



## WINE PROTEINS AND PROTEIN STABILITY

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### Subject Outline

- Prefermentation Processing Considerations**
- Fermentation and Post-Fermentation Processing Considerations**
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- Methods for Evaluation of Protein Stability**

Protein haze remains a significant problem for the wine industry and requires costly treatment with bentonite. Most of these costs are related to the loss of value of the wine recovered from the bentonite lees. The wine proteins responsible for haze are grape-related proteins.

The must and subsequent wine protein content are affected by the following:

- variety
- vintage
- maturity
- condition of the fruit
- pH
- processing methodology

Protein nitrogen content of wines varies between 10 and 275 mg/L. Despite the

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vast literature on protein instability, however, the actual protein levels at which wines will remain protein-stable are unknown (Waters et al., 2005).

The mechanism of protein haze formation is not fully understood. Slow denaturation of wine proteins is thought to lead to protein aggregation, flocculation into a hazy suspension and, finally, formation of visual precipitates. The importance of non-proteinaceous factors, such as proanthocyanidins (Koch and Sajak, 1959; Yokotsuka et al., 1991), in white wine protein haze formation have been suspected. Other factors, such as polysaccharides, alcohol levels, and pH, have also been implicated (Mesquita et al., 2001; Siebert et al., 1996).

Protein added to model wine does not precipitate or produce haze when heated, whereas visually obvious hazes occur when the same protein is added to a commercial wine. These observations led to the belief that precipitation involves two steps. Wine proteins exist as globular entities freely soluble in wine, together with other wine compounds.

The first step is to uncoil or denature the proteins. Other unknown wine components are essential to this step, and denaturation is accelerated by heating. The denatured protein then aggregates, thus their visual presence, depending on cross-linking through phenolic compounds, metal ions, and other wine solutes.

It appears that about half of the total wine protein is bound to a minor quantity of grape phenolics (flavonoids), and this portion is thought to be responsible for protein haze in white wines (Waters et al., 1995). Because of the relatively-high phenolic content, most, but not all, red wines do not demonstrate a potential for protein precipitation.

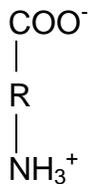
Proteins derived from yeast – as during fermentation and lees contact – do not

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play a role in protein instability. White wines contain relatively-large insoluble proteins which slowly precipitate from solution. Most white wines lack sufficient tannins to cause initial protein precipitation.

Protein haze may be due to the fraction of residual wine proteins that have been rendered prone to precipitation by the interaction with minor quantities of reactive phenols. Bentonite additions remove equal amounts of both unbound proteins, and those complexed with phenols. Wine proteins can be characterized based upon size and electrical charge. Figure 1 is a depiction of an amino acid making up a wine protein, where *R* is a functional group.

**Figure 1. Depiction of an Amino Acid Making Up a Wine Protein**



The solubility of wine proteins depends primarily on temperature, alcohol level, ionic strength, and pH. Changes in any parameter may affect the potential for protein precipitation. Soluble protein levels in juice, and in subsequent wines, increase with increasing grape maturity. Protein synthesis proceeds rapidly after véraison and parallels the rapid accumulation of sugar. The protein level of the fruit is frequently higher in warmer regions. Low crop levels have also been associated with higher protein and higher total nitrogen.

There are as many as eight separate protein fractions in wines, ranging from 11,000 to 28,000 molecular weight units. At a certain pH (the *isoelectric point*), the positive and negative charges of each protein fraction are equal. When these charges are equal, the protein is least soluble (Blade and Boulton, 1988).

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Control of protein instability is achieved through adsorption of wine proteins onto bentonite through a cation-exchange process (Blade and Boulton, 1988). Bentonite is prepared as a slurry, added batch-wise to wine, and agitation is provided, after which the bentonite is allowed to settle. Settling can be slow, and it can take up to a week before the wine may be racked off. Additionally, the bentonite sediment or “lees” occlude between 5 and 10% of the volume of the treated wine.

At the isoelectric or isoionic point (pI) of a protein, positive and negative charges are equal. The pH of wine is very close to the isoelectric point for many wine protein fractions. Wine proteins are least soluble at their isoelectric points. If the wine pH is above the isoelectric point of the protein fraction, the net charge on the fraction will be negative, and the protein will bind electrostatically with positively-charged fining agents.

Conversely, if the wine pH is lower than the isoelectric point, the net charge on the fraction will be positive. In this case, the protein will react with negatively-charged fining agents such as bentonite. The greater the difference between the wine pH and the isoelectric point of the protein fraction, the greater the net charge on the protein and the greater is its binding affinity toward charged fining agents.

**Table 1. Isoelectric points and percentage of associated proteins in Malvasia Istriana and White Riesling (Anelli, 1977)**

Variety	pH	Total Protein (%)
Malvasia Istriana	2.5	18
	2.8	11
	3.1	4
	4.6	30
	6.5	13
	7.1	5
	8.3	9
	8.7	10

Riesling	3.6	19
	3.9	53
	6.7	17
	7.1	11

Table 1 demonstrates the isoelectric points of the protein fractions in Malvasia Reisling. The 3.2 pH of Malvasia is below the isoelectric point of all the protein fractions of Riesling, but is below the isoelectric point of only about 67% of the protein fractions of Malvasia.

Bentonite is principally a negatively-charged fining agent. For Riesling at pH 3.2, 100% of the protein fractions are positively charged and accessible to be electrostatically bound to the negatively-charged bentonite. On the other hand, Malvasia at pH 3.2 has only about 33% of the protein fractions positively charged and accessible to the bentonite.

The charge characteristics of various protein fractions help to explain why some wines are easily protein-stabilized with bentonite, while others are not.

### **Prefermentation Processing Considerations**

The protein level extracted from the fruit is influenced by the initial grape handling methodology. Juice produced from whole-cluster pressing has lower protein

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levels compared with juice extracted from destemmed grapes. Stems play an important role in limiting protein diffusion. Mechanical harvesting that largely eliminates stems may be considered an important factor in contributing to the protein load in white wines.

Skin contact generally increases juice protein concentration, depending on the variety, temperature, and duration. Most of the protein extraction occurs during the first 10 hours of skin contact.

Settling and racking white grape juice before fermentation reduces the total nitrogen content 10-15%. Bentonite non-selectively removes proteins, peptides, and amino acids, and may adversely affect fermentation rates. As much as a 50% reduction in total nitrogen, including reductions in amino acids, may occur with bentonite fining. The amino acid content may be reduced 15-30% with bentonite treatment of 1 g/L, depending on the type of bentonite used.

### **Fermentation and Post-Fermentation Processing Considerations**

The use of bentonite during fermentation reduces non-protein nitrogen approximately two-fold, when compared with losses of protein nitrogen at each addition level to wine. Post-fermentation bentonite additions remove residual wine proteins, and nearly equal amounts of protein and non-protein nitrogen.

Using bentonite during fermentation also reduces subsequent wine lees volume. Increased levels of hydrogen sulfide during fermentation with bentonite contact may occur, likely due to the reduction in the FAN (free amino nitrogen) content of fermenting juice. The addition of nitrogen-containing fermentation adjuncts generally reduces this problem.

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Following fortification, wines may precipitate large quantities of proteinaceous lees. However, alcohol levels of 10-12% are seldom sufficient to cause complete protein precipitation.

The interaction between phenolic compounds and protein is important, because phenol complexation in red and white wines removes or reduces the concentration of some proteins in solution. In white wines, relatively-low phenolic levels usually do not remove enough protein, causing instability. High protein levels in some Pinot noir and other wines may lead to color instability by binding and co-precipitation with tannins and pigments. Pinot noir occasionally requires bentonite fining to attain protein stability, presumably due to insufficient tannins.

Wines fermented and/or aged in oak barrels frequently have lower concentrations of unstable protein and are much clearer than those held in stainless steel, because proteins react with wood tannins and precipitate. Champagne producers take advantage of this interaction by occasionally adding tannic acid to their wines to help bind potentially-unstable proteins resulting from both primary and secondary fermentation.

### *Effect of Aging on Nitrogen Components*

After fermentation, and prior to first racking, total nitrogen increases due to yeast autolysate. During aging, nitrogen increases are primarily attributed to amino nitrogen, which reaches maximum levels after about two months of storage on the lees. The balance is in the form of amide nitrogen and protein. Proteins from yeast autolysate do not contribute to protein instability.

### **Effect of Protein on Wine Stability**

Precipitation of soluble proteins in bottled wines creates an amorphous haze or deposit, which is formed most frequently in white wines or wines of low

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polyphenolic content. It is rarely encountered in wines with relatively-high levels of flavonoid phenols, particularly tannins, which complex with and precipitate proteins. Proteins may also serve as nuclei around which soluble iron, copper, and other heavy metals may deposit.

The nature of protein instability in wines has been difficult to elucidate due to the many factors involved. Differences in proteins occur due to cultivar, maturity, climate, molecular size, and electrical charge, as well as from interaction and precipitation with other components. Additionally, reliable methods of assaying soluble protein have not been developed.

The so-called protein haze in wine is likely a complex of protein, polysaccharide, and polyphenols. Unstable polysaccharide- and polyphenol complexes may explain why heat and other protein precipitation tests, as well as analysis of total protein, are not completely effective predictors of potential instability. A wine's total protein content is not a good index of stability, and thus it cannot be used to predict protein instability.

Protein clouding is due not only to the precipitation of thermally-labile proteins, but also to formation of insoluble protein-tannin complexes. The grape is the major source of protein in wine. About half of the total wine protein is bound to grape phenols, which are responsible for protein haze formation.

Yeast cells may excrete small amounts of protein during fermentation, but much larger amounts end up in the wine upon completion of fermentation as a result of autolysis. Proteins originating from yeast autolysate are not thought to be involved in instability.

### **Processing Considerations and Protein Stability**

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The treatment of fermenting and fermented wines with bentonite is a method of obtaining protein stability. Bentonite addition to wine has its disadvantages, including the formation of large volumes of lees, as well as possible detrimental impact on flavor. Alternative methods for protein stabilization have been investigated and include the following:

- tannin addition and use of immobilized tannins
- peptidases
- ultrafiltration
- fermentation with bentonite

### Tannin Addition and the Use of Immobilized Tannins

Tannins interact with proteins, resulting in precipitation. This is why wines stored in oak frequently clear readily. Immobilized tannic acid derivatives may be effective in stabilizing a white wine. Some white varieties, such as Pinot gris and Sauvignon blanc, are rather delicate in their aroma composition and/or require a significant concentration of post-fermentation bentonite. The pre-fermentation addition of tannin can precipitate proteins, lowering the post-fermentation bentonite requirement (see below).

### Peptidases

Although peptidases have the potential to reduce protein content, they are not currently being utilized in winemaking. Studies have demonstrated that when applied in high concentration and at temperatures greater than 40°C (104°F), treated wines or juices show reduced protein content.

### Ultrafiltration

Ultrafiltration (UF) is a tangential-flow membrane filtration process for separating molecules on the basis of size. Two flow streams are created: the *permeate*, consisting of the portions passing through the membrane, and the *retentate*,

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those fractions too large to pass. Depending on pore size, oxidized and polymerized phenols, proteins, yeast, and other compounds larger than nominal rating, can be removed in a single step with UF.

### Fermentation with Bentonite

Fermentation in contact with bentonite reduces the amount of bentonite fining needed after fermentation. The major concern is that bentonite reduces the FAN (free amino nitrogen) level. As such, supplemental addition to the fermentor is required.

### Enological Tannins

There are two chemical classes of tannins, hydrolysable and condensed. These two groups differ in nearly every characteristic, other than their ability to bind with proteins. Hydrolysable tannins bind with proteins mainly by hydrophobic interactions. Condensed tannins bind proteins through hydrogen bonding. Grape skins and seeds contain only condensed tannins. Hydrolysable tannins are derived from oak wood or as an additive to wines. Enological tannins available on the market may differ in a number of respects, including the following:

- extraction method
- purity
- processing method
- source, including wood, grape skins, and seeds
- toasting variation
- degree of oxidation

Tannins for wine addition can be derived from oak, chestnut, seedpods, etc. Most are water- or steam-extracted, dried, and milled. Different products undergo hydrolysis, pH and color adjustment, and sulfite addition, and may be finished by spray or freeze-drying.

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There is a vast array of tannins on the market, and many are suggested to perform different tasks. Tannins are added for the following purposes and problem corrections:

- redox buffer
- raisined fruit
- sun-damaged fruit
- unripe grape tannins
- structural/textural, mouthfeel modification
- increased substrate for microoxidation
- limit the activity of laccase
- help to precipitate proteins
- help to modify aromas, including vegetative aromas
- help increase aging potential
- possibly to help stabilize red wine color

Many believe that an addition of condensed and hydrolysable tannins is a desirable winemaking practice. Research on the tannin products available suggests that most contain less than 50% tannin. Regardless, post-fermentation use should involve careful laboratory fining trials.

The timing of tannin addition may be important, depending upon the purpose. Like many agents, their negative impacts are usually limited with earlier addition. Adding tannins before or during the early stages of fermentation allows for integration with the other structural elements.

It should be noted that grape proteins may be bound and precipitate with enological tannin addition. The degree of precipitation is dependent upon the grape variety and the season, among other factors. This is one reason why some winemakers use multiple additions during fermentation. Such a procedure can aid in protein stabilization.

### **Methods for Evaluation of Protein Stability**

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Winery operations may play a significant role in protein stability. Thus, bitartrate stabilization, malolactic fermentation, and acidification may render a previously-stable wine protein unstable, as a result of pH shifts. Both heat and additions of spirits may initiate denaturation, and induce subsequent flocculation and precipitation of wine protein and/or protein complexes. Protein stability must be determined after *all* cellar operations are completed, and before bottling.

### Determining Protein Stability

Tests performed for predicting protein stability generally involve heating, heating and cooling (at various temperatures and time durations), or the addition of a precipitation agent. The most common procedures call for subjecting wine samples either to heat or to a chemical oxidant, such as trichloroacetic acid, and subsequent examination for haze development.

Proteins are the most important foam-active components in champagne base wines. Thus, cuvée protein levels must be adjusted such that there is minimal precipitation in the bottle, while not detrimentally affecting carbonation.

In the case of sparkling wine, some choose to fortify cuvées in the laboratory by 1.1 to 1.5% before running a heat test. This may duplicate the additional alcohol achieved by bottle fermentations to produce a product with a final CO<sub>2</sub> pressure greater than 3.5 atmospheres.

Heat Stability Testing. Most predictive techniques involve some exposure of the wine to elevated temperatures for various periods of time. Precipitation of a colloid, such as a protein, is affected by temperature and duration of heating. Virtually all wine protein may be precipitated by heat.

Heating at 80°C for two hours generally gives the lowest dosage rates that accurately predict short- to medium-term stability, while reducing the risk of

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overfining. Chilling wine samples after heat treatment may increase visible haze formation.

**Chemical Precipitation Tests.** In addition to a wide array of laboratory methods involving heating, a number of chemical methods have been employed to predict stability. These include ethanol, ammonium sulfate, trichloroacetic acid, phosphotungstic acid (Bentotest), and tannic acid precipitation (see Zoecklein et al., 2005).



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