added to each bottle and then filled and crown capped. On

6/9/2020 one bottle of each wine was opened and analyzed for condensed and hydrolysable tannin, and a 50 mL centrifuge tube was saved for color analysis.

2.7. Color Analysis

The color of the wine samples was measured by a Chroma meter CR-410 (Konica Minolta, Sensing, Inc., Japan) using CIELAB L*, a*, b* values. 30 mL of wine was placed in a 50mm cylinder and measured from the top of the cylinder, 72 mm.

2.8. Data Processing

Data were collected and stored using Microsoft Excel (Redmond, WA). Mean comparisons of protein and tannin concentrations were performed using Tukey-Kramer HSD (multiple mean comparison) or one-tailed t-test ($p < 0.05$) (one mean comparison) on JMP statistical software (SAS Institute, Inc.).

3. Results and Discussion

3.1. Tannin Product Analysis

The exogenous condensed tannin product that was extracted from mature white grape seeds contained 26% tannin when measured via protein precipitable tannin assay. This is on par with other studies that found protein precipitable tannin in grape-derived exogenous tannin products to range from 12-50% (Alex James Fredrickson, 2015). This may seem low for a tannin product, but protein precipitable tannin only measures larger polymers of tannin that can be precipitated by BSA. When grape-derived exogenous tannin products were measured by HPLC it was found that 40% of the product was made up of monomeric and oligomeric phenolic material that is not considered tannin (Parker et al., 2007b).

The exogenous hydrolysable tannin product contained 63% protein precipitable tannin. The manufacturer indicated that this product contains 75-80% total hydrolysable tannin, so although that is higher than what was measured in this study, the manufacturer does not indicate how they measured total hydrolysable tannin and what components are included (i.e., protein precipitable tannin, monomeric, or oligomeric). Previous analysis of hydrolysable tannin found that four different products contained protein precipitable tannin ranging from 12-25% tannin (Alex James Fredrickson, 2015; Harbertson et al., 2012). Although this is higher than previously measured products, this product is specifically blended to have a high concentration of hydrolysable tannin. The previous studies did not indicate what the exogenous hydrolysable tannin product intended use was for. If they were used for finishing wines in the cellar, they could contain lower levels of hydrolysable tannin for a more subtle change in mouthfeel.

3.2. Grape native condensed tannin determination

The condensed tannin content of grapes shows significant amounts in skin and seeds for both samples of cv. Chambourcin from 2018 and 2019 (Table 1). The values are slightly higher than what was observed in previous studies of low condensed tannin hybrid cultivars, but cv. Chambourcin was not investigated. The values determined as part of this study are comparable to *Vitis vinifera* cultivars and hybrids that are known to have higher concentrations of condensed tannin (Harbertson et al., 2002b; Springer & Sacks, 2014). This indicates that the available condensed tannin in cv. Chambourcin fruit should not be considered a limiting factor for final condensed tannin concentration in the finished wine.

3.3. Exogenous condensed tannin addition timing

Exogenous condensed tannin additions had a wide range of effectiveness in terms of condensed tannin retention 6 months after pressing. The treatments were grouped into the must and fermentation additions (with skins), post pressing/1-week post pressing, and 1 to 3-month(s) post pressing (Figure 1). Additions at crush and during maceration are recommended by the manufacturer. The retention of condensed tannin ranged from 39-43% which is slightly less than previous investigations where exogenous condensed tannin added during crush resulted in retention values of 53% for *Vitis vinifera* cultivars (Alex J. Fredrickson et al., 2020; Harbertson et al., 2012). Although there was a significant increase in condensed tannin retention relative to the control, the fact that less than 50% of the exogenous protein precipitable condensed tannin added was retained is lower than expected. If the goal is to increase the final condensed tannin content in a wine, then adding condensed tannin at crush may not be optimal from an economic standpoint as exogenous condensed tannin products can be expensive at commercial production scales.

Condensed tannin additions made immediately after or up to one week after pressing had significantly higher retention rates ranging from 69-77%. This is similar to results from other studies in which condensed tannin retention doubled when additions were made to cv. Corot Noir at post alcoholic fermentation (60%) when compared to additions made to the must (27%) (Alex James Fredrickson, 2015). Additions made at least 1 month after pressing resulted in retention rates above 100%, indicating that the late additions can be very effective for protein precipitable condensed tannin retention. The reason for retention values exceeding 100% may be due to condensed exogenous condensed tannin products containing 40% oligomeric or smaller flavan-3-ols that, in the

presence of acetaldehyde, can polymerize and become part of the protein precipitable fraction (Es-Safi et al., 1999; Parker et al., 2007b; Peleg et al., 1999). Although we know that insoluble and soluble cell wall material has an impact on condensed tannin retention, it seems that allowing the insoluble material to settle and then removed by racking the wine before adding exogenous condensed tannin can result in a large increase in condensed tannin retention (Bindon et al., 2016a). Although sensory impact needs to be determined and addition rates optimized, adding condensed tannin after fermentation and racking off the gross lees results in high retention rates in cv. Chambourcin. There was no statistical difference in the L* (lightness from black (0) to white (100)), a* (green (−) to red (+)), and b* (blue (−) to yellow (+)) values of the wines, but there may have been a very small increase in L* and a* as condensed tannin concentration increased indicating that samples were whiter (light) and redder and in the condensed tannin addition samples. A previous study investigating color differences in cv. Cabernet Sauvignon wine with pre-fermentation condensed tannin additions relative to controls found very small differences even with a different color profile without diglucoside anthocyanins (Liu et al., 2013).

3.4. ACE

The use of ACE in 2018 increased native protein precipitable condensed tannin content in wine at 6 months post-press (Figure 2). Condensed tannin content increased 2.5-fold at 24 hours before pressing and 3-fold at crush over the control. Although the condensed tannin content is not “high”, The 146 mg/L CE in the ACE treatment at must is at a concentration that is considered in the lower range of wines made from many commercially successful cultivars grown in warm climates (Harbertson et al., 2008).

Previous studies using enzymatic treatment and hot press had little to no success at increasing native condensed tannin content (Manns et al., 2013). We were uncertain as to whether ACE would increase condensed tannin content as it has been shown that cell wall material can interact with condensed tannins causing them to precipitate, so exposing the matrix to potentially more soluble and insoluble cell wall material might reduce soluble condensed tannins (Bindon et al., 2010; Osete-Alcaraz et al., 2019b; Springer, Sherwood, et al., 2016b). Although there was likely more free cell wall material to react with the extracted condensed tannin, it seemed that that decreasing the surface area to perimeter ratio by reducing the particle size allows for more condensed tannin extraction (Sparrow, Smart, et al., 2016). In *Vitis vinifera* cv. Pinot noir, the use of ACE increased condensed tannin content over 7-fold relative to the control (Sparrow, Holt, et al., 2016). Although effective at increasing condensed tannin content, the use of ACE for low condensed tannin cultivars does not seem to have the same impact it does in cultivars that typically have higher amounts of condensed tannin in wines made using traditional winemaking methods.

3.5. Enzyme treatment

Enzymatic treatment of wines at pressing did not have an impact on condensed tannin retention of exogenous condensed tannin (Figure 3). The enzyme product contained a combination of pectinase and hemicellulose which in theory should be effective at depolymerizing some insoluble and soluble cell wall material left over after maceration. The combination of these enzymes has been effective at degrading cell wall material and improving the extraction of phenolic material during maceration (Moreno-arribas & Polo, 2009). The use of pectolytic enzymes on grape juice that was lightly

pressed, treated and racked off settled lees (then fermented with normal maceration) resulted in a 43% increase in condensed tannin content (Osete-Alcaraz et al., 2019b). However, when used in hybrid cultivars in which the use of a pectolytic enzyme was added directly to the must there was no increase in condensed tannin concentration (Manns et al., 2013). It is possible that using a combination of these enzymes with proteases to degrade soluble cell wall material may have been more effective at increasing condensed tannin retention. Also, in this study, the wine was not racked after enzyme additions and before exogenous condensed tannin addition, so insoluble cell wall material that had settled out may have still reacted with the added condensed tannin, thus reducing wine condensed tannin content (Bindon et al., 2016a). Optimizing the use of different enzymes and timing of additions, along with ACE and/or exogenous condensed tannin additions holds promise for increasing wine condensed tannin content and should be explored further.

3.6. Bentonite treatment

Before fermentation, the control juice contained 125 μg/mL BSA equivalents (BSAeq) while the bentonite treated juice contained 23 μg/mL BSAeq indicating that the majority of protein was removed with the bentonite treatment. However, at pressing the control had 61 μg/mL BSAeq of protein while the bentonite treatment contained 69 μg/mL BSAeq of protein, indicating that removing the protein from the juice does not account for all protein in the must. There was no significant difference in condensed tannin concentration due to the large variation between fermentation replicates (Figure 3). There was likely a slight increase in condensed tannin concentration, but not as much as adding exogenous condensed tannin or applying ACE. Although no sensory evaluation

was made on these wines, there was a change in mouthfeel which became flat or "water-like" when juice was fined with bentonite. A previous study that fined red Frontenac juice with bentonite had a similar reduction in protein, but as fermentation finished the protein level in the bentonite treated wines increased while in the control they decreased. The final difference in total protein was small (42.7 vs 26.8 mg/L BSAeq) and there was no difference in condensed tannin concentration (Nicolle et al., 2019). Another study treated Marechal Foch and Lemberger juice with bentonite and at bottling found large differences in protein in the Marechal Foch wine compared to unfined juice (138 vs 38 mg/L BSAeq). The fining of juice made no difference in the Lemberger total protein and a slight increase in condensed tannin (Springer, Chen, et al., 2016). Bentonite is effective at removing protein in juice, but when adding the treated juice back to the skins, protein content increases and has very little impact on the final condensed tannin concentration. With the amount of work that is required to press the skins, treat the juice, and then ferment normally, the results do not seem to warrant the extra effort.

3.7. Exogenous condensed tannin addition and ACE

The combination of exogenous condensed tannin addition and ACE was completed to help determine if these treatments used in combination could increase wine condensed tannin concentrations even more. Each treatment had a different condensed tannin concentration and trends were similar to what was observed in 2018 (Figure 4). The use of ACE at crush increased condensed tannin content 2.5-fold over the control. Exogenous condensed tannin addition at crush had a retention rate of 42% while addition 1-month post-press was over 100% as observed previously. In the L*a*b* values there was no difference in color (Figure 6). The treatments that combine ACE and exogenous

condensed tannin addition into one show an additive effect, indicating that combining ACE and condensed tannin additions will not impact retention. The use of these methods together could be an effective method for increasing condensed tannin content in low condensed tannin red wines.

3.8. Double exogenous hydrolysable tannin addition

Protein precipitable tannin was significantly higher in all double hydrolysable tannin additions relative to the control (Figure 7). Retention rates of the exogenous hydrolysable tannin product were between 6-23%, far below the previous experiments. The color of the wine in the treatments did not differ either. A similar study using the same oak-derived hydrolysable tannin at crush on *Vitis vinifera* cv Pinot noir at a rate of 100 mg/L found total condensed and hydrolysable tannin to be almost 12-fold higher than the control (Glenn Jeffries, 2018). This research did not describe in detail the method used for quantifying condensed and hydrolysable tannin, but it appeared to be based on ultraviolet and visible spectrophotometric measurements so doesn't specifically measure protein precipitable tannin or iron reactivity. Hydrolysable tannin additions to must increased condensed and hydrolysable tannin content over the control in Glenn Jeffries, (2018), but it is hard to compare with different analytical methods. Another study found that the addition of a hydrolysable tannin added at rates 150 and 600 mg/L at post-press to cv. Cabernet Sauvignon wines resulted in 1 and 1.5-fold increases over the control with retention rates above 100% (Harbertson et al., 2012). Interestingly, wines made with *Vitis vinifera* cultivars with exogenous hydrolysable tannin addition seem to result in significant increases in total condensed and hydrolysable tannin while this study observed relatively small increases. More work with exogenous hydrolysable tannins and cool

climate/hybrid cultivars needs to be done to understand the low condensed and hydrolysable tannin retention from this study.

4. Conclusion

Many exogenous condensed and hydrolysable tannin manufacturer's recommend adding tannin at crush to increase total, including both exogenous and native, condensed and hydrolysable tannin content in wine. However, we established that condensed and hydrolysable exogenous tannin additions in low tannin red wines from cv. Chambourcin even at levels above the manufacturer's recommendation retained less than 50% of the protein precipitable tannin that was added. With the high cost of exogenous condensed and hydrolysable tannin, winemakers must determine if this level of retention is economically acceptable for increasing wine tannin content. We did find, however, that wines with exogenous condensed tannin additions made after pressing and especially after racking off gross lees (1-month post-press and beyond) retained up to 77% and over 100% of protein precipitable tannin, respectively. One reason exogenous hydrolysable and condensed tannin additions are made at crush/during maceration is to avoid potential negative sensory impacts that later additions may have on a wine. If, however, the goal is to improve the hydrolysable and condensed tannin content and mouthfeel/complexity of a wine then this may be a more cost-effective solution.

The use of ACE at crush is an effective way to increase the amount of native condensed tannin extracted from pomace but may not be sufficient on its own as ACE only increased the amount of condensed tannin 2-3-fold over the control. When ACE was combined with exogenous condensed tannin additions retention remained higher relative to the control, indicating that combining condensed tannin treatments could be an

effective way to increase condensed tannin concentration without adding excessive amounts of exogenous condensed tannin.

Treating juice with bentonite was effective for removing protein from the juice, but when adding the fined juice back to the pomace, the protein level increased during fermentation. Condensed tannin levels may have increased slightly, but this time-consuming method is not recommended given the relatively small benefit.

The use of enzymes in aiding the breakdown of cell wall material after pressing also did not significantly improve retention of exogenous condensed tannin in the wine. Further exploration in the use of enzymes with ACE and exogenous condensed tannin additions is recommended to determine if one can improve extraction or retention of native and exogenous condensed tannins.

This study investigated possible ways to improve the hydrolysable and condensed tannin concentration in one cultivar, Chambourcin from one region, Missouri. Other cultivars and regions need to be studied for hydrolysable and condensed tannin extraction/retention to better understand if these methods can be applied broadly or if practices should be determined regionally.

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Table 1: Condensed tannin content of fruit. The condensed tannin content of grape berry components extracted in 70% acetone. Acetone was evaporated and made to the original volume with water. Extracted tannin was measured for protein precipitable tannin (Harbertson et al., 2002a). Condensed tannin content was reported in mg/g of protein precipitable tannin in berry components based on the method from Springer and Sacks, (2014).

Sample	mg of Condensed tannin/g Berry Component	Standard Error Mean
2018 cv. Chambourcin skins	0.42	0.05
2018 cv. Chambourcin seeds	0.99	0.05
2018 cv. Chambourcin pulp	0.03	0
2019 cv. Chambourcin skins	0.54	0.15
2019 cv. Chambourcin seeds	0.98	0.05
2019 cv. Chambourcin pulp	0.13	0.1

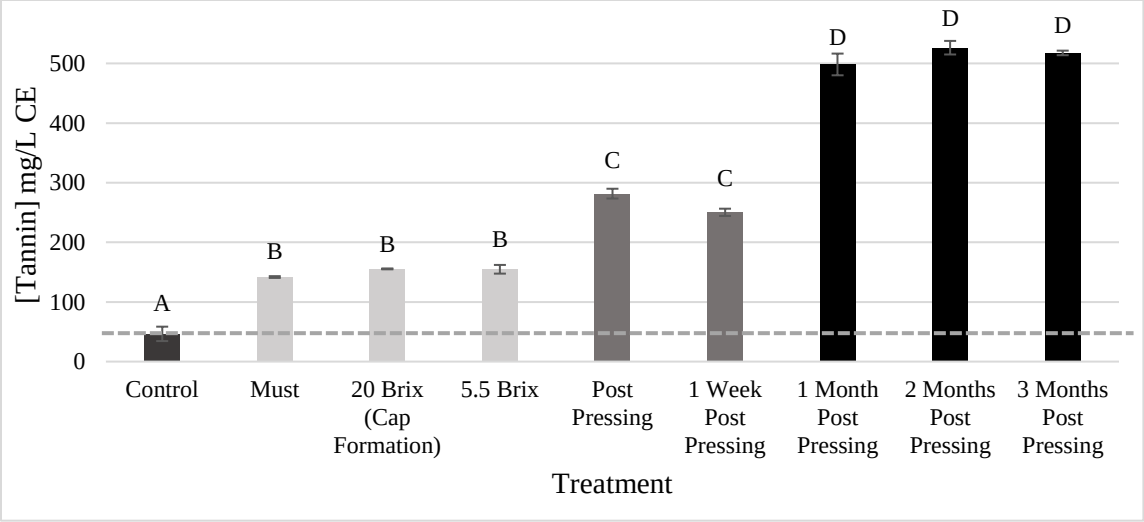

Figure 1: Condensed tannin content from exogenous condensed tannin addition timing. Exogenous condensed tannin addition timing condensed tannin concentration at 6 months post-press. 1400 mg/L (26% protein precipitable condensed tannin) of seed-derived exogenous condensed tannin made at various time points during the winemaking process. Condensed tannin measured via protein precipitable tannin assay at 6 months post pressing (Harbertson et al., 2002a). The dashed line is control level and means not followed by a common letter are significantly different according to Tukey-Kramer HSD.

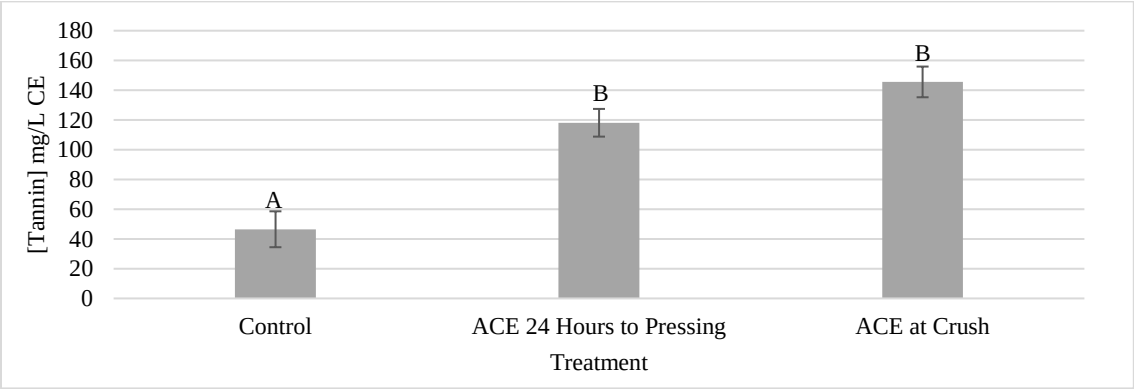

Figure 2: Condensed tannin content from ACE. ACE 2018 protein precipitable condensed tannin content at 6 months post-press. Accentuated cut edges (ACE) performed on cv. Chambourcin at Crush (ACE at Crush) and 24 hours to pressing (ACE 24 Hours to Pressing). Condensed tannin measured via protein precipitable tannin assay at 6 months post pressing (Harbertson et al., 2002a). Means not followed by a common letter are significantly different according to Tukey-Kramer HSD.

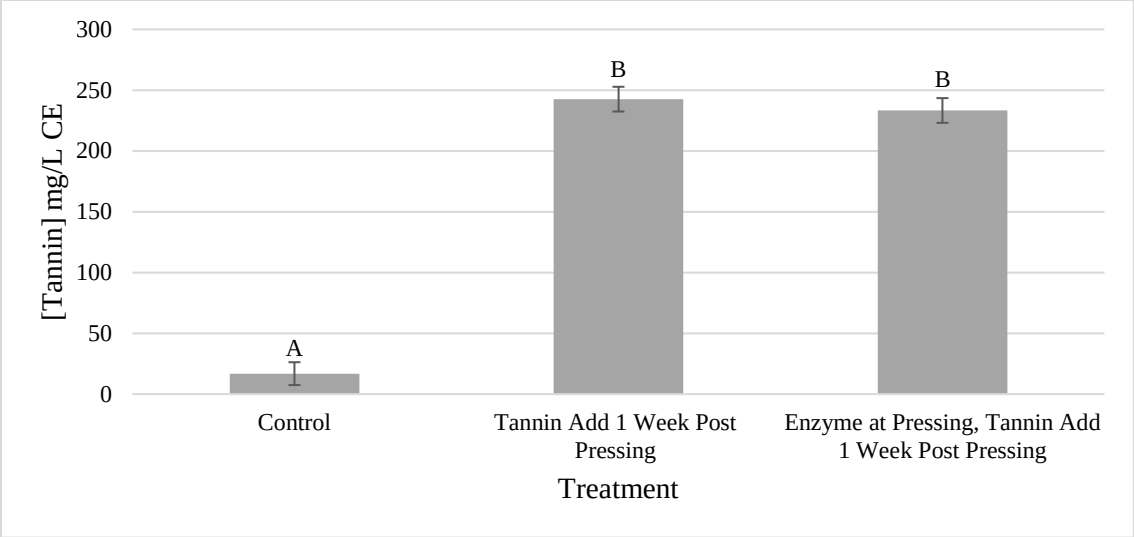

Figure 3: Condensed tannin content from enzyme pre-treatment at pressing. Protein precipitable tannin content at bottling (three months post pressing) of enzyme-treated wines at post-press. Enzyme addition of 0.379 g/L was added to enzyme treatment at pressing, then after one-week 1400 mg/L (26% protein precipitable tannin) of seed-derived exogenous condensed tannin was added (Enzyme at pressing, Condensed tannin Add 1 Week Post Pressing). At the same time, condensed tannin was added, a second treatment with only exogenous condensed tannin addition was completed (Tadd Add 1 Week Post Pressing). Condensed tannin content was measured via protein precipitable tannin assay at bottling (Harbertson et al., 2002a). Means not followed by a common letter are significantly different according to Tukey-Kramer HSD.

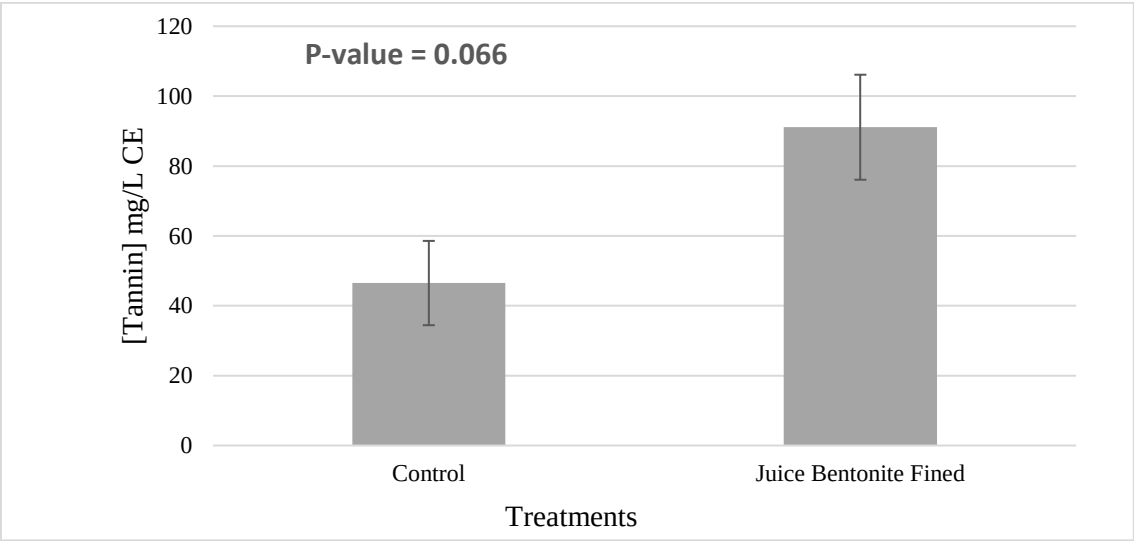

Figure 4: Condensed tannin content of bentonite treated juice. Juice of cv. Chambourcin was pressed and fined with hydrated Canaton bentonite (MoreFlavor, Pittsburg, CA) at a rate of 0.6 g/L and then racked off settled lees/bentonite. The juice was then added to the same pomace and fermented on skins the same as the control. Condensed tannin measured via protein precipitable tannin assay at bottling (Harbertson et al., 2002a). Mean comparison was performed with t-test ($p < 0.05$).

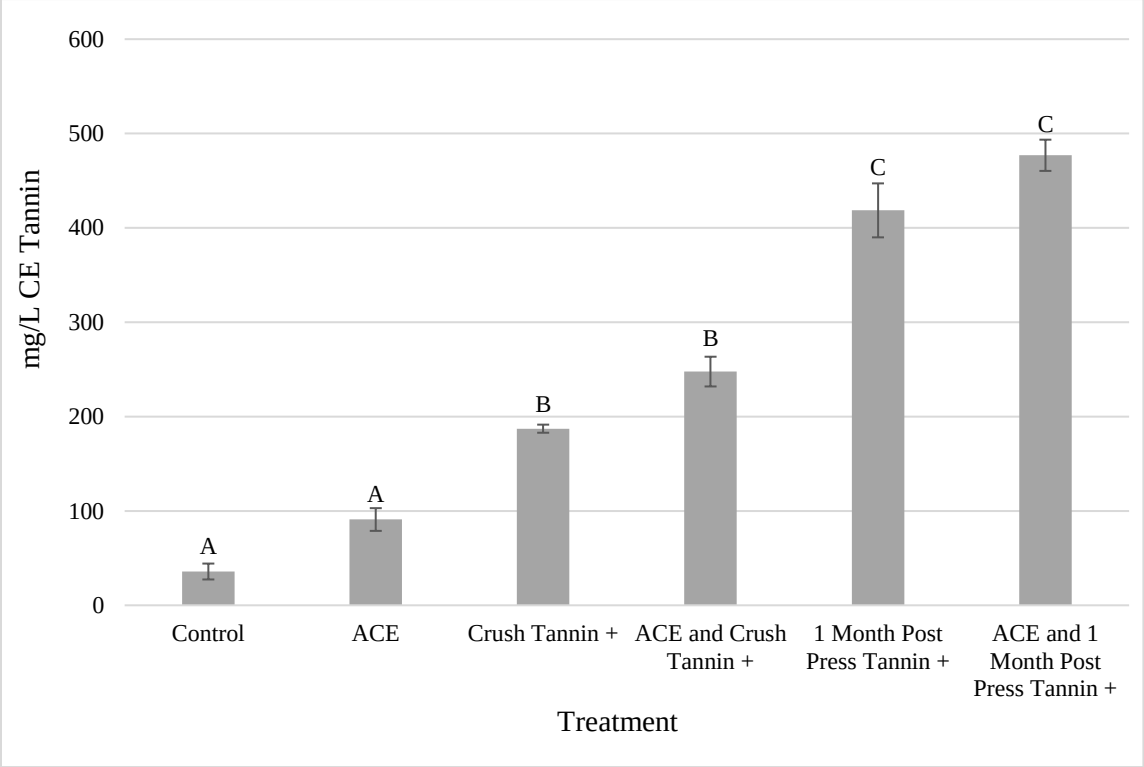

Figure 5: Condensed tannin content of exogenous condensed tannin addition and ACE. Combined exogenous condensed tannin addition timing and ACE. cv. Chambourcin in 2019 had 1400 mg/L (26% protein precipitable condensed tannin) of seed-derived exogenous condensed tannin made added at crush or 1 month post-press. Accentuated cut edges (ACE) was performed on cv. Chambourcin at crush. Combination treatments of exogenous condensed tannin additions and ACE were also performed. Condensed tannin measured via protein precipitable tannin assay at 8.5 months post-press (Harbertson et al., 2002a). Means not followed by a common letter are significantly different according to Tukey-Kramer HSD.

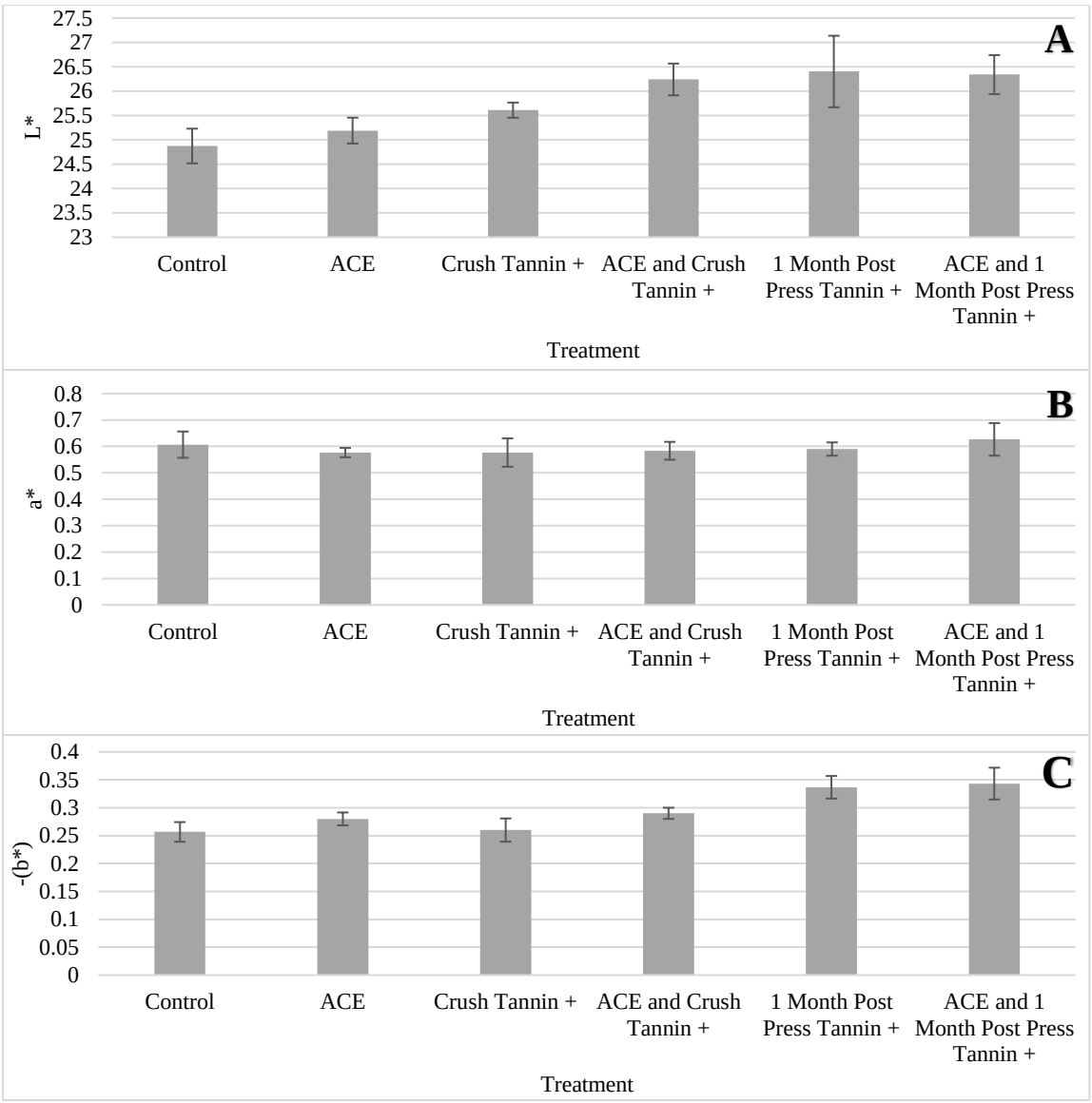

Figure 6: L*a*-(b*) values. L* (**A**), a* (**B**), and -(b*) (**C**) values of 2019 cv. Chambourcin combined exogenous condensed tannin addition timing and ACE treatments. The color of wine samples was measured by a Chroma meter CR-410 (Konica Minolta, Sensing, Inc., Japan). 30 mL of wine was placed in a 50mm cylinder and measured from the top of the cylinder 72 mm.

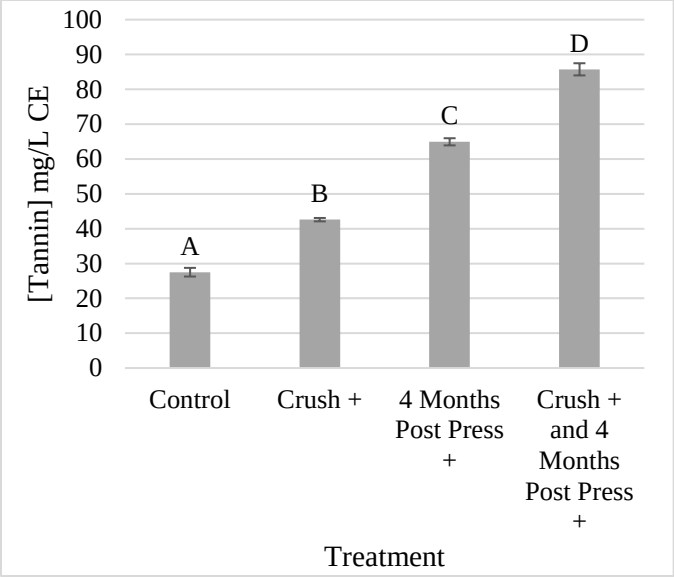

Figure 7: Hydrolysable and condensed tannin content from double exogenous hydrolysable tannin addition. Double exogenous hydrolysable tannin addition: 400 mg/L (63% protein precipitable tannin) additions of hydrolysable exogenous tannin made at crush (Crush +), 4 months post-press (4 Months Post Press +), or both addition time points (Crush + and 4 Months Post Press +). Hydrolysable and condensed tannin measured via protein precipitable tannin assay at 8.5 months post-press (Harbertson et al., 2002a). Means not followed by a common letter are significantly different according to Tukey-Kramer HSD.

VITA

Alex Fredrickson was born and raised in Tri-Cities, Washington around grapevines and wine (Columbia Valley). Although he showed little interest in winemaking as a child the seed was planted and as a Food Science student at the University of Idaho, he became interested in enology, in particular wine microbiology and chemistry. After receiving his B.S.F.S. in 2012 he worked his first wine harvest at E&J Gallo Winery in Healdsburg, CA where we developed a true passion for the industry. He then worked a second harvest at Church Road Winery in Hawkes Bay, New Zealand. During the harvest in NZ, his interest in the chemistry of wine grew and in particular understanding why certain processes were done in different wine regions that no one could scientifically explain, just that it makes the wine better.

Upon coming back to the USA, Alex began his graduate career in food science (enology) at Cornell University under the advisement of Anna Katharine Mansfield. Alex's research focused on exogenous condensed tannin additions and their impact on condensed tannin content and sensory attributes. Upon receiving his M.S. in 2015 he went back into the industry and was the harvest assistant winemaker at Canvasback Winery in Walla Walla, WA. In 2016 he worked a harvest in Tasmania, Australia at Moorilla Winery.

After completing the harvest, he started his own "urban farm winery" Wicked Water with co-owners Camila Tahim, and Megan Hall. After making award-winning wines, Alex was drawn back into research to obtain his Ph.D. in food science (enology) from the University of Missouri under the advisement of Misha Kwasniewski. Alex has recently accepted a position at E&J Gallo Winery-North Coast as an enology research

scientist. He hopes this will bridge his passion for winemaking and curiosity in research. He will begin his position at Gallo upon completion of his Ph.D.