CORRELATIONS BETWEEN TOTAL PROTEIN AND SPARKLING WINE FOAM PARAMETERS

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Ryan Michael Fifield

August 2021

ABSTRACT

Foam is a crucial factor in the perception of sparkling wine quality. Empirical data suggest that sparkling wines produced from grapes other than *Vitis vinifera* show greater foam height but this factor has received limited research attention. Marquette, Cayuga, and Chardonnay grapes were used to produce traditional method and still wine products. Still wines were treated with bentonite additions of 0.1 g/L , 0.3 g/L, and 0.5 g/L to create a range of protein concentration. Foam maximum volume, foam 50% collapse time, total protein, and malic acid were measured in all wines. Total protein had no significant impact on any foam parameters when traditional method and force-carbonated base wines were assessed together, but total protein had a significant effect on foam maximum volume in bentonite-treated wines, and foam stability showed a significant interaction between cultivar and total protein. Aspartic acid concentration had a negative correlation with foam volume.

BIOGRAPHICAL SKETCH

Ryan Fifield was raised in a town famous for dead grapevines (Anaheim), and took the necessary path to winemaking of earning a degree in English Literature before any scientific study (it is bottled poetry right?) Prior to grad school at Cornell he spent time as a burger flipper, bass player in garage bands, Hurricane Katrina shelter worker, group home caregiver, substitute teacher, a data entry clerk at an insurance company that insured other insurance companies (yes, it was boring), a behavior technician for autistic children, a bookseller, and many other jobs he can't remember. In other words, he was basically all over the place before finding wine. Since discovering the magical beverage that is wine, he has worked ten vintages throughout the beautiful state of California and completed enology programs at Santa Rosa Junior College as well as Fresno State. He would like to live long enough to simply make a wine of his own and serve it with a meal prepared from vegetables grown in the garden of an imaginary future home he shares with his amazing wife (and maybe some fresh baked bread as well).

ACKNOWLEDGMENTS

There are a lot of people to acknowledge for this work actually reaching completion. My name may be the one next that copyright symbol three pages back, but I find it extremely unlikely that I could have ever done this alone.

First, my advisor Anna Katharine Mansfield made it possible for me to be at Cornell at all and has been very patient with the stress and anxiety ridden Ryan that existed while here… so a major thank you for everything that she has done for me.

The rest of my committee, Justine Vanden Heuvel and Gavin Sacks, both were wonderful resources and just nice people that I liked talking to. I also very much loved getting the chance to almost duplicate the committee of a previous Cornell student that I admire.

Erin Norton, my analytical fairy godmother, was essential. She taught me the method for total protein analysis and facilitated permission from Keren Bindon at AWRI to allow me to use it for my work. One of the kindest ladies in the world, she never got annoyed with my constant questions about protein analysis.

The team at Agritech in Geneva, NY including Chris Gerling, Pam Raes, Cortni Stahl, Hannah Guchone, Molly Cappiello, and Luann Preston-Wilsey for assistance with getting wines made and lab equipment up and running.

Mohamed Hammami allowed me to use his foam analyzer (I really don't see how I could have done this work if he had said no).

Lynn Johnson in Cornell's Statistical Consulting Unit was the only reason that I was able to make R do anything that I wanted. I wouldn't have much to say in this paper without the stats and charts she helped me with.

Stephan Sommer at Fresno State was an excellent resource on EZ-faast protocol and general questions about amino acid analysis. I need to also thank Qun Sun at Fresno

State, the former Cornell student mentioned above, for encouraging me to apply to grad school in the first place and for always being happy for me each time I checked off one more requirement on my way to completing my degree.

I also, very unexpectedly, need to thank Randall Grahm. This was a terrible year. While he may have not been directly involved, I must admit that having one of my wine idols be so willing to chat on a regular basis throughout the entire pandemic kept me reminded of why I was putting myself through all of this work; a very bright spot in an extremely dark year that gave me some desperately needed motivation.

And lastly, my wife Nichole. I do not believe that it is a coincidence that since meeting her my career path has become more serious and that I have achieved more than I thought I was capable of. I am simply a better version of myself because I have her in my life, and I absolutely could not have made it through grad school without her support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 2:

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Sparkling wines effervesce when opened, and the foaming bubbles are an important component in the evaluation of their quality (Kemp et al. 2019). Examples of sparkling wine can be found throughout the world: French Champagne, Italian Prosecco, Spanish Cava, German Sekt, Cap Classique in South Africa, as well as other examples from the U.K., Australia, Canada, Russia, Hungary, and the United States (Buxaderas and López-Tamames 2012, Di Gianvito et al. 2019, Kemp et al. 2019). Producers of sparkling wines made from winegrape cultivars other than *Vitis vinifera* have reported excessive foaming and foam height when poured. Recent work supports the existence of overfoaming and documented greater foamability in interspecific hybrid-based, rather than in *V.vinifera*-based, wines (A. Lindstrom, unpublished data). Overfoaming can potentially impact repeat sales as a correlation has been demonstrated between analytical measures of foaming characteristics and perceived wine quality (Gallart et al. 2004). Aside from the difference in wine appearance, the level of effervescence has implications for the perceived aroma and the mouthfeel (Liger-Belair 2014). An upper limit on unacceptable carbonation does not appear to be documented, though typical sparkling wines can range from 2 - 12 g/L CO_2 (Kemp et al. 2019).

This study examined the potential causes of excessive foaming in non-*vinifera* wines.

1.2 Production Methods for Sparkling Wine

Sparkling wine can be produced in a variety of ways. The Méthode Champenoise used to produce Champagne in France goes by many names around the world outside of the Champagne region: classic method, traditional method, bottle fermented, and methode cap classique (Di Gianvito et al. 2019). In this method, a base wine is placed in a capped bottle with yeast and additional sugar to produce the carbon dioxide that will carbonate the finished product; the dead yeast, or "lees", remains in contact with the wine for a time before being removed; the finished product is sold in the same bottle that secondary fermentation occurred (Robinson 2006). A sparkling wine can undergo a secondary fermentation in this way or can alternatively can be force-carbonated and have carbon dioxide directly added to it like a soda to provide the bubbles (Culbert et al. 2017). In addition to the traditional method, the Charmat, transfer, and continuous methods also utilize a secondary fermentation. In the Charmat method, named for its co-creator Eugene Charmat, the secondary fermentation is completed in pressurized tanks that have stirring mechanisms for mixing up the yeast; wines made in this way will be filtered prior to bottling (Di Gianvito et al. 2019). The transfer method initially takes place in a bottle for secondary fermentation, but the wine is transferred to a tank after about two months in order to be filtered and bottled in a new bottle (Di Gianvito et al. 2019). The continuous method was developed in the former Soviet Union and is an adaptation of Charmat: base wine, sugar, and yeast enter a series of pressurized tanks where additional yeast is continually added, through tanks which contain material such as wood intended to retain dead yeast; and ultimately a relatively clear fizzy wine is

produced after a few weeks (Robinson 2006). There are also less common methods such as Méthode Ancestrale, or Pétillant Naturel, in which the base wine is simply bottled before all the sugar has been fermented to alcohol, and the small amount of sugar remaining produces a bubbly wine with less effervescence than the traditional method (Robinson 2006).

The choice of processing method will affect the composition and style of the final wine; those resulting from a bottle fermentation are typically described as being more sensorially complex (Culbert et al. 2017). Differences in the nitrogen content of wines relating to production choice have been found, with more amino acids in carbonated wines, and higher total protein in traditional method wines (Culbert et al. 2017, Condé et al. 2017b). The nitrogen content differences are potentially important, as greater foam stability has been observed in wines that did not undergo bottle fermentation (Culbert et al. 2017), and collar height, the height at which the foam lingers in the glass five minutes after being poured, has been found to be affected by choice of production method (Condé et al. 2017b). The different production methods also provide varying aging on the lees: traditional and transfer receiving months to years, Charmat only days, and carbonated potentially no time (Di Gianvito et al. 2019); this would lead to differences in the introduction of yeast derived proteins to the wine. In addition, there is great diversity found in wines with similar production methods but from different regions. For example, Cava and Champagne, wines both produced with in-bottle secondary fermentation, will have different foaming properties due to grape cultivar and regional regulations governing aging times (Pozo-Bayón et al. 2009). Aging requirements on Spanish Cava require 9 months, while French Champagne

requires 12 months (Di Gianvito et al. 2019). With these differences in mind, quality expectations for sparkling wine foam and bubbles will vary depending on region and method (Robinson 2006).

1.3 Foam formation and structure

Foam consists of gas bubbles trapped in a liquid or solid. In unopened bottles of sparkling wine, dissolved CO_2 reaches equilibrium with the CO_2 in the bottle headspace (Liger-Belair 2005). Sparkling wines are supersaturated with CO_2; when a bottle is opened, the CO_2 within the liquid is no longer in equilibrium with that in the headspace and it escapes (Liger-Belair 2005). Initial formation requires overcoming the energy of the Van der Waals forces between the liquid molecules; nucleation points allow the bubble to form (Liger-Belair et al. 2008). Bubbles that form in the wine will grow in size as they move towards the surface, as other molecules of carbon dioxide are absorbed during the trip up (Liger-Belair et al. 2008). Ultimate bubble size is related to length of travel time from bubble formation, as well as total concentration of dissolved CO_2 and wine temperature (Liger-Belair et al. 2008). Surfactants such as proteins and polysaccharides can adsorb to the rising bubbles, bringing them to the surface (Liger-Belair 2005). This is important, as major components of sparkling wine foam have been found to be proteins and polysaccharides (Brissonnet and Maujean 1991). As early as 1937, researchers noted the impossibility of foaming in 'pure' liquids (Blom 1937), as surface tension must be lowered to allow bubbles to form (Kemp et al. 2019). Although, surface tension alone does not explain potential foaming properties as it has been pointed out that solutions

of substances such as acetic acid will also lower surface tension, but do not readily foam (Preston and Richardson 2002). It has also been shown that a soap's foamability is not directly correlated with the surface tension in solution alone (Preston and Richardson 2002). Researchers have been able to predict a soap's observed foamability through a calculation involving surfactant concentration, surface tension, surface concentration, molecular weight, and diffusion coefficient (Verma et al. 2021); so, the surface tension is one of multiple factors that contribute to foaming. Proteins, having both hydrophobic and hydrophilic components, help create a film around escaping gas at the surface, and retain it within a liquid (Kannan et al. 2012). Not all escaping CO_2 will form a bubble or contribute to foam; some will simply escape from the bottle or glass of wine to the environment (Liger-Belair 2014). Factors such as wine temperature and the angle of the glass at pouring will considerably impact how much CO_2 escapes and how much remains to bubble out of the sparkling wine (Liger-Belair 2014). Wine components other than proteins and polysaccharides have been implicated in foaming, as model wines constructed with all major wine components minus the proteins, and known surface active molecules demonstrated less foamability but the same Bikerman coefficient. The Bikerman coefficient is a way of understanding a fluid's foam potential, found by dividing the volume of consistent foam a liquid is capable of producing by the flow rate of the gas used to sparge the sample (Robillard et al. 1993). This finding demonstrates that other components, such as ethanol and acids, may play a role in foam activity (Robillard et al.1993).

Once foam is formed, it has a finite lifetime and will be lost through Ostwald ripening, coalescence, and drainage (Medina-Trujillo et al. 2017a). Ostwald ripening describes

the diffusion of CO_2 from small bubbles which is then absorbed by larger ones (Medina-Trujillo et al. 2017a). Coalescence is when two bubbles become one due to the rupture of the wall separating them (Medina-Trujillo et al. 2017a). Variations in bubble size can place pressure on smaller bubbles, affecting how long they last (Robillard et al. 1993). Drainage is the loss of the liquid phase, including surfactants, around the gas bubble due to gravity (Medina-Trujillo et al. 2017a). Loss of surface molecules like proteins around the gas will cause a thinning of the layers around the bubble causing it to burst or to merge with a neighbor (Robillard et al. 1993).

1.4 Gushing

Excessive foaming of a carbonated beverage when opened is referred to as "gushing" (Vogt et al. 2017). Unlike the variations in style discussed above in regard to production method, gushing is considered a major defect in quality for carbonated beverages (Lusk 2016). Gushing affects sparkling wine around the world; an estimated two percent of German sparkling wine, for example, is affected by gushing (Kupfer 2018). Many reasons for this phenomenon have been suggested: cork dust, phenolic compounds, tartrate instability, incomplete riddling, and the presence of specific proteins (Kemp et al. 2015a). Bubble formation in wine requires a nucleation point, so the above mentioned molecules are hypothesized to provide opportunities for bubble formation. Gushing has been studied extensively in beer, and its causes in that industry are broken down into primary, from ingredients such as grains, and secondary, from production methods (Christian et al. 2011). It is believed that primary causes are in many cases connected with fungal infections of species of

Fusariam, *Aspergillus*, and *Penicillium* in raw materials (Vogt et al. 2017). *Penicillium* strains were also found to be capable of inducing gushing in sparkling water (Vogt et al. 2017). The specific cause of this gushing was identified as hydrophobins, which are proteins produced by fungi that were found at the surface layer of the beverages (Vogt et al. 2017). In addition to fungal derived hydrophobins, beer gushing may also be caused by lipid transfer proteins, which are plant derived (Hippeli and Elstner 2002). Secondary causes of gushing in beer are related to processes that will create or leave metal ions, calcium, or other particles in the beverage (Christian et al. 2011). Fatty acids have also been assessed for potential to cause gushing in beverages; those with a chain length of at least twelve carbons showed potential to produce gushing, while smaller chains did not (Christian et al. 2011). A study of *Botrytis'* relation to gushing found that wine made from infected fruit contained less total protein, and specifically lower amounts of pathogenesis-related proteins (PRPs) (Kupfer et al. 2017a). It has also been observed that fruit infected with *Botrytis cinerea* impacted the yeast derived protein PAU5 (Kupfer 2018). Research on gushing has shown that wines lacking PAU5 had more unstable foam, and greater likelihood to gush; nine out of ten bottles lacking the protein in a recent study displayed gushing (Kupfer 2018). *Botrytis* infection has also been correlated with gushing in another study, although without being able to correlate that with a significant difference in total protein content between infected and control fruit (Marchal et al. 2001). That study, however, used the Bradford method which has been criticized for inaccurate protein level measurement, and which does not identify specific proteins (Gazzola et al. 2015, Condé et al. 2017b, Liu et al. 2018).

1.5 Measuring Foam

Sparkling wine foam is generally discussed in terms of foam height and stability (Kemp et al. 2019). These characteristics have been measured by a variety of approaches that can be grouped into two main categories: those that sparge wine to measure foaming potential visually or with sensors, and those that replicate pouring to assess foam in a glass. The second group tends to incorporate more technology, such as the use of robotic arms to uniformly pour the wine and cameras for visual analysis (Kemp et al. 2019).

The Bikerman method, though uncommon, is notable as one of the earliest sparging methods; the coefficient obtained from the method is calculated by sparging a sample to achieve a stable foam volume for at least 2 minutes, then dividing foam height by the flow rate of gas required (Robillard et al. 1993). The ratio was established by Bikerman as a better way to describe a fluid's foaming potential than foam height alone (Bikerman 1938). Key findings using the Bikerman coefficient include the ability to predict and adjust juice foam height and Bikerman coefficient through a calculation involving proteins, acids present, glycerol, sugars, and SO_2 addition (Lopez Barajas et al. 1997). The foamability of finished still wines was later found to be similarly predictable (López-Barajas et al. 1999). Predicting final sparkling wine foam is more difficult, however, as there no direct correlations have been found between base wine and the final product when a secondary fermentation is used (Esteruelas et al. 2015).

The Mosalux apparatus method is similar to Bikerman but incorporates additional technology. It is technically an adaptation of the method developed by Rudin for measuring beer foam (Kemp et al. 2019), although Rudin still cites Bikerman as a source for the method (Rudin 1957). The apparatus runs a constant 7L/h flow of CO_2 through a glass cylinder containing the wine sample; infrared sensors capture the foam height and a computer is used to analyze the data obtained (Brissonnet and Maujean 1991). Current versions of the Mosalux include the ability to record values for foam stability, foam expansion, the average lifetime of bubbles, and the Bikerman coefficient (Kemp et al. 2019). The Mosalux device has been criticized for misrepresenting the actual foam height, and for inconsistency due to artifacts from previous samples (Robillard et al. 1993). Attempts to improve the method by fully sparging the glass cylinder with gas prior to each run improved repeatability for some (Robillard et al. 1993), while others still found the same inconsistencies (Gallart et al. 1997). The sample preparation required for Mosalux (complete degassing and filtration) can also lead to contradictory results when compared to other methods of foam analysis, such as the Computerised Artificial Viewing Equipment (CAVE) (Cilindre et al. 2010).

The Kruss DFA 100 foam analyzer is another sparging method that has been used in studies; it is very similar in concept to the Mosalux but requires one fifth of the sample size (Liu et al. 2018).

The Computerised Artificial Viewing Equipment (CAVE) system incorporates a robotic arm pourer, laser detectors, and multiple cameras to capture foam

characteristics (Cilindre et al. 2010). This system can capture foam speed, thickness, and final height (Kemp et al. 2019). FIZZeyeRobot is similar but goes slightly further than CAVE with algorithms that more rapidly and precisely interpret the foam images (Condé et al. 2017a).

As no single method of foam analysis is universally popular, it is difficult to compare results of different studies. A study of sparkling wine foam response to increasing sugar found a significant positive correlation to foam height when measured on a Mosalux, but this response was not seen when the same wine's foam was analyzed by a pouring method (Crumpton et al. 2018).

1.6 Wine proteins and foam characteristics

Food foams are stabilized when specific proteins are present: dairy foams are stabilized by the whey protein β-lactoglobulin, and egg foams from the albumin proteins ovoglobulin G2 and G3 (Brady 2013). The importance of surfactants like proteins in wine foaming has similarly been demonstrated in a number of studies. Filtered wines display less foaming ability (Robillard et al. 1993). While polysaccharides have also been shown to be present and play an important role in the surface layer of sparkling wines (Aguié-Béghin et al. 2009), the necessary presence of proteins specifically has been demonstrated by multiple researchers. A wine stripped of all proteins was incapable of producing any measurable foam (Vincenzi et al. 2014), and partial removal of total protein through bentonite reduced foam height and stability (Dambrouck et al. 2005, Vanrell et al. 2007). Lao found that pectic enzymes,

which alter the protein makeup of a wine, generally decreased both foam height and stability, as treated wines tended to have lower levels of protein and more phenolic compounds (Lao et al. 1999). Wines made from *Botrytis*-infected fruit contained less protein and displayed poor foamability (Cilindre et al. 2007). In addition to protein alterations, wines made from different grape cultivars have shown different foaming characteristics, which may be attributed to greater protein and amino content (Moreno-Arribas et al. 2000). As specific proteins have been found to play a role in beer foaming, the focus on protein content and wine foam isn't surprising.

The correlation between total protein content and expected foam characteristics is complicated, however, as research results have proven contradictory. Positive correlations have been found between greater protein levels and foamability (López-Barajas et al. 1998), as well as foam stability (Pueyo et al. 1995). In contrast, other researchers found no connection between total protein and foam height (Condé et al. 2017b). Correlations between time of foaming and protein have similarly been found to be both negative (Andrés-Lacueva et al. 1996) and positive (Condé et al. 2017b). Such contradictory results may be due to analytical method. Many of the studies on sparkling wine that attempted to correlate total protein with foaming characteristics did so through one of two methods: the Bradford method or the bicinchoninic acid assay. The Bradford method of protein analysis, though cited in many studies, is said to overestimate total protein and may lead to inaccurate correlations (Gazzola et al. 2015, Condé et al. 2017b, Liu et al. 2018). The KDS/BCA assay correlates much better with actual total protein present (Vincenzi et al. 2005, Gazzola et al. 2015), although it is still not free from criticisms and interferences, discussed below.

Total protein content may also be difficult to correlate with foaming as it does not provide proportions of specific proteins. Work done on protein in haze formation found that total content was a poor indicator of haze, as certain fractions had a larger impact (Moretti and Berg 1965). It was later found that in addition to specific protein fractions, the concentration of other substances such as sulfate, acids, phenolics, and polysaccharides all had an influence over how proteins would aggregate (Van Sluyter et al. 2015).

Similar to the progression of research on hazing, the influence of separate protein fractions on foaming is undergoing increased scrutiny. Wine protein fractions have shown stronger or weaker correlation with foam height depending on their size (Esteruelas et al. 2015). Studies in model wine suggest that changes in the concentrations of various protein fractions do not impact foam equally, and that a synergistic effect of grape and yeast proteins creates better foam than either protein alone (Coelho et al. 2011, Vincenzi et al. 2014). The contribution of yeast protein was also demonstrated by Medina-Trujillo, when wines sequentially inoculated with *S. cerevisiae* and *T. delbrueckii* produced more polysaccharides in the base wine and greater foam height in the final sparkling wine than wines made with *S. cerevisiae* alone (Medina-Trujillo et al. 2017b). Specific proteins produced by yeast, like PAU5 in *S. cervisiae*, have shown very strong correlation with foam stability (Kupfer et al. 2017b). It is currently understood that fractions with the ability to get to the surface of the liquid more quickly play a larger role in foam formation; stability appears to be connected with the ability of the proteins to form strong bonds (Condé et al. 2017b). To understand protein's influence on foaming properties, it is necessary to first

characterize proteins present, then separate the individual and cooperative effects of grape and yeast derived proteins to foam quality.

1.7 Wine protein content

Total protein content of a finished wine can vary by cultivar, vineyard location, and annual environmental conditions (Bayly and Berg 1967). Supplementing nitrogen in the vineyard has a positive correlation with the total protein found in the resulting wine, as well as the amount of bentonite needed to eliminate protein haze (Spayd et al. 1994). In addition, the ratio of protein bands (the block groupings of proteins of the same size in gel separation techniques) is similar within the same cultivar but varies among cultivars (Pueyo et al. 1993). During the growing season grape protein concentrations increase rapidly between veraison and harvest (Robinson et al. 1997), as protein accumulation mirrors sugar accumulation, and the concentration and composition of total protein varies by maturity (Murphey et al. 1989). Notable accumulation of protein pre-veraison has been seen with fungal infection, which appears to induce pathogenesis-related protein accumulation in green berries (Monteiro et al. 2007). PRPs account for roughly 75% of total protein found in mature grapes (Monteiro et al. 2007), and invertase, the enzyme responsible for cleaving sucrose into glucose and fructose, has also been found to be a major wine protein (Jégou et al. 2009). Even while PRPs tend to be most abundant, the diversity of PRPs found in the berries decreases considerably at harvest (Monteiro et al. 2007). Studies have found that grapes in riper vintages had more total protein, and a notable increase of only specific low molecular weight proteins such as thaumatin-like proteins (TLPs)

and chitinases (Esteruelas et al. 2015). Additional studies agreed that PRPs increased as fruit ripened (Giribaldi et al. 2007), and total proteins increased in grapes harvested at multiple maturity levels, but not equally for all fractions: TLPs and chitinases increased in relative proportion, while others remained fairly stable in concentration, decreasing in relative proportion (Liu et al. 2018). Similarly, a recent study showed a marked increase of TLPs in the weeks just before harvest (Tian et al. 2019), possibly as a defense against fungal attacks on the nearly ripe fruit. It has been speculated that high levels of hexose sugars may induce defense genes that cause higher protein concentrations (Monteiro et al. 2007). Ripening fruit with fungal infections are also known to contain greater levels of TLPs and chitinases (Ferreira et al. 2001).

Seasonal differences and stresses are important in determining final protein content. A study tracking the same vineyard in different vintages found that the year with lower ripeness and less risk for powdery mildew had more than double the diversity of proteins, but far less total protein (Monteiro et al. 2003). Pesticide sprays have been shown to alter protein content in the leaves of grapevines, and water stress has been observed to alter which proteins are present in the skin and pulp (Giribaldi and Giuffrida 2010). Vines that experience water stress have also been found to produce fruit with higher protein levels (Meier et al. 2016). Yield has also been shown to play a role in protein content: Cava vineyards with higher yields had higher levels of protein, and lower levels of polysaccharides, than their lower-yielding counterparts (Riu-Aumatell et al. 2002)

Additional changes in protein content occur during picking and processing. The juice from hand harvested fruit contained less total protein, and specifically fewer chitinases, than machine harvested fruit (Pocock et al. 1998), and protein fractions in grapes decreased during crush (Hsu and Heatherbell 1987). Protein content in musts of *V. vinifera* grapes ranged from 181 to 787 mg/L when using a 6.25X conversion from Kjeldahl total nitrogen (Santoro 1995).

Not all proteins present in harvested grapes survive fermentation and processing to appear in the final wine (Ferreira et al. 2001). Soluble protein content decreased by as much as 70% after fermentation (Yokotsuka and Singleton 1997). Changes in specific protein fractions have been indicated by variations in heat instability throughout fermentation (Vincenzi et al. 2011). Fractions may be "lost" or precipitated out through interactions with phenolics, enzyme degradation, and isoelectric points that create insolubility at wine pH (Ferreira et al. 2001). Reports of typical wine values vary, ranging from 10 to 275 mg/L in some accounts when the Bradford method is used with a BSA standard (Ferreira et al. 2001, Murphey et al. 1989), to as high as 500 mg/L when quantified through 1-D electrophoresis using BSA as a standard (Sauvage et al. 2010). Researchers have found that protein fractions identified in wines following primary fermentation could be traced back to grape, rather than yeast, origin, and were primarily chitinases and TLPs (Sauvage et al. 2010, Ferreira et al. 2000, Luguera et al. 1997), though some studies also identify grape vacuolar invertase as a major wine protein (Dambrouck et al. 2005, Blasco et al. 2011, Liu et al. 2018). Although thaumatin is known to be quite sweet, the TLPs that wind up in the final wine have not been found to contribute detectable sweetness at typical levels (Flamini

and De Rosso 2006). The proteins found in wine that can be traced back to the grape are fairly small in size; thaumatin-like proteins ranging from 18-24kDa, chitinases, 27-35kDa, and grape invertase, 66 kDa (Dufrechou et al. 2012). Liu found that measurable amounts of proteins believed to originate from yeast cell walls were seen in some, but not all, wines post primary fermentation, and speculated that initial YAN may play a role (Liu et al. 2018). Higher YAN can increase yeast biomass, leading to more yeast derived proteins later (Liu et al. 2018). In *V. vinifera* sparkling wines changes in protein content affect specific proteins more than others; concentrations of chitinases and TLPs are much lower in finished sparkling wine than in the base wine, while invertase stays nearly unchanged (Le Bourse et al. 2011). Any fining, such as bentonite, that occurs following primary fermentation will also alter protein content in the wine. Bentonite has been observed to adsorb proteins of different sizes at different rates, so changes will not be the same in all wines (Santoro 1995).

Secondary fermentation for sparkling wine production also alters final protein content through the inclusion of proteins released during yeast autolysis (Alexandre and Guilloux-Benatier 2006). The amount of time aging on lees will affect the protein content in the final wine (Alexandre and Guilloux-Benatier 2006). Limited aging (3-6 months) increases concentration of amino acids and some polysaccharides; at 9-12 months of aging, yeast cells are mostly releasing proteins (Alexandre and Guilloux-Benatier 2006). It should be noted that lower pH and colder storage temperatures slow this process (Alexandre and Guilloux-Benatier 2006).

1.8 Wine Amino Acid Content

In addition to protein, free amino acid content also varies by wine. Early in the growing season, the major amino acid is glutamine (Stines et al. 2000). Post veraison, proline levels increase markedly, prompting some researchers to suggest that the amino acid is used to offset osmotic stress during heavy sugar accumulation (Stines et al. 2000). The mature pulp of the berries of *V. vinifera* grapes, and the resulting wines, have proline and arginine as the most abundant amino acids (Stines et al. 2000). Seeds, however, have far less proline and arginine (Stines et al. 2000). A study of amino acid content in finished wines found that proline content accounts for anywhere from 30 to 85% of the amino acids present (Lehtonen 1996). The ratio of the dominant amino acid content has been found to vary by cultivar, a fact known since at least 1970 when Kliewer published a paper grouping grapes by their dominant amino acid (Kliewer 1970). Within *Vitis vinifera*, grapes like Cabernet Sauvignon (a proline accumulator) tend to have higher proline and others, like Gewurztraminer (an arginine accumulator), have higher arginine (Stines et al. 2000). The amino profile of non-vinifera species do not follow this pattern; the dominant aminos have been found to be different in each species (Lamikanra and Kassa 1999). While proline and arginine levels are still high, the aminos at highest concentration in *Vitis rotundifolia* were found to be histidine and alanine (Lamikanra and Kassa 1999). *Vitis labrusca* tends to have alanine as its most abundant amino acid (Lamikanra and Kassa 1999). Grapes that have alanine as one of their most abundant amino acids are typically assumed to have some amount of American parentage in their DNA (Lamikanra and Kassa 1999). Vineyard shading has been found to reduce the concentration of most amino acids in

grape berries (Guan et al. 2017). A correlation has also been established between amino acid content and anthocyanins; grapes with higher amino acid content tend to have lower color (Guan et al. 2017). More skin contact during winemaking was found to decrease amino acid levels, likely due to increased phenolic extraction (Gornischeff et al. 2020).

Specific amino acids have been implicated as playing a role in foam characteristics in sparkling wines, although reported effects vary. Asparagine has been correlated both negatively (Condé et al. 2017b) and positively with foam stability (Martínez-Lapuente et al. 2015). Tyrosine has been reported in one study as having a potentially positive effect on foam stability (Condé et al. 2017b).

Aside from potential effects on foaming characteristics, specific aminos can also play a role in aroma (Alexandre and Guilloux-Benatier 2006). Threonine, for example, is a precursor for the lactone sotolon, which is found in some sparkling wines (Alexandre and Guilloux-Benatier 2006).

1.9.1 Protein Measurement: Isolation and Purification

Prior to any measurement of protein, there is typically a step to precipitate protein out of solution and a second to clean or purify the obtained sample. Protein precipitation is commonly achieved by "salting out" with ammonium sulphate, by addition of organic solvents like ethanol and methanol, or by addition of acids such as trichloroacetic acid. (Moreno-Arribas et al. 2002). For the salting out approach, the anions and cations in salts interact with the water, which decreases the availability of

water to interact with the proteins, and allows for portions of opposite charge within the protein to interact, leading to precipitation (Novák and Havlíček 2016). Ammonium sulfate is commonly used because it splits into a cation and anion. Both are on the higher end of the Hofmeister series (Duong-Ly and Gabelli 2014), a ranking of ions by their influence on different processes in an aqueous solution; protein folding is a prominent one (Zhang and Cremer 2006). Ethanol addition works for precipitation because it decreases the polarity of the wine environment that the proteins are in, and they become less soluble as a result (Esteruelas et al. 2009). The major driving forces behind protein folding, and the stability of that folding, are hydrophobicity and optimal interactions of side groups; altering the environment to be less polar will undo that folding (Pace et al. 2004). Precipitation is generally followed by centrifugation or filtration to separate the soluble components from the now insoluble ones (Moreno-Arribas et al. 2002). The non-protein substances or lower weight components that are settled out with the proteins may also be removed prior to protein quantification. Dialysis against water, followed by freeze drying to concentrate the sample, is commonly used, and is considered to give good results (Moreno-Arribas et al. 2002).

1.9.2 Protein Measurement: Detection

Wine protein analysis has been constantly evolving in the hope of finding a universally accepted method, as many commonly used protein quantification methods have later been found to have interferences or to be unsuitable for wine analysis.

The Kjeldahl method of nitrogen determination is the legal standard for protein measurement in food products in the United States (Regenstein et al. 1984). Kjeldahl involves the digestion and distillation of a sample, nitrogen components being converted to ammonia in the process, and then a titration with acid (Sáez-Plaza et al. 2013). This method has been used at times to measure nitrogen in wine in a number of papers, for example: for total nitrogen (Kliewer 1970); as a benchmark to compare to other protein quantification assays (Smith et al. 2011); and as a way of testing out abilities of new equipment (Cerovic et al. 2015). It has however been pointed out that the Kjeldahl method is not ideal for measuring total protein in wines, as there are other non-proteinaceous compounds in wine that contain nitrogen (Condé et al. 2017b) which may return an erroneously high protein value (Brady 2013).

The Dumas method is an alternative to Kjeldahl based on sample combustion producing nitrogen oxides which are then titrated (Brady 2013); this is the official OIV method for quantifying total nitrogen in wines (OIV 2014). As both Dumas and Kjeldahl are total nitrogen and not a direct measure of proteins, the weaknesses of Kjeldahl for wine protein measurement apply to Dumas as well. Both tests require extraction prior to analysis to ensure that the nitrogen values obtained reflect only protein, and not other nitrogen within the wine. Also, a comparative study of Kjeldahl and Dumas in beer found that Dumas consistently gave higher results (Buckee 1994).

Color based assays that measure a change in color with a UV-spectrometer have made up the bulk of the methods used with wine research in the last few decades. The colorimetric biuret reaction of copper and protein peptide bonds forms the basis for a

few protein assays (Smith et al. 1985, Sapan et al. 1999). The exact composition of the proteins measured is not a factor in the binding and color formation with biuret (Sapan et al. 1999), and interferences include ammonium, glycerol, and Tris(hydroxymethyl)aminomethane (Schaffner and Weissmann 1973).

Lowry adapted the biuret method in a paper that became "the most highly cited paper in publishing history" (Kresge et al. 2005) when he added the Folin-Ciocalteau reagent (Smith et al. 1985, Kresge et al. 2005) making it 100X more sensitive than the biuret reaction (Schaffner and Weissmann 1973). Lowry's adaptation displays variability in the response to different proteins; the amino acids tyrosine and tryptophan play a large role in color formation (Sapan et al. 1999). The Lowry test is hampered by a slower reaction time, some unstable reagents, and a non-linear standard curve (Peterson 1979), and phenolic compounds have been found to interfere with results (Weetall et al. 1984).

Smith further improved sensitivity of the Lowry method by replacing the Folin-Ciocalteau reagent with bicinchoninic acid (BCA), which complexes with reduced copper (Smith et al. 1985). The BCA method shows a similar specific response and reaction based on protein composition found in assays like Lowry (Sapan et al. 1999). BCA reagent reacts with cysteine, cystine, tryptophan, and tyrosine; tri- and tetrapeptides not containing these amino acids also produced color change (Wiechelman et al. 1988). In comparison with assays like Lowry or Bradford, BCA has small protein-to-protein variation in response intensity (Reichelt et al. 2016). Precipitation with potassium dodecyl sulfate (KDS), typically used with BCA, does

not provide consistent quantification of glycoproteins: in some cases, as little as 15% of added glycoprotein was precipitated out (Smith et al. 2011). Interferences with the BCA method include phenolic compounds and reducing sugars such as glucose and fructose (Sapan et al. 1999, Smith et al. 2011). Other substances, such as phospholipids and biogenic amines, have also been found to cause errors (Reichelt et al. 2016). Depending on the choice of standard for BCA (BSA or another protein like yeast invertase), reported protein content can also vary widely (Smith et al. 2011). At high protein levels, peptides compete with BCA in binding to cuprous ion; as a result, absorbance levels will plateau and begin to decline with rising protein, giving false values (Huang et al. 2010).

Amido Black is another assay that attempted to overcome the interferences with biuret and Lowry, while still allowing a rapid quantification of proteins (Schaffner and Weissmann 1973). The assay makes use of trichloroacetic acid to precipitate the proteins, and staining with amido black acidic dye (Schaffner and Weissmann 1973). Amido Black is sensitive enough to allow for sample dilution to decrease interference, and is not affected by ethanol, phenolics, or glutathione; it is subject to potential interferences from polysaccharides (Weiss and Bisson 2001). It does not, however, have the same response level in red wine and juice as in water or white wine and juice (Weiss and Bisson 2001).

The Bradford method has often been used for quantifying total protein in wine studies (Marchal et al. 1997). The rapid test was designed as an improvement on the Lowry method, with its many interferences (Bradford 1976). The assay is based on the

binding of Coomassie brilliant blue dye and proteins, which will create a blue color that can be read on a spectrometer (Bradford 1976). The protein and dye complex has been noted to rely heavily on protein composition; there are strong reactions with lysine and arginine (Sapan et al. 1999). Coomassie Brilliant Blue exists as three forms: neutral, cationic, and anionic; anionic being the form that interacts with proteins (Marchal et al. 1997). The Bradford method has many interfering substances that alter results, with ethanol and phenolic compounds being the major ones in wine; these have been found to alter the absorbance by as much as 90% (Marchal et al. 1997).

A study that compared the Bradford assay's ability to quantify protein levels in spiked wines with an alternative method of hydrolyzing all proteins and summing the resulting amino acids present found similar weaknesses with the assay; summing amino acids resulted in a fairly consistent 91% recovery rate while Bradford's recovery rate varied with protein fraction from 20-50% (Waters et al. 1991). A non-wine study that compared the results of summing amino acids from hydrolyzed proteins with the Bradford, the BCA, and Lowry methods found that glycosylation of proteins was a major factor in test accuracy; non-glycosylated proteins gave results in a similar range for all tests (though slightly lower for Bradford and slightly higher in Lowry and BCA), while in glycosylated proteins the Bradford was at times half of the summed amino acids, and both the BCA and Lowry were up 60% higher (Fountoulakis et al. 1992). While there is disagreement among researchers about the percentage of proteins in wine that are glycosylated (Ferreira et al. 2001), their

presence would make many of the colorimetric methods of protein analysis less than ideal for wine studies.

Total protein analysis in wine by way of summing released amino acids after protein hydrolysis is not a common method in the literature, but the approach does appear to provide excellent recovery values (Waters et al. 1991). This approach requires first a hydrolysis step to break down the molecule, followed by some form of chromatography (Fountoulakis and Lahm 1998). It has been pointed out that variations or inaccuracies in protein results are mostly due to errors at the hydrolysis step (Fountoulakis and Lahm 1998). The different methods of hydrolysis make use of different amounts of time, temperature, hydrolyzing agents; the specific amino acids recovered will vary by hydrolysis approach (Fountoulakis and Lahm 1998). Hydrolysis is performed chemically, using either acid or base, or enzymatically (Fountoulakis and Lahm 1998). Complete hydrolysis by enzymatic methods typically requires the use of multiple enzymes; as their cleaving can be specific and needs long incubation times, chemical options have been used more frequently (Fountoulakis and Lahm 1998). Options for chemical hydrolyzing agents include HCl, methanesulfonic acid, *p*-toluenesulfonic acid, NaOH, and KOH, although the alkaline options are typically only used when quantification of tryptophan is desired (Fountoulakis and Lahm 1998). Of these, hydrolysis by hydrochloric acid is the most common method and has the advantage of evaporating off to allow for analysis of very small amounts of protein (Fountoulakis and Lahm 1998). The conditions required for acid hydrolysis of proteins do not allow for extraction of all amino acids (Otter 2012). Conventional acid hydrolysis, which is 6 M HCl at 110°C for 24 hours, destroys tryptophan

completely, tyrosine partially, and makes cysteine unmeasurable (Fountoulakis and Lahm 1998). Adaptations and alterations are possible with the conventional approach to achieve similar results; reaction rate will double every 10°C increase, so 160°C for 45 min equates to 110°C for 24 h (Fountoulakis and Lahm 1998). Prior to hydrolysis, proteins should have interfering substances removed; common methods for isolating proteins from a solution being precipitation, or dialysis and filtration (Le Bourse et al. 2010). It is most effective to dialyze the protein solution against water, especially with small proteins, as it increases reproducibility (Fountoulakis and Lahm 1998).

1.10 Quantifying Amino Acids

Historically, amino acid identification started with the discovery of aspartic acid in asparagus in the year 1806 (Toyo'oka 2013). Amino acid analysis began much later, with the work of Moore and Stein in the 1950's; they made use of a post column derivatization using ninhydrin and were awarded the Nobel Prize in 1972 for their work (Toyo'oka 2013). The ion exchange method they developed has been the standard against which new methods are compared (Hare et al. 1985). When the traditional method of Moore and Stein has been used in wine studies, the approach has been found to give reliable results but requires long analysis times and provides limited sensitivity (Callejón et al. 2010).

Chromatographic separation techniques that have been used in amino acid analysis include ion-exchange chromatography, reversed-phase chromatography, hydrophilic interaction chromatography, and gas chromatography; capillary electrophoresis and microchip electrophoresis have also been employed (Toyo'oka 2013). The ion

exchange of Moore and Stein was generally replaced in lab work by pre-column derivatization and reverse phase HPLC for amino acid analysis (Fountoulakis and Lahm 1998).

As an initial step in quantification, amino acids are derivatized and then analyzed by a chromatographic method (Toyo'oka 2013). Derivatization options vary by analytical method, and timing (before or after separation) can be different based on chromatographic method. In unknown complex mixtures, the post column derivatization used in liquid chromatography (though not possible in gas chromatography) has the advantage of removing any interfering analytes prior to the reaction (Fountoulakis and Lahm 1998). Ninhydrin is still considered the standard, but is not very sensitive; fluorescamine and OPA have been used as alternative post column options, and while more sensitive, neither provides measurements of proline (Fountoulakis and Lahm 1998).

Many wine studies have made use of liquid chromatography for amino acid analysis, as Lehtonen's review paper made clear by stating that liquid chromatography has been "the most important technique" (Lehtonen 1996). Despite the common use of liquid chromatography in studies, gas chromatography is also a good choice for amino acid analysis as it has high resolution and results tend to be reproducible on different instruments (Otter 2012). GC analysis has also been found to provide results comparable to ion exchange (Fountoulakis and Lahm 1998). In gas chromatography, derivatization makes molecules less polar and more volatile, leading to better separation (Bruheim et al. 2013). Derivatization of amino acids prior to gas chromatography removes most, if not all, of the active hydrogens atoms from reactive

groups; this process should be done as quickly as possible (Hušek 1998). Derivatization for GC has been primarily performed by one of two methods: alkylation or silylation (Kvitvang et al. 2014). Alkylation involves the replacement of an active hydrogen atom in side chains with an alkyl group; chloroformates are very commonly used alkylation derivatizing agents (Kvitvang et al. 2014). Chloroformate based derivatives have been found to work very well with GC as they esterify rapidly in liquid and form very stable derivatives (Bruheim et al. 2013). Their comparative low cost and ability to derivatize in a matrix of other substances without removal of water make them very good options (Hušek 1998). The methyl, ethyl and isobutyl chloroformates (MCF, ECF, IBCF) have been commonly used (Hušek 1998). Silylation agents include N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA); these are less than ideal options in amino acid analysis as their derivatives tend to be unstable (Krumpochova et al. 2015). Alkylation has been shown to provide higher reproducibility than silylation (Kvitvang et al. 2014).

EZ:Faast has been used in a number of wine studies in recent years for studies related to amino acids: in conjunction with LC-MS to study the connection between amino acid content and biogenic amine formation (Smit et al. 2013) and with GC-MS to better understand sluggish fermentations (Sommer et al. 2015) or to observe how foliar application of seaweed in the vineyard can alter the chemistry of the grape and resulting wine (Salvi et al. 2019).

EZ:faast combines solid phase extraction and simultaneous derivatization. Solid phase extraction (SPE) sorbs analytes of interest without interfering substances. Generally there is a pre-wash of the column, followed by the introduction of the sample to interact with the immobilized solid phase, a post-wash to remove undesirable substances, and a reagent that allows for release of the sample (Buszewski and Szultka 2012). The EZ:faast kit uses a series of solutions that alter the charge on amino acids, and affect how they interact with a solid substance, leading to their separation. The cation exchanger used in the kit is benzenesulfonic acid on silica gel (Sommer et al. 2015). The amino acids are first pushed into a negative charge to encourage cationic binding, followed by a basic reagent to flush them out (Badawy 2012).

The derivatization reagent in EZ:Faast is propyl chloroformate (Gornischeff et al. 2020). The amino acids are altered at both the carboxylic acid group as well as the amino group, which is seen as an advantage (Badawy 2012). The alteration that occurs is also different for each of the two sides of the amino acid; the carboxylic acid group is converted to a propyl ester while the amino group becomes a propyl formate carbamate (Krumpochova et al. 2015). The derivatives formed are stable for at least a day at room temp, and longer at colder temperatures (Badawy 2012).

A non-wine comparison of EZ:faast performance in GC-MS, reverse phase HPLC, and hydrophilic interaction liquid chromatography found GC-MS to be the most robust and precise; the only disadvantage being that specific amino acids could not be quantified (Krumpochova et al. 2015) Unless arginine quantification specifically was

desired, however, GC-MS was the preferred method with EZ:Faast (Krumpochova et al. 2015)

1.11 Malic acid and foamability

A wine's chemistry will affect how a protein is able to react, as a number of components may alter the protein itself. For example, increasing ethanol content increases rate of protein denaturation (Senée et al. 1999), and the presence of sulfate encourages unfolded proteins to aggregate (Marangon et al. 2011). Wine pH is very significant in determining how proteins will react with other proteins and polysaccharides. If the wine pH is below the isoelectric point of the protein (pI), the protein has a net positive charge; while when it is above the pI, the protein has a net negative charge (Waterhouse et al. 2016). The pI of most proteins is in the pH range of 4-6, meaning proteins found at wine pH levels are mostly positively charged (Waterhouse et al. 2016). When the pH is below the isoelectric point of a protein, but above the pKa of the acidic group on a polysaccharide, the two molecules will have an opposite charge, making them more likely to interact (Sadahira et al. 2016). As the pH drops further below a protein's isoelectric point, the molecule is more strongly positively charged; this can lead to interactions with oppositely charged molecules as well as repulsions with similarly charged proteins (Dufrechou et al. 2012).

Since pH affects protein-polysaccharide interactions, acidity would seem to be a significant factor in foaming. In fact, higher tartaric acid concentration causes greater foamability and stability (Kemp et al. 2015b), and sparkling wines with higher titratable acidity (TA) have greater foam stability (Condé et al. 2017a). Multiple

studies have found that malic acid specifically may play a role in foam quality; higher malic levels have been correlated with increased foam height (Andrés-Lacueva et al. 1996, López-Barajas et al. 1998, Girbau-Sola et al. 2002), and, in one study, with decreased foam stability (López-Barajas et al. 1998). The opposite effect has been noted with lactic acid (Kemp et al. 2015b). In contrast, Liu et al. (2018) found higher malic levels correlated negatively with foamability, and argued that despite that correlation malic itself may not play a role, but rather just be an indication of maturity and, consequently, the protein concentration. Work done in our lab suggested that malic acid can limit the influence of protein content on foaming; increased malic concentration diminished foam height, and at high malic levels there was no increase in foaming properties when protein increased (A. Lindstrom, unpublished data).

1.12 Protein and malic acid in non-vinifera wine grapes

Multiple studies suggest that non-*vinifera* wines may be higher in protein and malic acid content. Interspecific hybrid grape juices have higher protein concentrations than their *V. vinifera* counterparts (Springer et al. 2016). A study of *V. labrusca* cultivars Catawba, Concord, and Delaware reported protein content in the finished wine ranging from 50.8 - 54.7 mg/100mL; changes in free amino acid content were very similar to patterns established with *V. vinifera*, but in larger quantities (Kluba et al. 1978). In one study, wines made from the interspecific *V. riparia*-based hybrid Marquette contained three times as much protein as Cabernet Sauvignon (Norton et al. 2020). Chitinase production was found to be four times higher in *V. labrusca* than the levels found in *V. vinifera* (Pastorello et al. 2003). Many non-*vinifera* cultivars, including

interspecific hybrids, are known to possess greater ability to fight off fungal infections (Pedneault and Provost 2016). The higher protein is potentially related to disease resistance, as grapevines possessing stronger resistance to certain fungal infections show a greater expression of genes for TLPs (Yan et al. 2017). In addition, grapes that have been forced to overexpress specific TLP genes for research purposes showed greater resistance to powdery mildew (He et al. 2017), as did cultivars with higher chitinase activity (Giannakis et al. 1998). Higher protein levels are suspected to play a role in cold-hardiness of grapevines; expression of PRP genes was heightened during cold exposure (Wu et al. 2014). Chitinases have also been shown to play a role in protecting tissues from freezing damage (Fernandez-Caballero et al. 2009). Native North American cultivars and interspecific hybrids with native ancestry are known to possess adaptations that make them more cold resistant than *V. vinifera* (Londo and Kovaleski 2017).

In general, wine grapes grown in cool-climate regions of the Eastern United States have shown higher acid levels, particularly higher levels of malic acid (Gallander 1977), than those found in warmer climates. Total acid levels are also typically higher in hybrid grapes than in *V. vinifera* (Teissedre 2018). Malic acid levels have been found to fluctuate differently across grapevine species during the growing season; while not consistently lower than interspecific hybrids, *vinifera* species have been observed to degrade more malic and subsequently have a lower malic concentration than non-*vinifera* grapes (Burzynski-Chang et al. 2019). Recent work in our lab demonstrated that malic acid also tends to be higher in wines produced from interspecific hybrid grapes (A. Andrievsky, unpublished data). As both protein and

malic acid may be implicated in foam characteristics, the higher concentrations found in Eastern hybrid wines makes them likely suspects for the foaming issues experienced with hybrid-based sparkling wine.

REFERENCES

Aguié-Béghin V, Adriaensen Y, Péron N, Valade M, Rouxhet P, and Douillard R. 2009. Structure and chemical composition of layers adsorbed at interfaces with champagne. J Ag and Food Chem 57(21):10399-10407.

Alexandre H, and Guilloux-Benatier M. 2006. Yeast autolysis in sparkling wine–a review. Australian J Grape Wine Research 12(2):119-127.

Andrés-Lacueva C, López-Tamames E, Lamuela-Raventós R, Buxaderas S, and de la Torre-Boronat, M. 1996. Characteristics of sparkling base wines affecting foam behavior. J Ag and Food Chem 44(4):989-995.

Badawy AAB. 2012. The EZ: Faast family of amino acid analysis kits: application of the GC-FID kit for rapid determination of plasma tryptophan and other amino acids. *In* Amino Acid Analysis. Alterman M, and Hunziker P (eds), pp. 153-164. Humana Press, Totowa, NJ.

Bayly F, and Berg H. 1967. Grape and wine proteins of white wine varietals. Am J Enol Vitic 18(1):18-32.

Bikerman J. 1938. The unit of foaminess. Transactions of the Faraday Society 34:634-638.

Blasco L, Viñas M, and Villa T. 2011. Proteins influencing foam formation in wine and beer: the role of yeast. International Microbiology 14(2):61-71.

Blom J. 1937. Investigations on foam. J Institute of Brewing 43(3):251-262.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72(1-2): 248-254.

Brady JW. 2013. Introductory food chemistry. Comstock, Ithaca, NY.

Brissonnet F, and Maujean A. 1991. Identification of some foam-active compounds in champagne base wines. Am J Enol Vitic 42(2): 97-102.

Bruheim P, Kvitvang HF, and Villas-Boas SG. 2013. Stable isotope coded derivatizing reagents as internal standards in metabolite profiling. J chromatography A. 1296:196-203.

Buckee GK. 1994. Determination of total nitrogen in barley, malt and beer by kjeldahl procedures and the dumas combustion method collaborative trial. J Institute of Brewing. 100(2):57-64.

Burzynski-Chang E, Brown E, Reshef N, and Sacks GL. 2020. Malate Content in Wild Vitis spp. Demonstrates a Range of Behaviors during Berry Maturation. Am J Enol Vitic 71: 80-87.

Buszewski B, Szultka M. 2012. Past, present, and future of solid phase extraction: a review. Critical Reviews Anal Chem. 42(3):198-213.

Buxaderas S, and López-Tamames E. 2012. Sparkling wines: features and trends from tradition. *In* Advances in food and nutrition research. J Hendry (ed.) pp. 1-45. Academic Press, Cambridge, MA.

Callejón RM, Troncoso AM, and Morales ML. Determination of amino acids in grape-derived products: a review. Talanta 81(4-5):1143-52.

Cerovic ZG, Ghozlen NB, Milhade C, Obert M, Debuisson S, and Moigne ML. 2015. Nondestructive diagnostic test for nitrogen nutrition of grapevine (Vitis vinifera L.)

based on dualex leaf-clip measurements in the field. J Ag and Food Chem. 63(14):3669-80.

Christian M, Titze J, and Ilberg V. 2011. Chemical structure of model substances related to their gushing-inducing and-suppressing activity. J Am Society of Brewing Chemists, 69(3):170-179.

Cilindre C, Castro AJ, Clément C, Jeandet P, and Marchal R. 2007. Influence of Botrytis cinerea infection on Champagne wine proteins (characterized by two-dimensional electrophoresis/immunodetection) and wine foaming properties. Food Chem 103(1):139-149.

Cilindre C, Liger-Belair G, Villaume S, Jeandet P, and Marchal R. 2010. Foaming properties of various Champagne wines depending on several parameters: Grape variety, aging, protein and CO2 content. Analytica chimica acta 660(1-2):164-170.

Coelho E, Reis A, Domingues M, Rocha SM, and Coimbra, MA. 2011. Synergistic effect of high and low molecular weight molecules in the foamability and foam stability of sparkling wines. J Ag and Food Chemy 59(7):3168-3179.

Condé BC, Fuentes S, Caron M, Xiao D, Collmann R, and Howell KS 2017. Development of a robotic and computer vision method to assess foam quality in sparkling wines. Food Control 71:383-392.

Condé BC, Bouchard E, Culbert JA, Wilkinson KL, Fuentes S, and Howell KS. 2017. Soluble protein and amino acid content affects the foam quality of sparkling wine. J Ag and Food Chem 65(41):9110-9119.

Crumpton M, Rice CJ, Atkinson A, Taylor G, and Marangon M. 2018. The effect of sucrose addition at dosage stage on the foam attributes of a bottle-fermented English sparkling wine. J Science of Food and Ag 98(3):1171-1178.

Culbert JA, McRae JM, Condé BC, Schmidtke LM, Nicholson EL, Smith PA, Howell K, Boss PK, and Wilkinson KL. 2017. Influence of production method on the chemical composition, foaming properties, and quality of Australian carbonated and sparkling white wines. J Ag and Food Chem 65(7): 1378-1386.

Dambrouck T, Marchal R, Cilindre C, Parmentier M, and Jeandet P. 2005. Determination of the grape invertase content (using PTA− ELISA) following various fining treatments versus changes in the total protein content of wine. Relationships with wine foamability. J Ag and Food Chem 53(22):8782-8789.

Di Gianvito P, Arfelli G, Suzzi G, and Tofalo R. 2019. New Trends in Sparkling Wine Production: Yeast Rational Selection. *In* Alcoholic Beverages Volume 7: The Science of Beverages. Grumezescu AM, and Holban AM (eds.), pp. 347-386. Woodhead Publishing. Cambridge, UK.

Dufrechou M, Poncet-Legrand C, Sauvage FX, and Vernhet A. 2012. Stability of white wine proteins: combined effect of pH, ionic strength, and temperature on their aggregation. J Ag and Food Chem60(5):1308-1319.

Duong-Ly KC, Gabelli SB. 2014. Salting out of proteins using ammonium sulfate precipitation.*In* Methods in enzymology Volume 541, pp. 85-94. Academic Press. Cambridge, MA

Esteruelas M, Poinsaut P, Sieczkowski N, Manteau S, Fort MF, Canals JM, and Zamora F. 2009. Comparison of methods for estimating protein stability in white wines.Am J Enol Vitic. 60(3):302-311.

Esteruelas M, González-Royo E, Kontoudakis N, Orte A, Cantos A, Canals JM, and Zamora F. 2015. Influence of grape maturity on the foaming properties of base wines and sparkling wines (Cava). J of the Science of Food and Ag 95(10):2071-2080.

Fernandez-Caballero C, Romero I, Goni O, Escribano M I, Merodio C, and Sanchez-Ballesta MT. 2009. Characterization of an antifungal and cryoprotective class I

chitinase from table grape berries (Vitis vinifera Cv. Cardinal). J Ag Food Chem 57(19):8893-8900.

Ferreira RB, Monteiro S, Piçarra-Pereira MA, Tanganho MC, Loureiro VB, and Teixeira AR. 2000. Characterization of the proteins from grapes and wines by immunological methods. Am J Enol Vitic 51(1):22-28.

Ferreira RB, Piçarra-Pereira MA, Monteiro S, Loureiro VB, and Teixeira AR. 2001. The wine proteins. Trends in food science & technology 12(7):230-239.

Flamini R, and De Rosso M. 2006. Mass spectrometry in the analysis of grape and wine proteins. Expert Review of Proteomics. 3(3):321-31.

Fountoulakis M, Juranville JF, and Manneberg M. 1992. Comparison of the Coomassie brilliant blue, bicinchoninic acid and Lowry quantitation assays, using non-glycosylated and glycosylated proteins. J biochemical and biophysical methods. 24(3-4):265-74.

Fountoulakis M, and Lahm HW. 1998. Hydrolysis and amino acid composition analysis of proteins. J chromatography A. 826(2):109-34.

Gallander J F. 1977. Deacidification of eastern table wines with Schizosaccharomyces pombe. Am J Enol Vitic 28(2):65-68.

Gallart M, López-Tamames E, and Buxaderas S. 1997. Foam measurements in wines: comparison of parameters obtained by gas sparging method. J Ag Food Chem 45(12):4687-4690.

Gallart M, Tomás X, Suberbiola G, López-Tamames E, and Buxaderas S. 2004. Relationship between foam parameters obtained by the gas-sparging method and sensory evaluation of sparkling wines. J of the Science of Food and Ag 84(2):127-133.

Gazzola D, Vincenzi S, Pasini G, Lomolino G, and Curioni A. 2015. Advantages of the KDS/BCA assay over the Bradford assay for protein quantification in white wine and grape juice. Am J Enol Vitic 66(2):227-233.

Giannakis C, Bucheli CS, Skene KG, Robinson SP, and Scott NS. 1998. Chitinase and β-1, 3-glucanase in grapevine leaves: a possible defence against powdery mildew infection. Australian J Grape Wine Research. 4(1):14-22.

Giribaldi M, and Giuffrida MG. 2010. Heard it through the grapevine: Proteomic perspective on grape and wine. J Proteomics. 73(9):1647-1655.

Girbau-Sola T, López-Tamames E, Buján J, and Buxaderas S. 2002. Foam aptitude of Trepat and Monastrell red varieties in cava elaboration. 1. Base wine characteristics. J Ag Food Chem 50(20):5596-5599.

Giribaldi M, Perugini I, Sauvage FX, and Schubert A. 2007. Analysis of protein changes during grape berry ripening by 2-DE and MALDI-TOF. Proteomics 7(17):3154-3170.

Gornischeff A, Kruve A, and Rebane R. 2020. Characterization of wines with liquid chromatography electrospray ionization mass spectrometry: quantification of amino acids via ionization efficiency values. J Chromatography A. 1620:461012.

Giannakis C, Bucheli CS, Skene KG, Robinson SP, and Scott NS. 1998. Chitinase and β-1, 3-glucanase in grapevine leaves: a possible defence against powdery mildew infection. Australian J Grape Wine Research. 4(1):14-22.

Guan L, Wu B, Hilbert G, Li S, Gomès E, Delrot S, and Dai Z. 2017. Cluster shading modifies amino acids in grape (Vitis vinifera L.) berries in a genotype-and tissue-dependent manner. Food Research International 98:2-9.

Hare PE, John PS, and Engel MH. 1985 Ion-exchange separation of amino acids. *In* Chemistry and Biochemistry of the Amino Acids, pp. 415-425. Springer, Dordrecht, Netherlands.

He R, Wu J, Zhang Y, Agüero C B, Li X, Liu S, Wang C, Walker MA and Lu J. 2017. Overexpression of a thaumatin-like protein gene from Vitis amurensis improves downy mildew resistance in Vitis vinifera grapevine. Protoplasma 254(4):1579-1589.

Hippeli S, and Elstner EF. 2002. Are hydrophobins and/or non-specific lipid transfer proteins responsible for gushing in beer? New hypotheses on the chemical nature of gushing inducing factors. Zeitschrift für Naturforschung C. 57(1-2):1-8.

Hsu J C, and Heatherbell D A. 1987. Isolation and characterization of soluble proteins in grapes, grape juice, and wine. Am J Enol Vitic 38(1):6-10.

Huang T, Long M, and Huo B. 2010. Competitive binding to cuprous ions of protein and BCA in the bicinchoninic acid protein assay. The open biomedical engineering journal 4:271.

Hušek P. 1998. Chloroformates in gas chromatography as general purpose derivatizing agents. Journal of Chromatography B: Biomedical Sciences and Applications. 717(1-2):57-91.

Jégou S, Conreux A, Villaume S, Hovasse A, Schaeffer C, Cilindre C, Van Dorsselaer A and Jeandet P. (2009). One step purification of the grape vacuolar invertase. Analytica chimica acta 638(1):75-78.

Kannan A, Hettiarachchy N, and Marshall M. 2012. Proteins and peptides as foaming agents. *In* Food Proteins and Peptides. Hettiarachchy N (ed.) pp. 151-164. CRC Press, Boca Raton, USA.

Kemp, B., Wiles, B., & Inglis, D. (2015). Gushing of sparkling wine at disgorging: Reasons and remedies. Practical Winery and Vineyard Journal October:58-63.

Kemp B, Alexandre H, Robillard B, and Marchal R. 2015. Effect of production phase on bottle-fermented sparkling wine quality. J Ag Food Chem 63(1):19-38.

Kemp B, Condé B, Jégou S, Howell K, Vasserot Y, and Marchal R. 2019. Chemical compounds and mechanisms involved in the formation and stabilization of foam in sparkling wines. Critical reviews in food science and nutrition 59(13):2072-2094.

Kliewer WM. Free amino acids and other nitrogenous fractions in wine grapes. J Food Science. 1970 Jan;35(1):17-21.

Kluba R M, Mattick L R, and Hackler L R 1978. Changes in concentration of free and total amino acids of several native American grape cultivars during fermentation. Am J Enol Vitic 29(3):181-186.

Kresge N, Simoni R D, and Hill R L 2005. The most highly cited paper in publishing history: Protein determination by Oliver H. Lowry. J Biological Chem 280(28):e26-e28.

Krumpochova P, Bruyneel B, Molenaar D, Koukou A, Wuhrer M, Niessen WM, and Giera M. 2015. Amino acid analysis using chromatography–mass spectrometry: An inter platform comparison study. J Pharmaceutical Biomed Anal. 114:398-407.

Kupfer VM, Vogt EI, Ziegler T, Vogel RF and Niessen L. 2017. Comparative protein profile analysis of wines made from Botrytis cinerea infected and healthy grapes reveals a novel biomarker for gushing in sparkling wine. Food Research Int. 99:501-509.

Kupfer VM, Vogt EI, Siebert AK, Meyer ML, Vogel RF, and Niessen L. 2017. Foam-stabilizing properties of the yeast protein PAU5 and evaluation of factors that can influence its concentration in must and wine. Food Research Int. 102:111-118.

Kupfer V M 2018. The influence of plant-and yeast-derived proteins on gushing in sparkling wine. Thesis, Technische Universität München.

Kvitvang HF, Kristiansen KA, Lien SK, and Bruheim P. 2014. Quantitative analysis of amino and organic acids by methyl chloroformate derivatization and GC-MS/MS analysis. *In* Mass Spectrometry in Metabolomics, pp. 137-145. Humana Press, New York, NY.

Lamikanra O, and Kassa AK 1999. Changes in the Free Amino Acid Composition with Maturity of the Noble Cultivar of Vitis rotundifolia Michx. Grape. J Ag Food Chem 47(12):4837-4841.

Lao C, Santamaria A, López-Tamames E, Bujan J, Buxaderas S, and De la Torre-Boronat M C 1999. Effect of grape pectic enzyme treatment on foaming properties of white musts and wines. Food Chem 65(2):169-173.

Le Bourse D, Jégou S, Conreux A, Villaume S, and Jeandet P. 2010. Review of preparative and analytical procedures for the study of proteins in grape juice and wine. Analytica Chimica Acta 667(1-2):33-42.

Le Bourse D, Conreux A, Villaume S, Lameiras P, Nuzillard JM, and Jeandet P 2011. Quantification of chitinase and thaumatin-like proteins in grape juices and wines. Anal and Bioanal Chem 401(5):1541.

Lehtonen P. 1996. Determination of amines and amino acids in wine—a review. Am J Enol Vitic. 47(2):127-33.

Liger-Belair G. 2005. The physics and chemistry behind the bubbling properties of champagne and sparkling wines: a state-of-the-art review. J Ag Food Chem 53(8):2788-2802.

Liger-Belair G, Polidori G, and Jeandet P. 2008. Recent advances in the science of champagne bubbles. Chemical Society Reviews 37(11):2490-2511.

Liger-Belair G. 2014. How many bubbles in your glass of bubbly?. The Journal of Physical Chemistry B 118(11):3156-3163.

Liu PH, Vrigneau C, Salmon T, Hoang D, Boulet JC, Jégou S, and Marchal R. 2018. Influence of grape berry maturity on juice and base wine composition and foaming properties of sparkling wines from the Champagne region. Molecules 23(6):1372.

Londo JP, and Kovaleski AP. 2017. Characterization of wild North American grapevine cold hardiness using differential thermal analysis. Am J Enol Vitic 68(2):203-212.

López-Barajas M, Viu-Marco A, López-Tamames E, Buxaderas S, and de la Torre-Boronat MC. 1997. Foaming in grape juices of white varieties. J Ag and Food Chem 45(7):2526-2529.

López-Barajas M, López-Tamames E, Buxaderas S, and De la Torre-Boronat MC. 1998. Effect of vinification and variety on foam capacity of wine. Am J Enol Vitic 49(4):397-402.

López-Barajas M, López-Tamames E, Buxaderas S, Tomás X, and de La Torre M C. 1999. Prediction of wine foaming. J Ag Food Chem 47(9):3743-3748.

Luguera C, Moreno-Arribas V, Pueyo E, and Polo M C. 1997. Capillary electrophoretic analysis of wine proteins. Modifications during the manufacture of sparkling wines. J Ag Food Chem 45(10):3766-3770.

Lusk LT. 2016. Controlling beer foam and gushing. *In* Brewing Materials and Processes, pp. 175-198. Academic Press, Cambridge, MA.

Marangon M, Sauvage FX, Waters EJ, and Vernhet A. 2011. Effects of ionic strength and sulfate upon thermal aggregation of grape chitinases and thaumatin-like proteins in a model system. J Ag Food Chem 59(6):2652-2662.

Marchal, R., Bouquelet, S. and Maujean, A., 1996. Purification and partial biochemical characterization of glycoproteins in a Champenois Chardonnay wine. J Ag and Food Chemistry 44(7):1716-1722.

Marchal R, Seguin V, and Maujean A. 1997. Quantification of interferences in the direct measurement of proteins in wines from the Champagne region using the Bradford method. Am J Enol Vitic 48(3):303-309.

Marchal R, Tabary I, Valade M, Moncomble D, Viaux L, Robillard B, and Jeandet P. 2001. Effects of Botrytis cinerea infection on Champagne wine foaming properties. J Science of Food and Ag 81(14), 1371-1378.

Martínez-Lapuente L, Guadalupe Z, Ayestarán B, and Pérez-Magariño S. 2015. Role of major wine constituents in the foam properties of white and rosé sparkling wines. Food Chem 174:330-338.

Medina-Trujillo L, Matias-Guiu P, López-Bonillo F, Canals JM, and Zamora F. 2017. Physicochemical Characterization of the Foam of White and Rosé Base Wines for Sparkling Wine Production (AOC Cava). Am J Enol Vitic 68(4):485-495.

Medina-Trujillo L, González-Royo E, Sieczkowski N, Heras J, Canals JM, and Zamora F. 2017. Effect of sequential inoculation (Torulaspora delbrueckii/Saccharomyces cerevisiae) in the first fermentation on the foaming properties of sparkling wine. Euro Food Research and Tech 243(4):681-688.

Meier M, Jaeckels N, Tenzer S, Stoll M, Decker H, Fronk P, Dietrich H, and Will F. 2016. Impact of drought stress on concentration and composition of wine proteins in Riesling. Euro Food Research and Tech 242(11):1883-1891.

Monteiro S, Piçarra-Pereira MA, Teixeira AR, Loureiro VB, and Ferreira RB. 2003. Environmental conditions during vegetative growth determine the major proteins that accumulate in mature grapes. J Ag Food Chem 51(14):4046-4053.

Monteiro S, Piçarra-Pereira MA, Loureiro VB, Teixeira AR, and Ferreira RB. 2007. The diversity of pathogenesis-related proteins decreases during grape maturation. Phytochemistry 68(4):416-425.

Moreno-Arribas V, Pueyo E, Nieto FJ, Martın-Alvarez PJ, and Polo MC. 2000. Influence of the polysaccharides and the nitrogen compounds on foaming properties of sparkling wines. Food Chem 70(3):309-317.

Moreno-Arribas MV, Pueyo E, and Polo MC. 2002. Analytical methods for the characterization of proteins and peptides in wines. Analytica Chimica Acta. 58(1):63-75.

Moretti RH, and Berg H. 1965. Variability among wines to protein clouding. Am J Enol Vitic 16(2):69-78.

Murphey JM, Spayd SE, and Powers JR. 1989. Effect of grape maturation on soluble protein characteristics of Gewürztraminer and White Riesling juice and wine. Am J Enol Vitic 40(3):199-207.

Murphey JM, Powers JR, and Spayd SE. 1989. Estimation of soluble protein concentration of white wines using Coomassie brilliant blue G-250. Am J Enol Vitic 40(3):189-193.

Norton EL, Sacks GL, and Talbert JN. 2020. Non-Linear Behavior of Protein and Tannin in Wine Produced by Cofermentation of an Interspecific Hybrid (Vitis spp.) and Vinifera Cultivar. Am J Enol Vitic 71(1):26-32.

Novák P, and Havlíček V. 2016. Protein extraction and precipitation. *In* Proteomic profiling and analytical chemistry. pp. 51-62. Elsevier, Amsterdam, Netherlands.

OIV. 2014. Compendium of international methods of analysis of wines and musts.

Otter DE. 2012. Standardised methods for amino acid analysis of food. Brit J Nutrition 108:S230-S237.

Pace CN, Trevino S, Prabhakaran E, and Scholtz JM. 2004. Protein structure, stability and solubility in water and other solvents. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences. 359(1448):1225-35.

Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Fortunato D, Giuffrida MG, Garoffo LP, Calamari AM, Brenna O, and Conti A. 2003. Identification of grape and wine allergens as an endochitinase 4, a lipid-transfer protein, and a thaumatin. J Allergy and Clinical Immunology. 111(2):350-9.

Pedneault K, and Provost C. 2016. Fungus resistant grape varieties as a suitable alternative for organic wine production: Benefits, limits, and challenges. Scientia Horticulturae 208:57-77.

Peterson G L. 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal Biochem 100(2):201-220.

Pocock KF, Hayasaka Y, Peng Z, Williams PJ, and Waters EJ. 1998. The effect of mechanical harvesting and long-distance transport on the concentration of haze-forming proteins in grape juice. Australian J Grape and Wine Research 4(1):23-29.

Pozo-Bayón MÁ, Martínez-Rodríguez A, Pueyo E, and Moreno-Arribas MV. 2009. Chemical and biochemical features involved in sparkling wine production: from a traditional to an improved winemaking technology. Trends Food Sci & Tech 20(6-7):289-299.

Preston WC, and Richardson AS. 2021. The surface properties of soap solutions. The J of Physical Chem. 33(8):1142-50.

Pueyo E, Dizy M, and Polo MC. 1993. Varietal differentiation of must and wines by means of protein fraction. Am J Enol Vitic 44(3):255-260.

Pueyo E, Martín-Alvarez PJ, and Polo MC. 1995. Relationship between foam characteristics and chemical composition in wines and cavas (sparkling wines). Am J Enol Vitic 46(4):518-524.

Regenstein, JM, Regenstein C, and Kochen B. 1984. Food Protein Chemistry: an Introduction for Food Scientists. Academic Press, Orlando, FL.

Reichelt W N, Waldschitz D, Herwig C, and Neutsch L. 2016. Bioprocess monitoring: minimizing sample matrix effects for total protein quantification with bicinchoninic acid assay. J Industrial Microbio Biotech 43(9):1271-1280.

Riu-Aumatell M, López-Barajas M, López-Tamames E, and Buxaderas S. 2002. Influence of yield and maturation index on polysaccharides and other compounds of grape juice. J Ag Food Chem 50(16):4604-4607.

Robillard B, Delpuech E, Viaux L, Malvy J, Vignes-Adler M, and Duteurtre B. 1993. Improvements of methods for sparkling base wine foam measurements and effect of wine filtration on foam behavior. Am J Enol Vitic 44(4):387-392.

Robinson, J. 2006. The Oxford Companion to Wine, pp. 656-659. 3d ed. Oxford University Press, New York.

Robinson SP, Jacobs AK, and Dry IB. 1997. A class IV chitinase is highly expressed in grape berries during ripening. Plant physiology. 114(3):771-8.

Rudin AD. 1957. Measurement of the foam stability of beers. J Institute of Brewing 63(6):506-509.

Sadahira MS, Rodrigues MI, Akhtar M, Murray BS, and Netto FM. 2016. Effect of egg white protein-pectin electrostatic interactions in a high sugar content system on foaming and foam rheological properties. Food Hydrocolloids 58:1-10.

Sáez-Plaza P, Navas MJ, Wybraniec S, Michałowski T, and Asuero AG. 2013. An overview of the Kjeldahl method of nitrogen determination. Part II. Sample preparation, working scale, instrumental finish, and quality control. Critical Reviews in Anal Chem.43(4):224-72.

Salvi L, Brunetti C, Cataldo E, Niccolai A, Centritto M, Ferrini F, and Mattii GB. 2019. Effects of Ascophyllum nodosum extract on Vitis vinifera: Consequences on plant physiology, grape quality and secondary metabolism. Plant Phys Biochem. 139:21-32.

Santoro M. 1995. Fractionation and characterization of must and wine proteins. Am J Enol Vitic 46(2):250-254.

Sapan CV, Lundblad RL, and Price NC. 1999. Colorimetric protein assay techniques. Biotech and Applied Biochem 29(2):99-108.

Sauvage FX, Bach B, Moutounet M, and Vernhet A. 2010. Proteins in white wines: Thermo-sensitivity and differential adsorbtion by bentonite. Food Chem 118(1):26-34.

Spayd SE, Wample RL, Evans RG, Stevens RG, Seymour BJ, and Nagel CW. 1994. Nitrogen fertilization of white Riesling grapes in Washington. Must and wine composition. Am J Enol Vitic 45(1):34-42.

Schaffner W, and Weissmann C. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal Biochem 56(2):502-514.

Senée J, Robillard B, and Vignes-Adler M. 1999. Films and foams of Champagne wines. Food Hydrocolloids 13(1):15-26.

Smit AY, du Toit WJ, Stander M, and du Toit M. Evaluating the influence of maceration practices on biogenic amine formation in wine. LWT-Food Science and Technology. 53(1):297-307.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano M, Fujimoto EK, Goeke NM, Olson BJ, and Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 150(1)76-85.

Smith MR, Penner MH, Bennett SE, and Bakalinsky A T. 2011. Quantitative colorimetric assay for total protein applied to the red wine pinot noir. J Ag Food Chem 59(13):6871-6876.

Sommer S, Wegmann-Herr P, Wacker M, and Fischer U. 2015. Rationale for a stronger disposition of Chardonnay wines for stuck and sluggish fermentation. S African J Enol Vitic 36(1):180-90.

Springer LF, Chen LA, Stahlecker AC, Cousins P, and Sacks GL. 2016. Relationship of soluble grape-derived proteins to condensed tannin extractability during red wine fermentation. J ag and food chem. 64(43):8191-9.

Stines AP, Grubb J, Gockowiak H, Henschke PA, Høj PB, and Van Heeswijck R. 2000. Proline and arginine accumulation in developing berries of Vitis vinifera L. in Australian vineyards: Influence of vine cultivar, berry maturity and tissue type. Australian J Grape Wine Research 6(2):150-158.

Teissedre P L. 2018. Composition of grape and wine from resistant vines varieties. OENO One 52(3):211-217.

Tian B, Harrison R, Morton J, and Jaspers M. 2019. Changes in pathogenesis-related proteins and phenolics in Vitis vinifera L. cv.'Sauvignon Blanc' grape skin and pulp during ripening. Scientia horticulturae 243:78-83.

Toyo'oka T. 2013. Amino acid analysis: current topics and trends. Analytical and Bioanalytical Chemistry. 25(405):7905-6.

Van Sluyter SC, McRae JM, Falconer RJ, Smith PA, Bacic A, Waters EJ, and Marangon M. 2015. Wine protein haze: mechanisms of formation and advances in prevention. J Ag Food Chem 63(16):4020-4030.

Vanrell G, Canals R, Esteruelas M, Fort F, Canals JM, and Zamora F. 2007. Influence of the use of bentonite as a riddling agent on foam quality and protein fraction of sparkling wines (Cava). Food Chem 104(1):148-155.

Verma A, Kumar N, and Raj R. 2021. Direct prediction of foamability of aqueous surfactant solutions using property values. J of Molecular Liquids. 323:114635.

Vincenzi S, Mosconi S, Zoccatelli G, Dalla Pellegrina C, Veneri G, Chignola R, Peruffo A, Curioni A, and Rizzi C. 2005. Development of a new procedure for protein recovery and quantification in wine. Am J Enol Vitic 56(2):182-187.

Vincenzi S, Marangon M, Tolin S, and Curioni A. 2011. Protein evolution during the early stages of white winemaking and its relations with wine stability. Australian J Grape Wine Research 17(1):20-27.

Vincenzi S, Crapisi A, and Curioni A. 2014. Foamability of Prosecco wine: Cooperative effects of high molecular weight glycocompounds and wine PR-proteins. Food Hydrocolloids 34:202-207.

Vogt EI, Kupfer VM, Vogel RF, and Niessen L. 2017. Evidence of gushing induction by Penicillium oxalicum proteins. J Applied Microbio. 122(3):708-718.

Waterhouse AL, Sacks GL, and Jeffery DW. 2016. Understanding Wine Chemistry. John Wiley & Sons, West Sussex.

Waters EJ, Wallace W, and Williams PJ. 1991. Heat haze characteristics of fractionated wine proteins. Am J Enol Vitic. 42(2):123-7.

Waters EJ, Pellerin P, Brillouet JM. 1994. A Saccharomyces mannoprotein that protects wine from protein haze. Carbohydrate Polymers. 23(3):185-91.

Weetall HH, Zelko JT, Bailey LF. 1984. A new method for the stabilization of white wine. Am J Enol Vitic. 35(4):212-5.

Weiss KC, and Bisson LF. 2001. Optimisation of the Amido Black assay for determination of the protein content of grape juices and wines. J Science Food and Ag 81(6):583-589.

Wiechelman KJ, Braun RD, and Fitzpatrick JD. 1988. Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. Anal Biochem 175(1):231-237.

Wu J, Zhang Y, Yin L, Qu J, and Lu J. 2014. Linkage of cold acclimation and disease resistance through plant–pathogen interaction pathway in Vitis amurensis grapevine. Functional Integrative Genomics 14(4):741-755.

Yan X, Qiao H, Zhang X, Guo C, Wang M, Wang Y, and Wang X. 2017. Analysis of the grape (Vitis vinifera L.) thaumatin-like protein (TLP) gene family and

demonstration that TLP29 contributes to disease resistance. Scientific Reports 7(1):4269.

Yokotsuka K, and Singleton VL. 1997. Glycoproteins: Characterization in a hybrid grape variety (Muscat Bailey A) juice, fermenting must, and resultant red wine. Am J Enol Vitic 48(1):100-114.

Zhang Y, and Cremer PS. 2006. Interactions between macromolecules and ions: the Hofmeister series. Current Opinion Chem Bio 10(6):658-63.

CHAPTER 2

Correlations Between Total Protein and Sparkling Wine Foam Parameters

2.1 Introduction

Sparkling wines effervesce when opened, and the foaming bubbles are an important component in the evaluation of their quality (Kemp et al. 2019). Proteins and polysaccharides are major components of sparkling wine foam (Brissonnet and Maujean 1991). Proteins, having both hydrophobic and hydrophilic components, help create a film around escaping gas at a wine's surface and retain it within the liquid (Kannan et al. 2012).

The necessary presence of proteins for wine foam has been demonstrated by multiple researchers: a wine stripped of all proteins was incapable of producing any measurable foam (Vincenzi et al. 2014), and partial removal of total protein through bentonite fining reduced foam height and stability (Dambrouck et al. 2005, Vanrell et al. 2007). Studies in model wine also suggest that changes in the concentrations of various protein fractions do not impact foam equally, and that a synergistic effect of grape and yeast proteins creates better foam than either protein alone (Coelho et al. 2011, Vincenzi et al. 2014).

While protein is important to wine foam, the correlation between total protein content and expected foam characteristics is complicated, and research results have proven contradictory. Positive correlations have been found between greater protein levels and foamability (foamability is defined as the ability of a substance to produce foam, and is generally taken as the maximum foam height reached when a wine sample is sparged with gas) (López-Barajas et al. 1998), as well as foam stability (Pueyo et al.

1995). In contrast, other researchers found no connection between total protein and foam height (Condé et al. 2017). Correlations between foam duration and protein have similarly been found to be both negative (Andrés-Lacueva et al. 1996) and positive (Condé et al. 2017).

Many of the studies on sparkling wine that attempted to correlate total protein with foaming characteristics did so through one of two methods: the Bradford method or the KDS/BCA assay. The Bradford method of protein analysis, though frequently cited, may overestimate total protein and lead to inaccurate correlations (Gazzola et al. 2015, Condé et al. 2017, Liu et al. 2018), as it many substances cause intereferences that alter results. Ethanol and phenolic compounds are the major ones in wine; these have been found to alter the absorbance by as much as 90% (Marchal et al. 1997). The KDS/BCA (potassium dodecyl sulfate/bicinchoninic acid) assay gives a more accurate measure of total protein (Vincenzi et al. 2005, Gazzola et al. 2015), but has other weaknesses. Precipitation with KDS does not provide consistent quantification of glycoproteins; in some cases, as little as 15% of added glycoprotein was precipitated (Smith et al. 2011). Phenolic compounds and reducing sugars such as glucose and fructose interfere with the assay (Sapan et al. 1999, Smith et al. 2011), and substances such as phospholipids and biogenic amines have been found to cause errors (Reichelt et al. 2016). A study that compared the Bradford assay's ability to quantify protein levels in spiked wines with an alternative method of hydrolyzing all proteins and summing the resulting amino acids found similar weaknesses; summing amino acids resulted in a fairly consistent 91% recovery rate, while Bradford's recovery rate varied with protein fraction from 20-50% (Waters et al.

1991). A non-wine study that compared the summation of amino acids from hydrolyzed proteins with the Bradford, BCA, and Lowry assays found that glycosylation of proteins was a major factor in test accuracy; non-glycosylated proteins gave results in a somewhat similar range for all tests (though slightly lower for Bradford and slightly higher in Lowry and BCA), while for glycosylated proteins the Bradford assay measured as little as half of the summed amino acids, and both the BCA and Lowry assay results were 60% higher (Fountoulakis et al. 1992). Though researchers disagree on the percentage of glycosylated proteins in wine (Ferreira et al. 2001), their presence makes many colorimetric methods protein analysis methods less than ideal.

Total protein content of a finished wine can vary by cultivar, vineyard location, and annual environmental conditions (Bayly and Berg 1967). Soluble protein content decreases by as much as 70% after fermentation (Yokotsuka and Singleton 1997), and other protein fractions may be lost through precipitation interactions with phenolics, enzyme degradation, or as a result of isoelectric points that create insolubility at wine pH (Ferreira et al. 2001). In *V. vinifera*-based sparkling wines, changes in protein content affect some proteins more than others; concentrations of chitinases and thaumatin like proteins (TLPs) are much lower in finished sparkling wine than in their base wine oforigin, while invertase stays nearly unchanged (Le Bourse et al. 2011). Secondary fermentation for sparkling wine production also alters final protein content through the inclusion of proteins released during yeast autolysis (Alexandre and Guilloux-Benatier 2006). Bentonite has been observed to precipitate proteins of different sizes at different rates, so its effects will vary by wine (Santoro 1995).

Multiple studies suggest that non-*vinifera* wines may be higher in protein content. Interspecific hybrid grape wines had greater protein concentrations than their *V. vinifera* counterparts in one study (Springer et al. 2016). In another, wines made from the interspecific *V. riparia*-based hybrid Marquette contained three times as much protein as Cabernet Sauvignon (Norton et al. 2020). Many non-*vinifera* cultivars, including interspecific hybrids, are known to possess greater resistance to fungal infections (Pedneault and Provost 2016). Increased protein is potentially related to disease resistance, as grapevines possessing stronger resistance to certain fungal infections show a greater expression of genes for TLPs (Yan et al. 2017). In addition, grapes that have been forced to overexpress specific genes for TLPs for research purposes have shown greater resistance to powdery mildew (He et al. 2017), as have cultivar with higher chitinase activity (Giannakis et al. 1998). Higher protein levels may play a role in the cold-hardiness of grapevines; expression of PRP genes was heightened during cold exposure (Wu et al. 2014), and chitinases have been shown to play a role in protecting tissues from freezing damage (Fernandez-Caballero et al. 2009). Native North American cultivars and interspecific hybrids with native ancestry possess adaptations that make them more cold resistant than *V. vinifera* (Londo and Kovaleski 2017).

As protein may be implicated in foam characteristics, the higher concentrations found in Eastern hybrid wines make protein concentration a potential cause for the foam-related issues experienced with hybrid-based sparkling wine. Producers of sparkling wines made from winegrape cultivars other than *Vitis vinifera* have reported excessive foaming and excessive foam height when poured. This can decrease consumer

perception of quality, and potentially impact repeat sales. Preliminary work supports the existence of overfoaming and documented greater foamability in a selection of interspecific hybrid-based wines when compared to a regional Chardonnay-based sparkling wine (A. Lindstrom, unpublished data). This study examined the potential interactions of total protein content and foam parameters in sparkling wines to understand if the greater foaming observed in wines produced from interspecific hybrid winegrape cultivars is due to greater protein concentration in those wines.

2.2 Materials and Methods

Winemaking

Fruit:

Marquette was hand- harvested on 9/17/19 at Cornell's teaching and demonstration vineyard (Penn Yan, NY), and stored overnight in a cold room at 2° C. The grapes were crushed and pressed the following day with a basket press (Mori PZ82) at Cornell AgriTech's Vinification and Brewing Lab (Geneva, NY). Fifty mg/L SO_2 was added after pressing using a 103.2g/L solution of potassium metabisulfite (KMBS) (Presque Isle Wine Cellars, North East, PA). Soluble solids were adjusted to 20 Brix with sucrose. Yeast assimilable nitrogen (YAN) was adjusted to 150ppm with 0.3g/L GoFerm and 0.25g/L Fermaid (Scott Laboratories, Petaluma, CA).

Juice from two white cultivars was sourced in bulk from local wineries. Cayuga White was machine harvested, crushed, and pressed with a DeFranceschi MO40 bladder

press on 9/27/19 at Lucas Vineyards (Interlaken, NY); 50ppm SO_2 as KMBS was added during crushing. PEC5L pectinase (Scott Labs) was added to increase settling. Settled juice was brought to Cornell AgriTech, where soluble solids were adjusted to 20 Brix with sucrose and YAN to 186 with 0.3g/L GoFerm.

Chardonnay from Leonard Oaks Vineyard (Medina, NY) was picked by hand and pressed in a bladder press on 10/1/19. Twenty-five ppm SO_2 was added at crushing using a 57% KMBS mixture. Juice was transported to Cornell AgriTech, where YAN was adjusted to 152 with 0.3g/L GoFerm and 0.25g/L Fermaid.

Fermentation:

All juice was inoculated with yeast strain EC1118 at the manufacturer's recommended rate of 25g/hL and fermented in 18.9L glass carboys in a storage room set at 20°C. Primary fermentation was considered complete when Brix was less than 0.5% as determined by Clinitest tablets (Presque Isle Wine Cellars, North East, PA); all were complete within 10-14 days.

Treatments:

Post fermentation, replicate fermentations of each cultivar were blended to ensure homogeneity, then split into the following eight lots: a control (C), traditional method sparkling produced with in-bottle secondary fermentation (TM), and bentonite treatments with additions of 0.1. 0.3, and 0.5 g/L, performed in duplicate. All but the TM lots received 20ppm SO_2 prior to bentonite treatment and cold stabilization.

Bentonite (KWK Krystal Klear, Presque Isle Wine Cellars, North East, PA) was prepared in a 6% slurry in DI water then hand-mixed into each carboy with a plastic rod. Treatment addition rates were chosen based on the range used in similar studies (Weiss and Bisson 2001, Dambrouck et al. 2005). Wines were stored in a cold room set at 4°C for one week, then racked off the bentonite and into clean carboys.

Wines were cold stabilized at 2 °C for 3.5 months after seeding with potassium bitartrate (Presque Isle Wine Cellars, North East, PA) at the manufacturer's recommended dose of 2 g/gal.

Cold stability was confirmed prior to bottling by conductivity on a Checkstab a2012LF (Delta Acque, Florence, Italy).

All still wines received 20ppm SO_2 via a 57% KMBS addition prior to bottling in 750mL, 29mm opening glass bottles with screw cap closures (Waterloo Container, Waterloo, NY).

In TM wines, 0.3 g/L Go Ferm and enough sucrose to bring residual sugar to 24g/L was added prior to being bottling in 29mm glass sparkling wine bottles capped with standard screw caps (Waterloo Container, Waterloo, NY). Wines remained in bottle for six months, stored on their side at 20 °C. A 21-day hand-riddling took place at 20 °C , and bottles were disgorged and recapped on 11/18/20.

Chemical and Physical Analyses

Sampling:

Three samples were prepared and analyzed from each lot for each assay. In the case of the still wines, there was an assumption of uniformity among the bottles in each lot, so three samples were pulled from the same bottle. Traditional method lots were sampled in the same fashion, but due to the potential variability caused by separate secondary fermentations, two bottles of each traditional method wine were each sampled in triplicate. Prior to analysis, the TM wines were degassed for 5 min with a vacuum flask during continuous stirring via magnetic stirbar.

***Protein analysis*:**

Protein analysis was performed by summation of amino acid values obtained by hydrolyzing proteins and analyzing the hydrolysates by GC. Wine samples of ~1.5 mL were first spun at 13.4K RPM for 5 min in microcentrifuge tubes using a Scilogex D2012 centrifuge. One mL of each sample was then transferred to a 15mL Falcon tube (Celltreat Scientific product #229410, Pepperell, MA) and proteins were precipitated with the addition of 5 mL 95% ethanol (Acros Organics, Fair Lawn, NJ). The Falcon tubes were vortexed and allowed to settle overnight at 4°C. The pellet was precipitated by centrifuge (Eppendorf 5810R) at 4°C at 3750 RPM for 20 minutes. The supernatant was poured off and the pellet was resuspended in 0.75 mL of deionized water, then purified by dialysis.

Dialysis was performed using Thermo "slide-a-lyzer" devices with a 3.5KDa cutoff (Thermo #88403 Waltham, MA). Dialysis ran at 4°C for 36 hours with DI water changes every 3-4 hours during the day, and one longer period of 12-14 hours overnight in between days. There was a total of six water changes.

Post dialysis, samples were transferred to a 10x75mm pyrolysis tube (#9820-10 Pyrex, San Nicolas de la Garza, Nuevo Leon Mexico). Samples were then concentrated in a freeze dryer (Millrock Technology MAX53) using an initial freeze period of -40°C for four hours and five minutes, followed by a drying period of twelve hours (Table 1.)

Table 1: 12 Hour Freeze Drying Cycle used on Wine Samples Post-Dialysis

Drying Stage	Time[a]	Temperature[b]	Vacuum[c]
1	1	0	300
2	2	5	350
3	2	10	400
4	2	15	500
5	2	20	400
6	1	24	300
7	2	24	200

[a]Time is measured in hours
[b]Temperature is measured in °C
[c]Vacuum is measured in mTorr

An Eldex Hydrolysis/Derivatization Station (Eldex Laboratories Inc. Napa, CA) was used to hydrolyze the proteins to amino acids. Pyrolysis tubes were placed in a reaction vial (Eldex #1163) with 350 microliters of 6N HCl (EMD Millipore Darmstadt, Germany) and 15 microliters of 5% phenol (LabChem Zellenople, PA). To remove oxygen from the headspace, vials were submitted to four rounds of nitrogen introduction followed by vacuum. The reaction vial was then heated at 150°C for 65 minutes in the Eldex hydrolysis workstation oven.

Post heating, tubes were placed in a clean reaction vial and placed under a vacuum for ten minutes. Prior to EZ:Faast analysis, 100 microliters of a 0.2mM norvaline solution was added to each pyrolysis tube as an internal standard (reagent 1 in the EZ Faast kit).

The amino acids obtained were then extracted and derivatized using an EZ:Faast kit from Phenomenex (KGO-7167). Derivitized samples were analyzed with an Agilent 6890N gas chromatograph equipped with a flame ion detector (GC-FID) and a Zebron-AAA column provided with the EZ:Faast materials. The GC program followed the EZ:Faast recommendations from Phenomenex: split injection at a ratio of 1:15, inlet temperature set to 250°C; the helium carrier gas set at a flow rate of 1.1 mL/min; an oven program that started at 110 °C and in 30 °C/min ramps to a final temperature of 320 °C; and an detector temperature of 250 °C. Chromatograms were analyzed using Agilent's OpenLab software version Rev. B.04.03 [16].

Foam Analysis

A Kruss DFA100 (Hamburg, Germany) fitted with a CY4572 (40 mm prism) column and a FL4502 filter was used to assess foam characteristics. A 50 mL sample of each wine was placed within the column, then sparged with compressed air for 30 seconds at a rate of 0.25 L/min. Foam parameters were recorded for 2 min after gas flow stopped. Max foam volume and the ratio of the max foam volume to gas volume (FC) were recorded as indications of foamability. Head retention value and time to 50% foam collapse were recorded as indications of stability.

Malic Acid Analysis

Malic acid was measured by HPLC. Samples were first prepared by pipetting 500 microliters of the wine and 500 microliters of HPLC grade water together in a microcentrifuge tube (#L250803 Laboratory Product Sales Inc., Rochester, NY). This was vortexed, and then filtered through a 13 mm diameter 0.22 micrometer syringe filter (#229746 Celltreat Pepperell, MA) into a vial (#5182-0715 Agilent Technologies, Santa Clara, CA).

A Shimadzu Prominence HPLC was used with a SIL-10ADVP autosampler and LC-20AB pump. The column was a Phenomenex Resex ROA organic acid 300 X 7.8 mm with a 50 X 7.8 mm guard column. The mobile phase was a 0.005N Sulfuric acid solution. The oven (model CTO-20AC) was set to 45°C. A 20 microliter sample size was used for each injection. The assay had a 35 minute run time and a 0.5mL/minute flow rate. The diode array detector (model SPD-M20A) was set at 45°C. The refractive index detector (model RID-10A) was also set at 45°C.

2.3 Results

Grape and Wine Chemistry

Harvest chemistry of all three cultivars is shown in Table 2. Titratable acidity (TA) was highest in Marquette and lowest in Cayuga at both the juice and wine stage (Tables 2 & 3). All cultivars fermented to dryness at similar ethanol levels.

Table 2: Chemistry of Juice Samples after Pressing

Cultivar	Brix	pH	Titratable Acidity[a] (g/L)
Cayuga	19.7	2.97	9.6
Chardonnay	19.9	3.16	9.9
Marquette	17.4	2.87	12.8

[a]expressed as Tartaric Acid Equivalents (TAE)

Table 3: Chemistry of Control Wines

Cultivar	pH[a]	Malic Acid (g/L)[ab]	Tartaric Acid (g/L)[ab]	Ethanol (%)[ab]
Cayuga	3.15	4.36	2.95	11.70
Chardonnay	3.25	6.01	2.22	11.63
Marquette	3.03	5.59	3.90	11.74

[a]Values reported are the average of triplicate measurements
[b]Malic Acid, Tartaric Acid, and Ethanol values were measured by HPLC

Protein Concentration

Initial protein concentration was different in each cultivar. In this study, Chardonnay had higher total protein values than Cayuga or Marquette (Fig 1). In general, wine protein decreased with increased bentonite addition, but the amount varied by cultivar, with the largest reduction in Chardonnay, followed by Cayuga and Marquette (Fig 1).

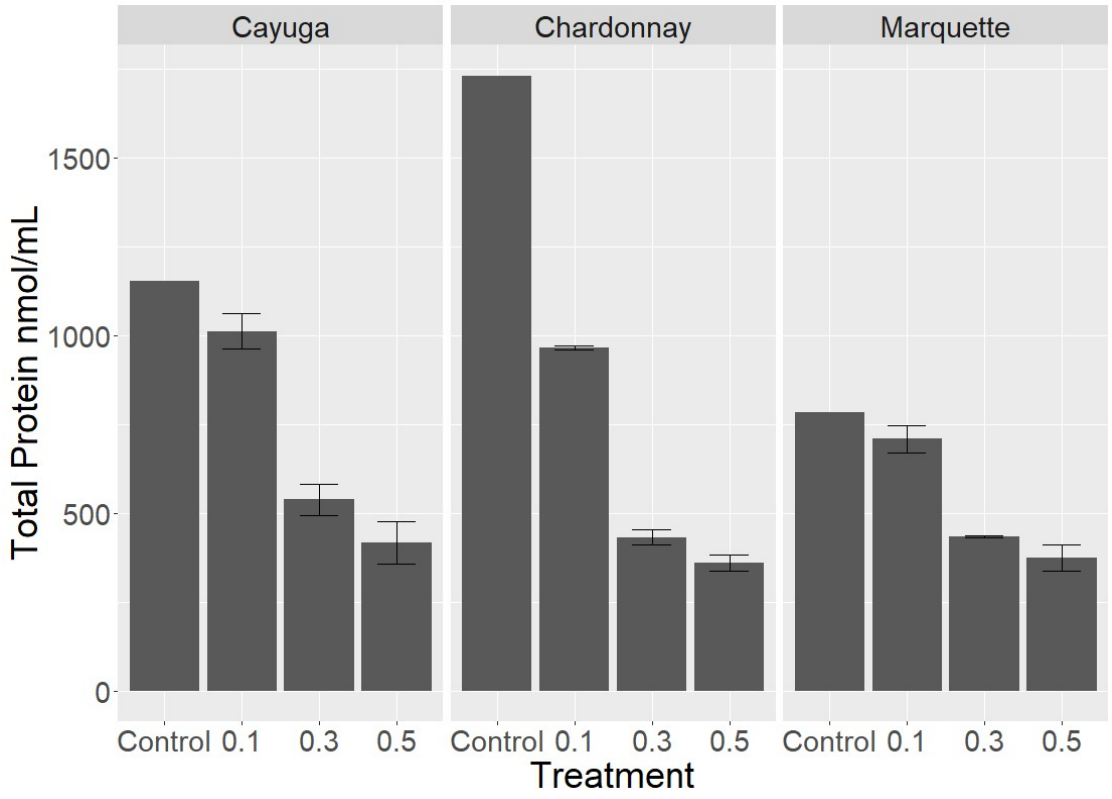

Figure 1: Total Protein remaining in monovarietal wines following bentonite additions at three different levels. Treatment levels are in g/L. Error bars indicate the standard error between duplicate observations.

At an addition of 0.1 g/L, 34% of the initial protein was removed from Chardonnay, but only 12% and 9% and from Cayuga and Marquette, respectively. Despite different initial protein concentrations and removal rates, the 0.5 g/L addition left Chardonnay and Marquette with similar protein concentrations, with Cayuga around 50 nmol/mg higher (Table 4). In all cultivars, there was no significant percent difference in protein concentration between the 0.3 and 0.5 g/L treatments, and the impact these treatments had on foam parameters were also not significant (Table 4). Among TM wines, only the Marquette displayed an increase in total protein content compared to control levels.

Table 4: Total Protein in Monovarietal Wines, and Foam Maximum Volume and Time to 50% Collapse as Measured by KRUSS DFA 100

Cultivar	Treatment[ab]	Total Protein (nmol/mL)[ac]	Maximum Foam Volume (mL)[a]	Time to 50% Collapse of Foam (s)[a]
Cayuga	Control	1152.0 ±185.1a[d]	94.2 ±2.9a	47.3 ±1.5a
	0.1	1011.0 ±95.3a	89.0 ±2.0a	43.8 ±0.6a
	0.3	537.7 ±75.9b	71.3 ±2.5b	38.5 ±0.8b
	0.5	415.6 ±84.6b	62.1 ±2.8b	36.8 ±0.8b
	TM	1017.0 ±183.4a	58.3 ±13.9b	37.9 ±0.9b
Chardonnay	Control	1730.5 ±71.2a	87.5 ±2.9a	49.5 ±4.2a
	0.1	965.7 ±158.0b	76.4 ±5.8ab	49.8 ±3.6a
	0.3	431.7 ±94.0c	66.4 ±4.0b	39.4 ±0.5b
	0.5	359.5 ±51.1c	62.7 ±3.7b	37.2 ±0.9b
	TM	1310.2 ±217.3d	66.0 ±5.8b	37.1 ±0.7b
Marquette	Control	783.4 ±113.0ac	106.1 ±11.6a	61.6 ±2.5a
	0.1	708.1 ±85.5a	79.5 ±8.2b	50.8 ±10.0b
	0.3	434.0 ±42.0b	71.2 ±4.2b	38.6 ±0.5c
	0.5	373.4 ±78.1b	73.6 ±8.6b	39.0 ±2.1c
	TM	1009.8 ±182.6c	46.7 ±1.5c	37.4 ±0.5c

[a]Values shown are the average of triplicate measurements and duplicate treatments
[b] 0.1, 0.3, 0.5 indicate the g/L addition of bentonite, TM indicates traditional method
[c] Units refer to the molar summation of the amino acids hydrolyzed from proteins precipitated in wine
[d] Different letters indicate significant difference at $p < 0.05$ within column and cultivar.

Differences in Amino Acid Composition

The specific amino acid composition of the proteins revealed few differences between wines, with the exception of aspartic acid/asparagine and serine. In all cultivars, the aspartic acid/asparagine content made up a greater percentage of the protein

composition in traditional method and bentonite-treated wines than in the control (Table 5). As this assay reports aspartic acid and asparagine as one value, it is unclear how their concentrations vary across treatments.

Table 5: Aspartic Acid/Asparagine Percentage of Total Protein

Cultivar	Treatment[ab]	Aspartic Acid / Asparagine Percent of Total Protein (%)[a]
Cayuga	Control	3.48 ± 0.64
	TM	6.74 ± 1.50
	0.1	12.02 ± 4.10
	0.3	11.88 ± 4.26
	0.5	16.69 ± 9.53
Chardonnay	Control	4.13 ± 0.59
	TM	5.32 ± 0.94
	0.1	6.22 ± 1.19
	0.3	10.05 ± 2.00
	0.5	17.64 ± 4.23
Marquette	Control	4.83 ± 0.88
	TM	8.45 ± 1.85
	0.1	8.82 ± 1.70
	0.3	12.40 ± 3.00
	0.5	17.85 ± 4.99

[a]Values shown are the average of triplicate measurements and duplicate treatments
[b] 0.1, 0.3, 0.5 indicate the g/L addition of bentonite,
TM indicates traditional method

The aspartic acid value was weakly negatively correlated with foam maximum volume (-0.1227); it was the only amino acid that was negatively correlated with any foam parameter.

Total protein impact on foam parameters

Foam characteristics did not correlate with total protein content across all wines (Table 4). In all cultivars, there was no significant difference in observed foam volume and collapse between the 0.3 g/L and 0.5g/L addition rates, and Chardonnay and Cayuga showed no significant difference in either parameter between control wines and those treated with 0.1 g/L bentonite. Collapse values in TM wines were not significantly different from the 0.3 g/L or 0.5 g/L values for any of the cultivars. Total protein was not significantly correlated with foam max volume or 50% collapse. When TM wines were excluded, total protein correlated with max foam volume ($p = 0.000748$) regardless of cultivar. The correlation of total protein with foam collapse, however, had a significant interaction with cultivar ($p = .002028$). For both parameters, significance varied by cultivar (Figs. 2 & 3).

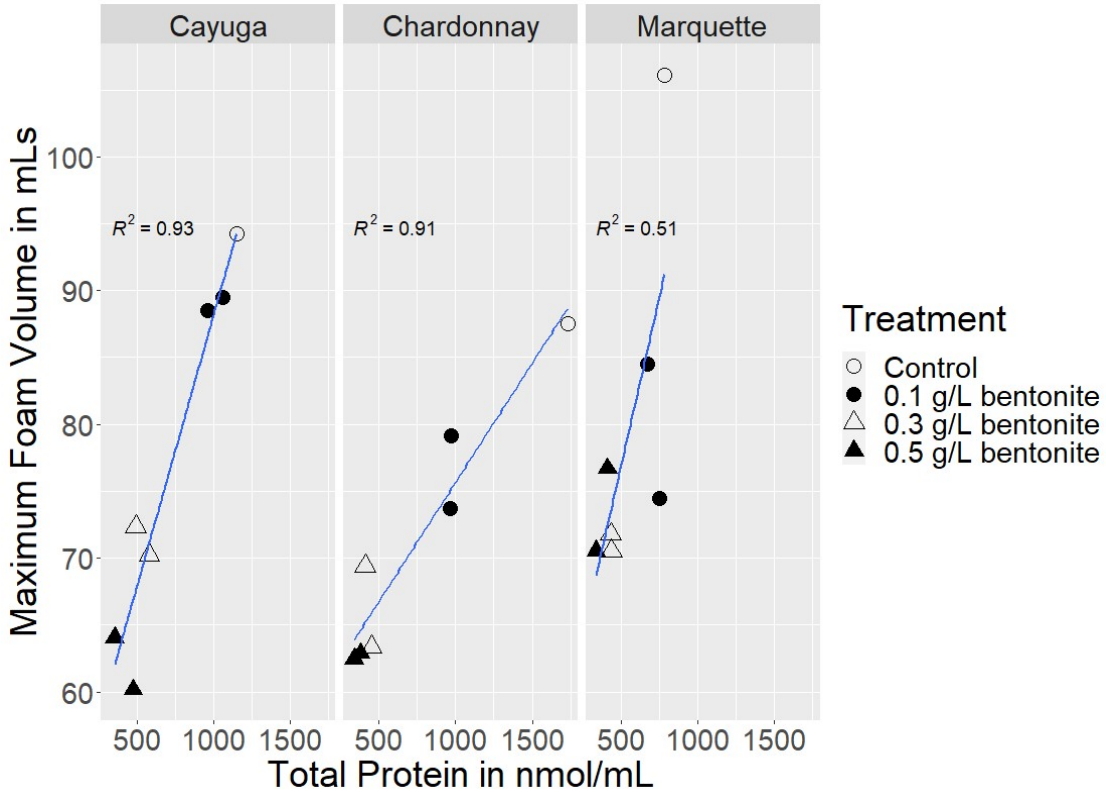

Figure 2: Maximum Foam Volume in three bentonite-treated monovarietal wines by Total Protein.

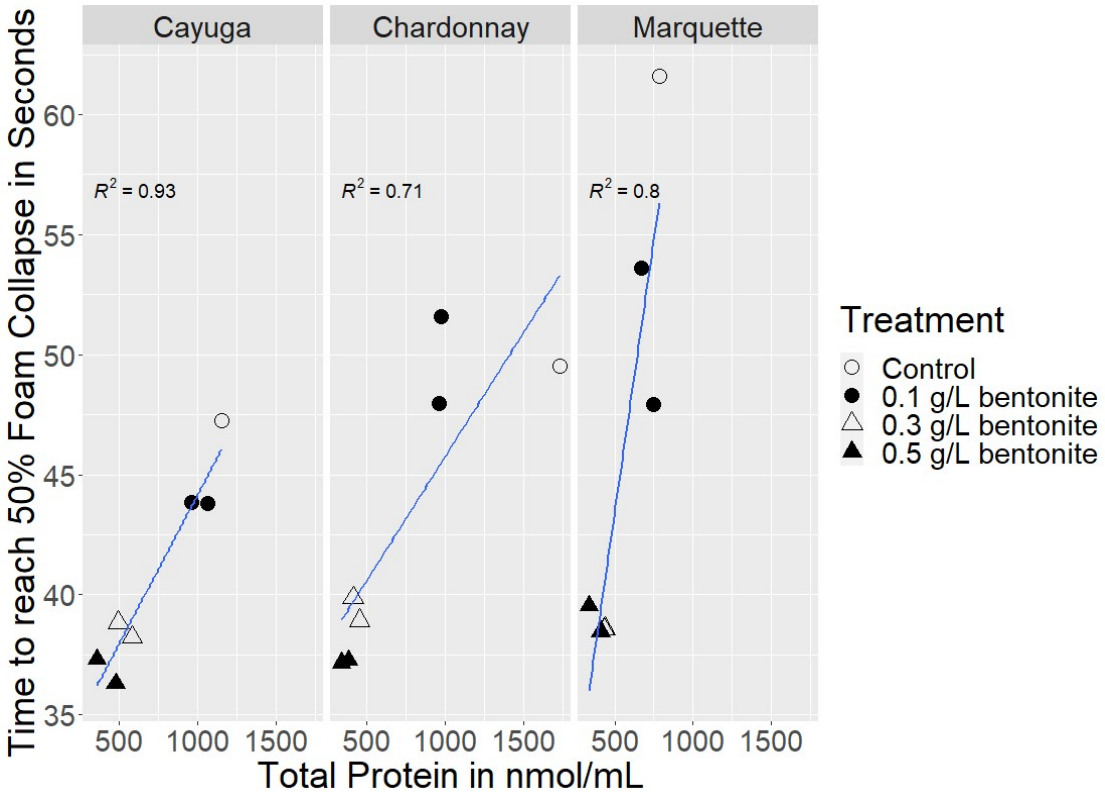

Figure 3: Time to 50% Foam Collapse in three bentonite-treated monovarietal wines by Total Protein.

2.4 Discussion

Protein Content

The higher protein concentration in Chardonnay was initially surprising, as prior work suggested non-*vinifera* grapes would have as much or more (Pastorello et al. 2003, Norton et al. 2020, Kluba et al. 1978, Springer et al. 2016). It is likely that the relative protein rankings observed are not broadly representative of these three cultivars, likely due to the specifics of how these wines were made; a larger sample of wines made from these cultivars would likely show greater variation. If the average amino content of the Chardonnay control wine is multiplied out by each amino's molar mass to obtain an estimate of mg/L values, the wine would be around 197 mg/L, placing it in a

normal range for the cultivar. Also, in this study the pectolytic enzymes used during Cayuga winemaking likely reduced protein concentration, as has been observed previously (Lao et al. 1999). The Marquette was harvested at lower ripeness than the other two cultivars (Table 2); as protein accumulation has been shown to be greater at higher maturity (Murphey et al. 1989), the lower protein in the Marquette in this study may simply be due to harvest date.

As expected, bentonite caused a reduction in total protein that was generally greater with larger additions (Table 4). The three cultivars displayed different responses to bentonite treatment. Application of the 0.1 g/L bentonite treatment removed total protein ranging from an average of 9.6% in Marquette to 34.8% in Chardonnay. Final removal rates at the 0.5 g/L bentonite rate were 52.3% in Marquette, 63.9% in Cayuga, and 72.8% in Chardonnay. It is possible these differences in removal point to different amounts of specific proteins present in the base wines, as it has been shown that bentonite removal rate is strongly connected with protein type (Achaerandio et al. 2001). In that study, the same rate of addition removed 100% of BSA protein from a model wine but only 39% of lysozyme protein from the same medium. The gel separations or other methods of identifing specific proteins necessary to confirm this supposition were beyond the scope of this project.

Additionally, competing molecules in the wines may have affected bentonite adsorption; potassium and calcium, for instance, may cause interference with protein removal (Blade and Boulton 1988). These compounds were not measured in the study wines.

The total protein levels observed following 0.3g/L and 0.5g/L bentonite additions were

very similar (Figure 1). Bentonite is highly effective at removing many proteins from wine but has little to no effect on specific TLPs and larger glycosylated proteins (Jaeckels et al. 2017). It is likely that these compounds made up the majority of total protein in wines with higher bentonite doses.

Among TM wines, total protein content increased after the second fermentation in Marquette, but not in the other two wines. Yeast autolysis is said to be impacted by temperature, yeast strain, and pH (Alexandre and Guilloux-Benatier 2006). Base wines for all three cultivars were approximately the same % ethanol, and all wines were stored at the same temperature during the secondary fermentation and storage. The Marquette did have higher acidity and lower pH (Table 3), although lower pH would be expected to slow the rate of autolysis, not accelerate it (Alexandre and Guilloux-Benatier 2006). Autolysis can vary by cultivar, and protein content can fluctuate over time (Alexandre and Guilloux-Benatier 2006). The protein values presented for TM wines should therefore be viewed as a snapshot; given enough time, the shift in total protein when compared to each base wine may look similar between the cultivars. Although two bottles of each TM wine were tested and similar results obtained, bottle-to-bottle variation may also be partially responsible for differences observed.

Differences in Amino Acid Composition

Compositional analysis of total protein following bentonite treatment revealed very few consistent changes (data not shown). As a percentage of amino acids contributing to total protein, only aspartic acid/asparagine increased in all treatments (Table 5). It

is possible that the higher aspartic acid/asparagine percentage corresponds with an increase in the relative proportion of glycosylated proteins. Glycosylation in yeast mannoproteins are N-linked between protein and sugar by asparagine, or O-linked through the amino acids serine and threonine (Waters et al. 1994). Grape derived glycoproteins are all N-linked, meaning they require asparagine as a linkage (Marchal et al. 1996). Aspartic acid's proportion of protein values obtained post-hydrolysis has been previously used as an indication of potential asparagine N-linked sites within a protein (Waters et al. 1994).

Given that yeasts release highly glycosylated mannoproteins into wine during traditional method production (Alexandre and Guilloux-Benatier 2006), and that bentonite has less impact on larger glycosylated proteins (Salazar et al. 2010, Jaeckels et al. 2017) it seems reasonable to speculate that the observed proportional increase in aspartic acid/asparagine is due to changes in the protein/glycoprotein ratio, though additional analysis is required to confirm this. It has been shown that wines become more heat stable as protein/glycoprotein ratio gets smaller (Fusi et al. 2010); if a similar relationship existed and could be determined for foaming parameters, this could be a useful metric for sparkling wine producers.

Foam Volume

Foam volume generally correlated to protein concentration within each cultivar (Fig. 2), in agreement with previous studies that showed a reduction in foaming parameters after the addition of bentonite (Dambrouck et al. 2005, Vanrell et al. 2007). The major exception to this was the TM lots, which had protein content values close to or above

the control wines, and foam volume values that were among the lowest recorded for each cultivar (Table 4). Attempts to perform regression analysis when TM lots were included gave fairly weak R^2 values of 0.22 for Cayuga, 0.44 for Chardonnay, and 0.13 for Marquette.

Aspartic acid was the only negative correlation with foam parameters (-0.1227 with foam maximum volume). Because acid hydrolysis will cause aspartic acid and asparagine co-elute in the EZ:Faast GC-FID method, it is difficult to know for certain which amino acid is actually present. As a free amino acid, asparagine has been found to correlate with foam stability negatively (Condé et al. 2017) but has also been positively correlated with foam maximum height and foam stability (Martínez-Lapuente et al. 2015). Aspartic acid as a free amino acid has been correlated positively with both maximum foam height and foam stability (Martínez-Lapuente et al. 2015). Based on potential glycoprotein presence mentioned above, and asparagine's negative associated with foam parameters, the correlation (-0.1227) is likely due to asparagine. If that is the case, foam maximum volume may be reduced when there is a higher proportion of glycoproteins present in the wine. This would help to explain why the traditional method wines did not fit the pattern of other treatments and had much lower foam volume despite high total protein.

Foam Collapse

The time to 50% collapse of foam was generally longer in wines with higher total protein concentration; as with foam volume, the TM lots did not fit this rule, and displayed very similar collapse rates to the highest bentonite doses despite having high

total protein (Table 4). As with foam volume, regression analysis with the TM lots showed poor correlation, with R^2 values of 0.47 for Cayuga, 0.19 for Chardonnay, and 0.031 for Marquette; correlation was much stronger when TM wines were excluded (Fig. 3.) In the wines studied, foam stability was greater when foam volume was greater; in other words, the more foam produced, the longer it took to collapse. Protein content, however, was also generally greater when foam volume was higher, so increased stability may simply reflect protein content.

Cultivar Differences

The impact of bentonite fining on foaming parameters was different for the three cultivars. Comparing control wines to the high dose bentonite rate of 0.5g/L, Marquette experienced a greater percent loss of foam stability with more bentonite (-30% in volume, -37% collapse), while Cayuga lost more volume (-34% volume, -22% collapse), and Chardonnay showed near equivalent reduction in both volume and collapse (-28% volume, -25% collapse). These differences likely point to different protein composition in each cultivar, as foam height is affected by variance in protein size, and foam stability by protein structure (Martin et al. 2002).

2.5 Conclusion

Foam parameters were generally reduced as total protein decreased, but total protein alone was not predictive of either foam volume or foam collapse. All effects varied by cultivar, and both foam volume and foam collapse values had a stronger relationship

with total protein when cultivars were plotted individually. Foam collapse displayed a significant interaction with cultivar in regards to total protein impact on collapse time. Protein composition was more important than total protein in prediction of foam characteristics, as a greater percentage of aspartic acid and /or asparagine as total protein, and their potential indication of increased glycoprotein, seemed most indicative of foam parameters. Further work is needed to confirm relationship of glycoproteins on foam formation.

REFERENCES

Achaerandio I, Pachova V, Güell C, and López F. 2001. Protein adsorption by bentonite in a white wine model solution: effect of protein molecular weight and ethanol concentration. Am J Enol Vitic 52(2): 122-126.

Alexandre H, and Guilloux-Benatier M. 2006. Yeast autolysis in sparkling wine–a review. Australian J Grape Wine Research 12(2):119-127.

Andrés-Lacueva C, López-Tamames E, Lamuela-Raventós R, Buxaderas S, and de la Torre-Boronat, M. 1996. Characteristics of sparkling base wines affecting foam behavior. J Ag and Food Chem 44(4):989-995.

Bayly F, and Berg H. 1967. Grape and wine proteins of white wine varietals. Am J Enol Vitic 18(1):18-32.

Blade, W.H. and Boulton, R., 1988. Adsorption of protein by bentonite in a model wine solution. Am J Enol Vitic 39(3):193-199.

Brissonnet F, and Maujean A. 1991. Identification of some foam-active compounds in champagne base wines. Am J Enol Vitic 42(2): 97-102.

Coelho E, Reis A, Domingues M, Rocha SM, and Coimbra, MA. 2011. Synergistic effect of high and low molecular weight molecules in the foamability and foam stability of sparkling wines. J Ag and Food Chemy 59(7):3168-3179.

Condé BC, Bouchard E, Culbert JA, Wilkinson KL, Fuentes S, and Howell KS. 2017. Soluble protein and amino acid content affects the foam quality of sparkling wine. J Ag and Food Chem 65(41):9110-9119.

Dambrouck T, Marchal R, Cilindre C, Parmentier M, and Jeandet P. 2005. Determination of the grape invertase content (using PTA− ELISA) following various fining treatments versus changes in the total protein content of wine. Relationships with wine foamability. J Ag and Food Chem 53(22):8782-8789.

Fernandez-Caballero C, Romero I, Goni O, Escribano M I, Merodio C, and Sanchez-Ballesta MT. 2009. Characterization of an antifungal and cryoprotective class I

chitinase from table grape berries (Vitis vinifera Cv. Cardinal). J Ag Food Chem 57(19):8893-8900.

Ferreira RB, Piçarra-Pereira MA, Monteiro S, Loureiro VB, and Teixeira AR. 2001. The wine proteins. Trends in food science & technology 12(7):230-239.

Fountoulakis M, Juranville JF, and Manneberg M. 1992. Comparison of the Coomassie brilliant blue, bicinchoninic acid and Lowry quantitation assays, using non-glycosylated and glycosylated proteins. J biochemical and biophysical methods. 24(3-4):265-74.

Fusi M, Mainente F, Rizzi C, Zoccatelli G, and Simonato B. 2010. Wine hazing: A predictive assay based on protein and glycoprotein independent recovery and quantification. Food Control, 21(6): 830-834.

Gazzola D, Vincenzi S, Pasini G, Lomolino G, and Curioni A. 2015. Advantages of the KDS/BCA assay over the Bradford assay for protein quantification in white wine and grape juice. Am J Enol Vitic 66(2):227-233.

Giannakis C, Bucheli CS, Skene KG, Robinson SP, and Scott NS. 1998. Chitinase and β-1, 3-glucanase in grapevine leaves: a possible defence against powdery mildew infection. Australian J Grape Wine Research. 4(1):14-22.

He R, Wu J, Zhang Y, Agüero C B, Li X, Liu S, Wang C, Walker MA and Lu J. 2017. Overexpression of a thaumatin-like protein gene from Vitis amurensis improves downy mildew resistance in Vitis vinifera grapevine. Protoplasma 254(4):1579-1589.

Jaeckels, N., Tenzer, S., Meier, M., Will, F., Dietrich, H., Decker, H., & Fronk, P. 2017. Influence of bentonite fining on protein composition in wine. LWT 75: 335-343

Kannan A, Hettiarachchy N, and Marshall M. 2012. Proteins and peptides as foaming agents. *In* Food Proteins and Peptides. Hettiarachchy N (ed.) pp. 151-164. CRC Press, Boca Raton, USA.

Kluba R M, Mattick L R, and Hackler L R 1978. Changes in concentration of free and total amino acids of several native American grape cultivars during fermentation. Am J Enol Vitic 29(3):181-186.

Lao C, Santamaria A, López-Tamames E, Bujan J, Buxaderas S, and De la Torre-Boronat M C 1999. Effect of grape pectic enzyme treatment on foaming properties of white musts and wines. Food Chem 65(2):169-173.

Le Bourse D, Conreux A, Villaume S, Lameiras P, Nuzillard JM, and Jeandet P 2011. Quantification of chitinase and thaumatin-like proteins in grape juices and wines. Anal and Bioanal Chem 401(5):1541.

Liu PH, Vrigneau C, Salmon T, Hoang D, Boulet JC, Jégou S, and Marchal R. 2018. Influence of grape berry maturity on juice and base wine composition and foaming properties of sparkling wines from the Champagne region. Molecules 23(6):1372.

Londo JP, and Kovaleski AP. 2017. Characterization of wild North American grapevine cold hardiness using differential thermal analysis. Am J Enol Vitic 68(2):203-212.

López-Barajas M, López-Tamames E, Buxaderas S, and De la Torre-Boronat MC. 1998. Effect of vinification and variety on foam capacity of wine. Am J Enol Vitic 49(4):397-402.

Marchal, R., Bouquelet, S. and Maujean, A., 1996. Purification and partial biochemical characterization of glycoproteins in a Champenois Chardonnay wine. J Ag and Food Chemistry 44(7):1716-1722.

Marchal R, Seguin V, and Maujean A. 1997. Quantification of interferences in the direct measurement of proteins in wines from the Champagne region using the Bradford method. Am J Enol Vitic 48(3):303-309.

Martin AH, Grolle K, Bos MA, Stuart MA, van Vliet T. 2002. Network forming properties of various proteins adsorbed at the air/water interface in relation to foam stability. J Colloid and Interface Sci. 254(1):175-83.

Martínez-Lapuente L, Guadalupe Z, Ayestarán B, and Pérez-Magariño S. 2015. Role of major wine constituents in the foam properties of white and rosé sparkling wines. Food Chem 174:330-338.

Murphey JM, Spayd SE, and Powers JR. 1989. Effect of grape maturation on soluble protein characteristics of Gewürztraminer and White Riesling juice and wine. Am J Enol Vitic 40(3):199-207.

Norton EL, Sacks GL, and Talbert JN. 2020. Non-Linear Behavior of Protein and Tannin in Wine Produced by Cofermentation of an Interspecific Hybrid (Vitis spp.) and Vinifera Cultivar. Am J Enol Vitic 71(1):26-32.

Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Fortunato D, Giuffrida MG, Garoffo LP, Calamari AM, Brenna O, and Conti A. 2003. Identification of grape and wine allergens as an endochitinase 4, a lipid-transfer protein, and a thaumatin. J Allergy and Clinical Immunology. 111(2):350-9.

Pedneault K, and Provost C. 2016. Fungus resistant grape varieties as a suitable alternative for organic wine production: Benefits, limits, and challenges. Scientia Horticulturae 208:57-77.

Pueyo E, Martín-Alvarez PJ, and Polo MC. 1995. Relationship between foam characteristics and chemical composition in wines and cavas (sparkling wines). Am J Enol Vitic 46(4):518-524.

Reichelt W N, Waldschitz D, Herwig C, and Neutsch L. 2016. Bioprocess monitoring: minimizing sample matrix effects for total protein quantification with bicinchoninic acid assay. J Industrial Microbio Biotech 43(9):1271-1280.

Salazar, F.N., Zamora, F., Canals, J.M. and López, F., 2010. Protein stabilization in sparkling base wine using zirconia and bentonite: influence on the foam parameters and protein fractions. J. Int. Sci. Vigne Vin, 44:51-58.

Santoro M. 1995. Fractionation and characterization of must and wine proteins. Am J Enol Vitic 46(2):250-254.

Sapan CV, Lundblad RL, and Price NC. 1999. Colorimetric protein assay techniques. Biotech and Applied Biochem 29(2):99-108.

Smith MR, Penner MH, Bennett SE, and Bakalinsky A T. 2011. Quantitative

colorimetric assay for total protein applied to the red wine pinot noir. J Ag Food Chem 59(13):6871-6876.

Springer LF, Chen LA, Stahlecker AC, Cousins P, and Sacks GL. 2016. Relationship of soluble grape-derived proteins to condensed tannin extractability during red wine fermentation. J ag and food chem. 64(43):8191-9.

Vanrell G, Canals R, Esteruelas M, Fort F, Canals JM, and Zamora F. 2007. Influence of the use of bentonite as a riddling agent on foam quality and protein fraction of sparkling wines (Cava). Food Chem 104(1):148-155.

Vincenzi S, Mosconi S, Zoccatelli G, Dalla Pellegrina C, Veneri G, Chignola R, Peruffo A, Curioni A, and Rizzi C. 2005. Development of a new procedure for protein recovery and quantification in wine. Am J Enol Vitic 56(2):182-187.

Vincenzi S, Crapisi A, and Curioni A. 2014. Foamability of Prosecco wine: Cooperative effects of high molecular weight glycocompounds and wine PR-proteins. Food Hydrocolloids 34:202-207.

Waters EJ, Wallace W, and Williams PJ. 1991. Heat haze characteristics of fractionated wine proteins. Am J Enol Vitic. 42(2):123-7.

Waters EJ, Pellerin P, Brillouet JM. 1994. A Saccharomyces mannoprotein that protects wine from protein haze. Carbohydrate Polymers. 23(3):185-91.

Weiss KC, and Bisson LF. 2001. Optimisation of the Amido Black assay for determination of the protein content of grape juices and wines. J Science Food and Ag 81(6):583-589.

Wu J, Zhang Y, Yin L, Qu J, and Lu J. 2014. Linkage of cold acclimation and disease resistance through plant–pathogen interaction pathway in Vitis amurensis grapevine. Functional Integrative Genomics 14(4):741-755.

Yan X, Qiao H, Zhang X, Guo C, Wang M, Wang Y, and Wang X. 2017. Analysis of the grape (Vitis vinifera L.) thaumatin-like protein (TLP) gene family and demonstration that TLP29 contributes to disease resistance. Scientific Reports 7(1):4269.

Yokotsuka K, and Singleton VL. 1997. Glycoproteins: Characterization in a hybrid grape variety (Muscat Bailey A) juice, fermenting must, and resultant red wine. Am J Enol Vitic 48(1):100-114.

Chapter 3

Future Work

The general trends observed in this study would be more meaningful if multiple examples of each cultivar had been assessed. Another major issue was the fact that the initial processing of the fruit was not uniform. All juice was fermented at Cornell Agritech's Vinification and Brewing Lab, but the steps prior to that were different for each of the three cultivars. While the cultivar was a significant factor in how protein impacts foam collapse, it is difficult to know for certain how much of the difference is attributed to cultivar, and how much is processing, without cultivar replicates. Additional wines may have allowed for pattern recognition within or across the same cultivars.

In addition, it would be beneficial to assess other cultivars beyond these three. The primary goal was to find a way to assist producers of sparkling wines made from non-*vinifera* cultivars. More *V. vinifera* and non-*vinifera* cultivars would need to be included in order to establish differences in the foam of different species of grapes.

One of the easiest things to change, and that will likely be addressed by future students, is the inclusion of multiple vintages. As the conditions of the growing season play a large role in determining the final total protein content of grapes, multiple vintages of data from the same vineyards would be important in separating what cultivar impact and vintage conditions.

For this study, and the study of wine foam in general, it would be extremely helpful to test commercial wines that are determined to have "good" foam and "bad" foam.

Within the literature on this topic, researchers often comment on how specific chemical compounds or production practices result in “better” foam, but there is no definition of what “better” is. Having clear analytical standards, like foam maximum volume or time to foam collapse, to characterize “better” foam is important for a baseline understanding of quality.

The total protein measurements on this study correlating poorly with foam parameters across treatments and cultivars. Some additional analytical measurements could help explain this phenomenon. A gel separation of the proteins, to look at the comparative differences in proteins present in different wines, would be informative for future studies. Assays of protein glycosylation would help clarify the results of the current study, as the ratio of glycoproteins may play a role in foaming parameters.