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Protein Stability Determination in Juice and Wine

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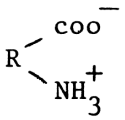
It is important to remember that initial judgments are made by sight. If there is a deficiency of clarity (as may result from protein instability), it may be presumed that there is a deficiency in other desirable attributes. This may result in lost wine sales. In order for the Virginia Wine industry to compete successfully, the products must not only be highly palatable but also stable.

The major source of protein in wine is the grape. Variety, vintage, maturity, condition of the fruit, pH, and processing methodology affect the must and subsequent wine protein content. Protein nitrogen content of wines varies between 10-275 mg/1. Despite the vast literature on protein instability, however, the actual protein levels at which wines will remain protein-stable are unknown.

It appears that about 1/2 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 (Somers and Ziemelis 1973).

Proteins derived from yeast - as during fermentation and lees contact - do not 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. There are as many as eight protein fractions which range from 11,000 to 28,000 molecular weight units (Boulton 1980). The figure below is a depiction of a wine protein.



At a certain pH, the positive and negative charges of each protein fraction are equal. When these charges are equal, the protein is least soluble (Boulton , 1981)

The pH value where the positive and the negative charges are equal is known as the isoelectric point, or isoionic point, of the protein.

The greater the difference between the wine pH and the isoelectric point of the protein fraction, the greater is the net charge on the protein fraction and the greater is the binding with fining agents (Boulton 1980). The isoelectric properties of proteins influence not only their natural tendency to precipitate but also the affinity of the protein fraction to be removed with various agents.

Determining Protein Stability

Formation of protein haze in bottled wine is always a concern for the winemaker and consumer alike.

Table 1 summarizes responses from a survey conducted during a wine stability workshop conducted in Virginia.

Table 1

Protein Stability Tests Utilized by 9 Virginia Wineries

Winery	Procedure
A	Heat 60°C for 72 hours - or Bentotest
B	Membrane filter 0.45µm - heat 145°F 3 days, cool 24 hours – observe
C	No test performed
D	Heat 120°F 24 hours – observe
E	Filter, heat 120°F 24 hours chill to 35°F 24 hours -observe - heat 48 hours at 140°F – observe
F	Heat 100°F 3 days - observe each day
G	No test performed
H	Heat 120°F 3 days - observe at the end of 3 days
I	Filter, heat > 150°F for 5-15 minutes then inspect, cool to room temperature - inspect - refrigerate – inspect

From this it is apparent that there is no Virginia industry standard by which protein stability is evaluated, nor is there an evaluation standard for the wine industry as a whole. There are several reasons for this lack of harmony. 1) Stability is a relative term. For example, one winemaker, knowing that his wine will be shipped across the country and stored in a warm warehouse for a long time, would probably (or be wise to!) set a different standard from a producer selling a few bottles out the door. 2) It has been difficult to devise a protein stability

test which is easy, inexpensive, quick, and will accurately predict how long a wine will remain stable after bottling.

As seen from our survey, most of our winemakers use techniques involving some exposure of the wine to elevated temperatures for varying time periods. Heat tests are performed simply because they are easy to run - not necessarily because they are the best predictors of protein instability.

Heat Tests

Precipitation of protein is affected not only by the exposure temperature but also by the duration of heating. Since all wine protein may be precipitated by heat, there are varying degrees of stability with regard to proteins. For example, heating a sample at 40°C for 24 hours precipitates about 40% of the wine proteins whereas holding at 60°C for the same time period precipitates 95-100% of the protein (Pocock and Rankine, 1973). The time necessary for haze formation decreased with increasing temperature.

Some winemakers utilizing heat tests recommend chilling the wine sample following heat treatment. Visible haze formation is slightly greater than that seen in samples without subsequent cooling. Berg and Akiyoshi (1961) recommend holding the sample at 49°C (120°F) for four days followed by cooling to -5°C (23°F) for 24 hours. The samples are observed cold upon warming the wine to room temperature, haze and/or precipitate formation is again evaluated. Ribereau-Gayon and Peynaud (1961) considered wines heated to 80°C (176°F) for 10 minutes to be stable if no haze developed upon cooling.

The most significant question is: what is the relationship between time / temperature and bottle stability when one utilizes a heat test? As discussed under Lab vs. Cellar Treatments below, this is a difficult question to answer.

Heat Test Procedure

I. Equipment:
- (1) Two 4 ounce bottles
- (2) Incubator set at the desired test temperature
- (3) 0.45 µm membrane and filter housing
- (4) High intensity light source

II. Procedure:
- (1) Membrane-filter sufficient quantity of wine to fill two 4-ounce sample bottles.
- (2) Fill sample bottles, labeling the first as “room temperature” and the second as “one day at the test temperature”.
- (3) Examine each sample under high-intensity light and record impressions relative to initial clarity.
- (4) Place “one day heat sample” in incubator, noting temperature. The other sample remains at room temperature.

(5) At 24 hours, examine each carefully. Refrigerate the heated sample for 2-24 hours and examine.
(6) Clouding and/or precipitate formation in heated sample versus a clear control sample is indicative of protein instability.

Precipitation Test

In addition to a wide array of laboratory methods involving heating, a number of chemical methods have been employed to bring about precipitation of wine proteins. These include precipitation of protein using ethanol, ammonium sulfate, trichloracetic acid, phosphomolybdic acid, phosphotungstic acid, and tannic acid. Most of these precipitation tests are much more severe than heat tests - causing a denaturation and precipitation of all protein fractions.

The "Bentotest", developed by Jakob (1962), uses a solution of phosphomolybdic acid prepared in hydrochloric acid to denature and precipitate wine proteins. Precipitation is proportional to the amount of protein present. Like other protein stability tests, the bentotest can be used to determine bentonite addition levels required for protein stability. The Bentotest reagent is available in kit form from Fritz Merkel, Herzelstrasse 5, 673 Neustadt an-der-Weinstrasse, West Germany. A copy of the analytical procedure is available through my office Department of Horticulture, VPI & SU, Blacksburg, VA 24061.

It has been demonstrated that the Bentotest is more sensitive than a heat test technique using 70°C and 15 minutes exposure (Rankine and Pocock, 1973).

The Trichloroacetic Acid Test, or TCA test (Berg and Akiyoshi, 1961), involves the use of 1 ml of this reagent added to 10 ml of wine. The solution is then heated in boiling water for 2 minutes, after which it is cooled to room temperature. Presence of haze is indicative of protein. The methodology for this procedure was adapted from Berg and Akiyoshi (1961). The TCA test has the advantage of being more severe than heat tests, quick and economical. I recommend it.

TCA Test Procedure (Adapted from Berg and Akiyoshi, 1961).

A. Equipment:
(1) Boiling water bath
(2) Pyrex test tubes (20 ml capacity)
(3) High intensity light source
(4) Pipets (1 ml)
(5) Nephelometer (Coleman Model 9 nephlocolorimeter or equivalent). Those wineries without nephelometers may run this test by simply examining the clarity of the treated sample compared to the control sample using a high-intensity lamp.

B. Reagents:
(1) (55%) Trichloroacetic acid made by dissolving 55 grams of TCA in distilled water and bringing it to 100 ml.

C. Procedure:
(1) Fill two test tubes with 10 ml of wine to be treated.

(2) Examine both samples for clarity under a high-intensity lamp.

(3) To one sample add 1 ml of 55% trichloroacetic acid and transfer to boiling water bath for two minutes.

(4) At the end of reaction period, remove sample and visually compare its clarity with that of the control sample. Haze in the heated sample is indicative of protein instability.

(5) After removal from boiling water bath, allow samples to stand for 15 minutes for reaction to complete.

(6) Consult operators manual for nephelometer setup and operation.

(7) Determine “nephelos units” of sample(s). Berg and Akiyoshi, using the Coleman Model 9 unit, report the following results on all wine types studies:

Potential For Protein Precipitation	Nephelo Reading
“stable”	< 19 nephelos
“variable stability”	19-40 nephelos
“unstable”	> 40 nephelos

Nephelometric principle passage of a light ray through a turbid medium results in scattering and apparent energy loss in the incident beam. In fact, energy is not lost but undergoes directional changes as a result of scattering. This scattering effect may be measured at any angle relative to the plane of incident light. As seen in the figure below, nephelometric measurements are made at 90° to the incident light beam:

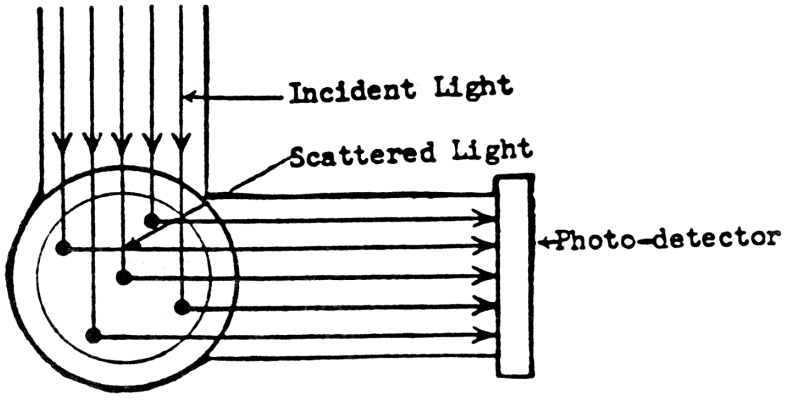


Figure 1
Schematic Representation of a Nephelometric Measurement

The degree of scattering depends primarily upon particle number, size, and shape. These parameters are, themselves, dependent upon several variables, including temperature, pH, concentration of reagents, and mixing procedures.

Lab vs. Cellar Treatments

It is entirely possible for a white wine which has passed a heat test to demonstrate protein precipitation in the bottle. As noted in the above discussion, the parameters of temperature and time can give significantly varied results.

As stated, as much as 1/2 of the total white wine proteins are believed to be bound to flavonoid phenols. As wines oxidize (as occurs with time and the inclusion of molecular oxygen), these phenols polymerize or bind together. As this polymerization continues, this phenol-protein complex becomes more dense -i.e., heavier. When the density of the complex is about the same density of the wine, a visible haze is noted. When the density increases further, the phenol-protein complex begins to fall from solution as a precipitate.

Winemakers often perform a stability analysis on their wine under one set of conditions and then process the wine under a different set of conditions. For example, producers utilizing a gravity filler without a vacuum corker may get 1 ml of oxygen per liter or more in their wine. The result is a possible increase in the phenol polymerization rate and a subsequent formation and precipitation of the protein-phenol complex.

Cellar stability treatments must be performed under the exact conditions that were being followed in the laboratory trials -otherwise bottle instabilities may occur. For example, the same bentonite (from the, same lot, i.e., bag) must be used for cellar and laboratory activities. Bentonite must be hydrated using the same exact methodologies for both cellar and lab. Additionally, the use of Waring type blenders for laboratory preparations exert a shear force which affects the bentonite suspension and which cannot be duplicated in the cellar. This will cause variation between laboratory and cellar treatments.

Another less obvious difference between laboratory and cellar treatments with bentonite is that of contact time. Bentonite reacts almost immediately in binding proteins. Proteins are bounded electrostatically. In time (a day or more), they will begin to "sluff off" the bentonite platlet. Be sure that laboratory fining trials that have demonstrated protein stability are duplicated by the same winebentonite contact time in the cellar. (For additional information on bentonite use for obtaining protein stability, see Zoecklein 1988).

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