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White wine protein instability: a review

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Abstract. The presence of protein compounds in wine leads to decreased organoleptic quality of finished products such as clarity and hue. These compounds have important nutritional values. A large part of the proteins present in wine are closely related to the pathogenesis process. Disturbance in wine samples occurs as a consequence of the presence of several non-protein factors such as the presence of phenolic compounds, metals and pH. To date, a wide range of procedures are known for the protein of wine precipitation in order to determine the total protein content of wine samples, using solutions such as: trichloroacetic acid (TCA), acetone, ethanol, etc. Numerous techniques specific to the process of protein stability are also known and developed. The best known techniques for determining protein stability are: thermal with the addition of chemicals, bentonite, tannin, ProtoCheck, acetone respectively combined (hot test + tannin, etc.).

Keywords: nephelometry, proteins, smectitic material, bentotest, vinification, suspensions.

1. Introduction

Proteins are present in wine in small quantities, their level being closely related to the grape variety used and the technological processes of vinification and maturation [1]. Proteins may be responsible for the colloidal instability of wine, with the formation of amorphous sediment. This type of sediment is present in wine in the form of flocculating suspensions. Their presence gives the wine an unsightly appearance (mist type or turbidity) and can result in significant economic losses for winemakers [1-3]. This problem is a major one for white wine, because its clarity is an essential organoleptic parameter. At higher temperatures, the presence of light scattering particles occurs due to the phenomena of protein self-aggregation [4]. The formation of protein colloids involves factors such as: wine

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storage temperature, pH, ionic strength, protein composition of wine, organic acids, ethanol, polyphenolic substances, the amount of sulfates [5, 6]. Even if the presence of colloids does not present a risk to the health of the consumers [7], it is not approved from a sensory point of view [8]. The most important proteins related to protein instability may be those of *Vitis vinifera* type which include chitinases and thaumatin [9]. These types of proteins can be slowly denatured and aggregated during the wine storage process. This phenomenon must be kept under control by removing them from the wine through the stability process [9]. Protein instability is prevented by the removal of unstable proteins using fining agents in wine. These deproteinizing materials are substances that have an electric charge in their composition. When are in contact with wine, they flocculate and precipitate the particles that have an opposite electric charge involved in the protein disorder [10]. To treat this type of defect, various alternative clarification techniques with deproteinizing agents have been studied, such as: ultrafiltration [11], 'flash' type pasteurization [12] and the addition of organic and inorganic chemical compounds [7, 13]. Wine proteins can be characterized by polyacrylamide gel electrophoresis (PAGE) [14] and PAGE under denaturing conditions (SDS-PAGE) [13, 15]; using isoelectric focusing (IEF) analysis of proteins grape must or wine [1, 13], based on agarose or polyacrylamide gels either in a tube or on a plate, or denatured PAGE (SDS-PAGE) [13]. Other methods such as capillary electrophoresis (EC) or lithium dodecyl sulfate polyacrylamide gel (LDS-PAGE) are also used [13] or fractionation of liquid protein chromatography (FPLC) [13], as shown in **Figure 1** [13]. In order to determine the level of proteins in wine, spectrophotometric and technical methods can be used for the analysis of colloids in white wine samples: zetametry, gel electrophoresis, PCS photon correlation spectroscopy for sizing sub-micron particles, turbidity [16].

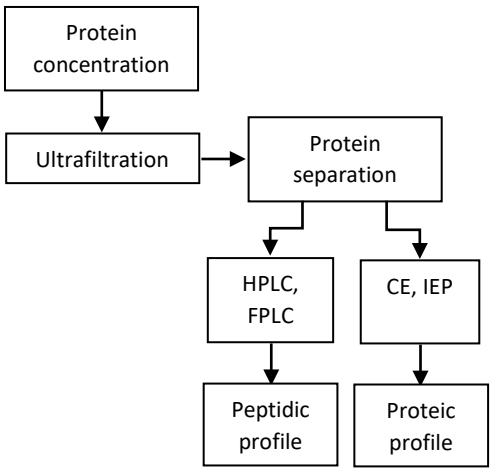


Fig. 1. Schematic representation of a protein characterization workflow in winemaking.

2. Proteins responsible for protein instability

The protein compounds of wine are made up of proteins from *Vitis vinifera* grapes and those obtained from the autolysis process, *Saccharomyces cerevisiae* type [13]. Ribeiro et al. [10] indicated that the proteins responsible for the instability of the wine are those related to pathogenesis [13]. Pathogenesis-related proteins are essential for their development. As a defense mechanism against various pathogens, pathogenesis-related proteins are synthesized immediately [2]. Soluble proteins have a molecular weight in the range of 6-200 kDa and the isoelectric point (pI) of 2.5-8.7 [17], as shown in **Table 1**. The concentration of each protein fraction depends on: grape variety, degree of maturity of the raw material, soil, respectively, climatic conditions and winemaking processes [13, 18]. The protein level in untreated wine can vary in the range of 15-300 mg / L [2], sometimes even up to 700 mg / L [13]. The proteins responsible for the turbidity of the wine can withstand the technological processes of vinification, because they are very resistant to the proteolytic activities of the protease in grapes and yeasts [13].

Table 1. Identification of isoelectric point (pI) and molecular weight of proteins present in wine, by grape variety [13]

Grape variety	Isoelectric point (pI)	Molecular weight (kDa)
Macabeo, Parellada	3.0-5.6	14-94
Riesling	3.1-9.2	11-88
Chardonnay		
Verdeca	3.6-9.0	6-200
Pinot Noir		
-	4.0-8.2	10-70
Muscadine	4.6-8.8	12-41

The isoelectric point for proteins is the pH value where the molecule has no charge, being characteristic of each protein and depends on the composition of amino acids. As can be seen in the graph in **Figure 2** at pH values lower than the isoelectric point (pH <pI), the protein is positively charged with absolute load values increasing as the pH decreases; at pH higher than the isoelectric point (pH> pI) the protein assumes a negative charge [19]. At the pH of the wine, the proteins tend to be positively charged, but this charge tends to decrease until it disappears as it approaches the isoelectric point [19]. Waters et al. [20] has shown that most of the proteins responsible for the instability of wines are those produced by yeasts.

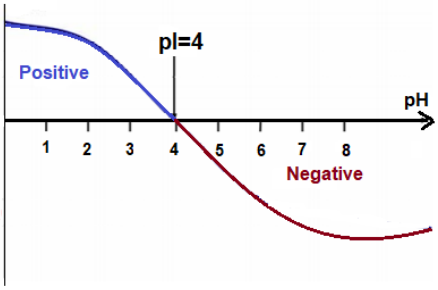
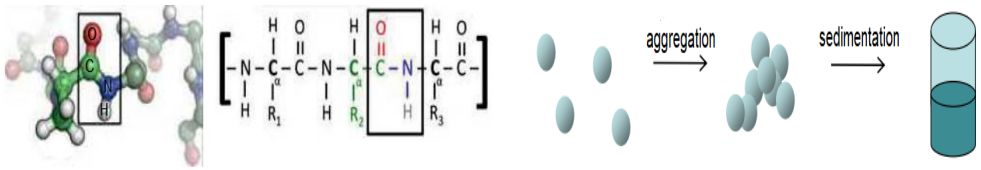


Fig. 2. The relationship between isoelectric point, medium pH and electrical charge of proteins

For the instability of proteins in white wines, it was observed that the wine sediments (**Figure 3**) formed at higher temperatures contained two main electrophoretic fractions with different behaviors at the same temperature [13].



A. The molecular structure of a protein [21] B. Sedimentation of protein colloids[16]

Fig. 3. Schematic representation for protein sedimentation in wine

Wine biopolymers were also divided and analyzed, noting that protein fragments with isoelectric points and low molecular weights were much more sensitive to heat treatment, being responsible for protein instability [13]. The proteins responsible for white wine tubidity have glycoproteins in their structure and have a molecular weight of 12.6-30 kDa, respectively, pI 4.1-5.8 [13]. It is possible that protein fractions with lower kDa are also responsible for diminishing the clarity of the wine.

3. Protein precipitation / cassation mechanism

The sediment consists mainly of protein substances in a percentage of about 80%. In addition to these, there are also polyphenols, polysaccharides and mineral substances. In this set, however, the protein-tannin combination (**Figure 4**) represents the predominant proportion [22]. A protein subjected to a high temperature has broken hydrophobic bonds in its composition, and the molecule becomes disordered. Protein denaturation is accompanied by insolubilization with the formation of "haze" in the wine. Tannins have the property of binding by hydrophobic protein bonds through the formation of insoluble complexes (**Figure 4**) [21]. Protein precipitation and protein cassation occur according to the mechanism of flocculation of hydrophilic colloids, following the elimination of the main stability factors (electric charge and solvation) [22]. Proteins lose their solvation, and from lyophiles they become lyophobic under the action of alcohol,

high temperature and tannins with a high degree of polycondensation, after which precipitation takes place under the action of cations and in the presence of air [22]. The proteins responsible for the turbidity phenomenon are considered those of the PR (PR-pathogenesis-related) type [23]. Chitinases and TLPs (thaumatin-like proteins) [9, 24] have in their composition a large number of disulfide bonds with the formation of stable globular structures that have high resistance against pathogenic compounds [23]. Proteins that do not form the "haze" type phenomenon that are present in must and white wine are: mannoproteins, glycoproteins and invertase from grapes, proteoglycans rich in arabinose and galactose or arabinogalactan proteins-AGP [10, 23]. The presence of these types of proteins that are not responsible for the turbidity of wine, can affect the quality of white wine if they are subjected to higher temperatures [25] and can interact with the aromatic compounds of wine [23].

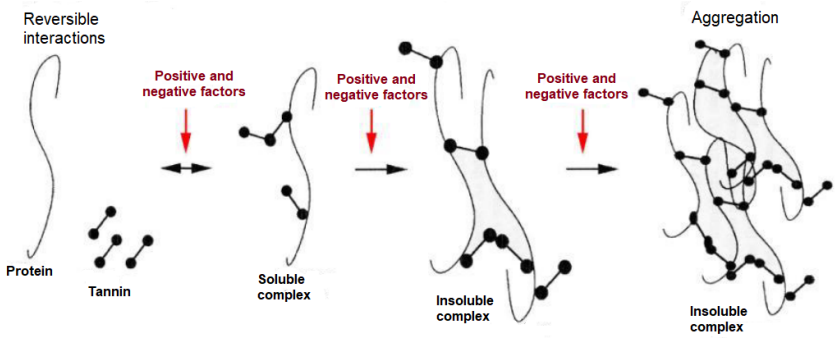


Fig. 4. Schematic representation for the mechanism of precipitation of wine proteins[11]

3.1. The main determinants of wine stability

The origin of the taste and bouquet in wine is achieved by redox processes (made of dissolved O_2) being a slow process and the stabilization of the wine naturally can take 3-15 years [26]. The resulting product is clear, transparent, with a well-defined color, specific to the type and assortment of wine. Young wines are the most difficult to stabilize, being necessary to accelerate the physico-chemical and biological processes in a relatively short time of several months [26, 27].

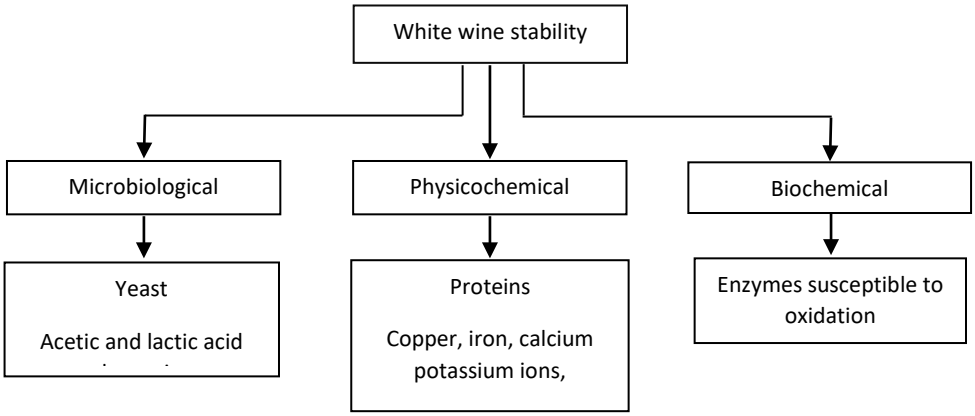


Fig. 5. Classification of the stability of young white wine [28].

According to the classification of the protein stability of the young wine indicated in **Figure 5**, the factors that can influence the combination of the different substances present in the wine can be determined.

3.2. Protein precipitation of wine

There are many protein precipitation procedures applicable to wine. The purpose is to determine the total protein content of wine samples. These specific protein precipitation procedures can be followed using different types of tests:
3.1. KDS precipitation. In this type of protein precipitation test the reaction between potassium chloride and 10% sodium dodecyl sulphate (SDS) takes place resulting in the formation of the KDS compound. This compound acts as a stabilizing agent due to its ionic nature, strengthening intermolecular interactions between proteins that cause the formation of precipitation [29].
3.2. Acetone precipitation. The proteins of the wine are precipitated by the addition of acetone in the wine sample in a ratio of 2: 1. The action of acetone consists in decreasing the dielectric constant of the medium in which the proteins are immersed, there is a decrease in their repulsive force and increasing the interaction between them with the formation of precipitation [29, 30].
3.3. Trichloroacetic / acetone precipitation. Another method of protein precipitation is the use of trichloroacetic acid (TCA) solution of 10% concentration and acetone, in a ratio of 1: 2. The resulting solution is added to the wine sample tested and centrifuged [29, 30].

4. Techniques for determining protein stability

To reduce the risk of formation of different colloids in white wine, various techniques have been implemented to determine protein stability, presented in **Scheme 1**, frequently used in winemaking. These types of tests are performed to

define the dose of clarifying agent required in stabilization treatments [20]. Techniques for determining the protein instability of wine include: determining the total protein content present in the wine or test methods by decreasing the protein solubility in the wine by involving a high temperature [31] or addition of chemicals. The following are indicated as protein chemical precipitates: TCA [32], phosphomolybdic acid or benthotest, ethanol or tannin solution. Protein precipitation (similar to thaumatin) does not occur during the thermal test when higher temperatures are used. The ethanol solution test precipitates a large number of polysaccharides, but is not effective [13]. A much higher temperature (90 ° C) [15] leads to the formation of a natural precipitate, in terms of biochemical composition, being the most suitable test to determine the level of stability for a wine sample [13]. Various tests provide information on instability indices, requiring certain doses of priming agents to obtain the stability of the wine depending on its results. [13]. Protein stability techniques are not closely related to the total protein concentration in wine, as individual protein fractions act differently [15]. However, analyzes for the protein content of wine are limited and do not take into account the role that the presence of other wine compounds may have in protein instability [13, 33].

4.1. Protein separation technique by SDS-PAGE method

For the separation of proteins it is preferred to use a common electrophoretic technique, namely SDS-PAGE electrophoresis [30, 34] (SDS polyacrylamide gel electrophoresis); this method is based on the separation of charged molecules in an electric field.

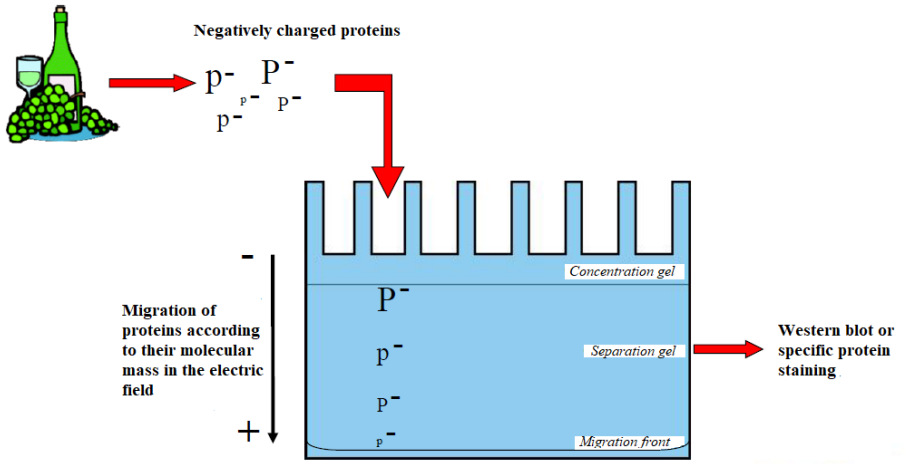
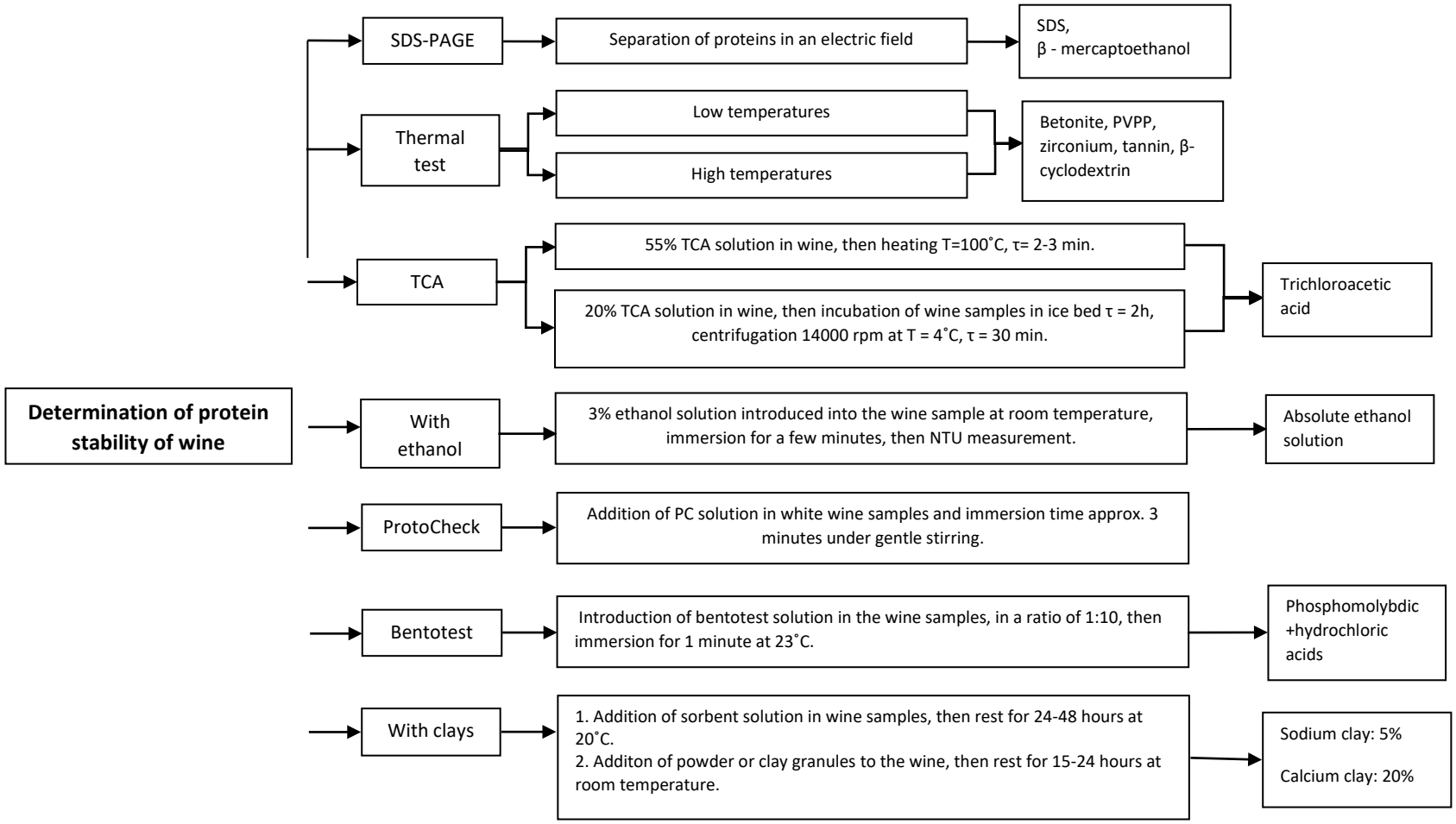


Fig. 6. Electrophoresis technique and SDS-PAGE gel staining [35]

The principle of operation for this technique presented in (Figure 6) is the presence of an electrophoresis tank connected to two terminals (a cathode (-) and an anode (+)) provided by an electric generator: the molecules that are introduced into this field will migrate,



Scheme 1. Working techniques for determining the protein stability of white wine.

depending on their load, to the complementary pole. The peculiarity of the SDS-PAGE technique involves the exposure of samples with a high protein content to a denaturing pre-treatment. Thus proteins are subjected to the action of two compounds [36]:
- β-mercaptoethanol: this compound exerts a denaturing action on oligomeric compounds by denaturing disulfide bridges that disrupt the three-dimensional structure. The SDS (sodium dodecyl sulfate) compound has the ability to attach to the periphery of protein chains, while providing a negative charge. The proteins covered by SDS will all have a negative charge. SDS-influenced protein compounds will migrate to the anode (+): actual protein loading is no longer involved; only their molecular mass will influence their migration.

4.2. Heat stability tests

Heat tests consist of the application of one or more pairs of temperatures [37] for example, heating a sample of wine to 80 ° C for 5 minutes or treating a sample of white wine at the same temperature, varying only the time (**Table 2**) in the same working environment (in the water bath or thermostat) [28]. It is not yet a standard protocol for performing the heat test [5, 38], because many studies have used different time periods and temperatures (**Table 2**).

Table 2. Experimental conditions for thermal protein stability

High temperatures	***Low temperatures***	***Bibliographical references***
35-20˚C, τ= 190 h	-	[20]
35˚C, τ= 720 h		
60˚C, τ= 24 h	-	[11]
60˚C, τ= 95 h	4˚C, τ= 6 h	[32]
80˚C, τ= 5 minutes	-	[11, 45]
80˚C, τ= ½ h	-	[46, 47]
80˚C, τ= 2 h	0˚C, τ= 2 h	[4, 48]
	4˚C, τ= 2 h	[49]
	4˚C, τ= 16 h	[20]
	20˚C, τ= 3 h	[20, 25]
80˚C, τ= 3 h	20˚C, τ= ½ h	[50, 51]
80˚C, τ= 6 h	4˚C, τ= 16 h	[5, 52]
80˚C, τ= 6 h	4˚C, τ= 12 h	[11, 20]
90˚C, τ= 1 h	4˚C, τ= 6 h	[32]
	4˚C, τ= 18 h	[53]

-where: τ= heating / cooling time period

This type of test involves heating a sample of wine to a pre-set temperature over a period of time. The thermal test is based on the denaturation of the proteins in the wine and to induce precipitation [39] with "haze" formation. This test is applied to simulate turbidity formation and is considered appropriate to determine the appropriate doses of priming agent required to remove heat-unstable proteins, but other wine compounds are less affected [40] (metal cations and wine pH) [39]. Wine proteins have different sensitivities in the presence of heat resulting in the formation of precipitates [41-43]. The most used test is the one described by

Pocock et al. [44] where the wine is heated at 80 ° C for 6 h. Ribéreau et al. [31] suggested that the wine should be heated to 80 ° C for 10 minutes; another working protocol [15] it could be heating the wine to 90 ° C for 60 minutes, but compared to other tests, this type of hot test leads to the formation of a precipitate with a composition similar to the natural one [39].
The most used process for hot testing is heating at 80 ° C for ½ - 2 h in a bath of heated water to allow the degradation of proteins in the solution, and during the heating of the wine samples are kept in a slight stirring. After heating, the samples will cool to room temperature. At this stage the proteins are coagulated and precipitated. Determine the degree of turbidity using net nepholometric / turbidimetric units (NTUs) by the difference between the NTUs after the respective stability test before heating (Eq. 1). The aim is to estimate the level of colloidal instability reached by the samples. The reference equation is as follows [29]:

$$\Delta\text{NTU NET} = \text{NTU after thermic treatment} - \text{NTU before} \quad (1)$$

So far there is a variation in protein conformation, which can expose hydrophobic groups, which triggers protein aggregation, protein compounds are fully developed at higher temperatures (70 ° C) [24] and are more likely to aggregation at a higher pH (pH=4) [6, 39]; thus, the cooling time can be essential for the results of the heat tests and the variations of the conditions can compromise the accuracy and repeatability of the results [54] White wines heated to 80 ° C τ = ½ - 6 h then chilled τ = ½ - 18 h at 0˚C, 4˚C or 20 ° C, extended heating times, prolonged cooling time or a cooling temperature lower - all these parameters led to a gradual increase of colloids formed after the hot test [39]. By heating a wine sample for 6 hours and cooling τ = 18 h to 4 ° C (24 h test), as is indicated in Table 2, require a higher dose of clay material (approximately 0.3 g / L) compared with heating τ = 2 h [13] [39]. The formation of turbidity in wine in these types of thermal tests can be explained by applying a certain higher temperature (above 20˚C) resulting in the exposure of several active protein sites - the binding of active polyphenolic compounds to the "haze" of wine [55]; the cooling process could lead to decreased solubility of more complex protein-polyphenol compounds [39, 56]. One of the disadvantages of this type of protein stability is the increased oxidation of the phenolic compound at higher temperatures [32], and may cause protein precipitation and interfere with test results [10].

4.3. Trichloroacetic acid (TCA) test

The working principle consists in the ability of TCA to completely precipitate proteins in wine [45]. The procedure consists in adding 1 mL of a 55% TCA solution [57] in 10 mL wine sample; the next step of this test is to heat the wine sample mixed with TCA solution to 100 ° C for a few minutes, then cool [31] before nephelometric measurement of the resulting wine. Another working method is to add 10 mL TCA of 55% concentration in 100 ml of wine. The mixture of wine

and TCA will be heated for 2 minutes in a water bath at a temperature of 100 ° C; turbidity measurement will be performed after 15 minutes at a temperature of 21-23˚C [39]. Another working technique is to heat the wine sample for 3 minutes, followed by cooling for 1-2 hours [11]. However, a lower concentration of the TCA solution of up to 20% was also used [58] then added to the wine samples to be analyzed.

4.4. Tannin solution test

Certain colloids are likely to form during wine storage where protein compounds could precipitate by binding to phenolic ones [32]. Another research conducted by Yokotsuka together with his collaborators [56] observed how the ability of phenolic compounds to bind to protein increased with the level of polymerization. This test is influenced by various intrinsic factors of wine such as: pH, integral protein content, iron, copper and potassium. But it is not a good predictor to determine the optimal amount of clarifying agent needed to stabilize wines [15, 17].

4.5. Ethanol test

This test consists in adding a volume of absolute ethanol to a wine sample. The turbidity formed in the presence of ethanol is measured nephelometrically immediately after its introduction into the wine. It is not only composed of thermo-unstable proteins, as it also precipitates polysaccharides (mainly mannoproteins) [29]. The ethanol test consists in reducing the average dielectric constant, resulting in a reduction in protein solubility [15, 32]. The disadvantage of this test is the overestimation of the risk of protein occurrence, but the advantage of the test is that it provides an immediate, fast result.

4.6. ProtoCheck test

The test called ProtoCheck (PC) is an international patent belonging to the University of Udine, which was developed for white wines [59]. The main features of this test are: it has become standardizable, is fast, has a high specificity (checking the electropositivity of proteins) and can be used directly in the wine storage area [60]. The addition of the reagent to the white wine samples causes the rapid neutralization of the proteins alone, without interacting with the other components of the wine (tannins or polysaccharides) [61].

Fig. 7.1. Measurement of turbidity on the sample before and after reagent addition [60]

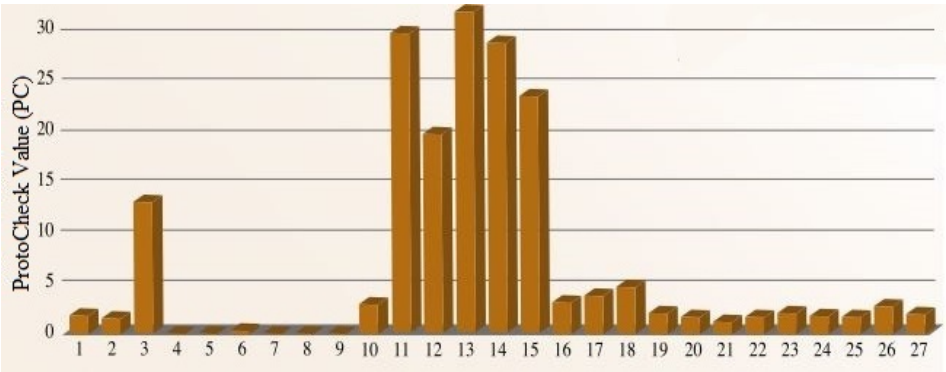


Fig. 7.2. Perform the ProtochecK test on a sample of 27 samples of white wine [60]

As can be seen in **Figures 7.1** and **7.2** the addition of the reagent causes the rapid neutralization of the proteins alone, without interacting with the other wine-specific components. For white wines from experiments performed, a wine sample with values close to 0 is considered stable. The superior results are related to variable risks of instability; 0.5 to a few units are medium-low instabilities, while a higher number indicate significant potential instabilities (**Figure 7.2**). The degree of performance of this test is very fast, respectively its simplicity of execution makes it directly applicable in the wine storage area [60]. This type of test ensures the nephelometric measurement for the wine sample in the initial state (NTU_1), then the wine is modified by adding anionic reagent (in a ratio of 2: 1) and after one minute a second reading will be performed (NTU_2). The result of the dilution PC will be given by Eq. 2 [59]:

$$PC= (NTU_2 - NTU_1/1.5) \quad (2)$$

The test involves the interaction of an organic anionic polyelectrolyte that neutralizes the positive electrical charge of the proteins, bringing them to the

isoelectric point, thus determining their insolubility and the formation of liquid turbidity [59, 61]. This test takes advantage of the fact that proteins are the colloids in wine that have a positive charge and are therefore specific for these types of substances [61]. This type of test verifies the electropositivity (+) of potentially unstable proteins present in the wine with a mixture of stabilized anionic adjuvants (-) [61]. The specific reaction that takes place does not interact with other substances and causes the browning of the wine which can be measured nephelometrically. The ProtoCheck test kit allows testing directly on the filtered / unfiltered white wine sample [57].

4.7. Protein stability test with benthot test

The bentot test uses a solution of phosphomolybdic acid which is added to that of hydrochloric acid. To the filtered wine sample will be added a reagent based on phosphomolic acid, in a ratio of 1:10 (reagent solution / wine sample) and "instantaneous" disturbances in the form of clusters / clouds [62]. This test precipitates wine proteins by neutralizing the protein load, thus leading to molybdenum ion aggregation [32]. This working procedure consists in the complete precipitation of the proteins in the wine sample. But the disadvantage of this test is the overestimation of the clarifying / priming agent used to stabilize the wine [10, 13, 63].

4.8. Protein stability test using clay materials

The treatment of must and wine with bentonite is a frequently used and internationally recognized practice [4, 17, 64]. Bentonite is used to improve the clarity respectively, the stability of the wine preventing the formation of precipitation in the wine storage stage [17]. Exchangeable cations in its T-O-T type lamellar structures (tetrahedral-octahedral-tetrahedral layers) [65] strongly influences some properties, such as specific surface area, exchange capacity and adsorption capacity [25, 28, 47]. Smectite clays can be administered in wine in the form of gel or bentonite milk (in water or wine)[22], in the form of powder or granules; administered in the form of a gel has a concentration of about 5% for sodium bentonite or 20% in the case of calcium or 10% when the two types of bentonite are mixed [22]. The advantage of administering bentonite as a gel is given by the lower degree of dilution of the wine. The disadvantage of this form is the difficulty of dispersing the bentonite sample in the corporation in the wine mass. Administration in powder form is very rarely practiced when using sodium bentonite [22]. To obtain a homogeneous dispersion, it is recommended that bentonite be introduced in very small quantities into water or wine under continuous stirring. "Bentonite milk" is left to stand for 24 hours to inflate bentonite particles [17, 28], being then used to "bentonize" the wine [22]. The introduction of the bentonite solution is done slowly under constant stirring, so that the bentonite, which is still in a colloidal state, is spread evenly. Regardless of the

form in which it is administered, the mixing of bentonite must be very fast, in order to achieve the best possible homogenization. After homogenization, the wine will be left to rest for 5-20 days depending on the size of the vessel and the degree of disturbance for clarification [22].

5. Spectroscopic methods for the degree of influence of protein stability tests

The conventional stability tests described above require a long period of time. Therefore, the use of infrared spectroscopy (NIR) to obtain predictable information about the level of protein instability present in wine seems to be promising. The need to use NIR to estimate the development of "haze" of protein instability in white wines obtained from thermal and colloidal stability tests was examined for the first time by Versari and his collaborators [66]. They tested a very large sample of white wine using the near and infrared spectral region. Partial least squares (PLS) regression analysis was used to construct predictive models of the acquired turbidity spectra. nephelometric values obtained from the addition of ethanol to wine could be obtained from NIR spectra with short wavelengths [13]. Another method that can be used to test the level of protein disorder is the UV-VIS method [67, 68]. Protein disorders produced by heat tests were measured spectrophotometrically at a wavelength of 520 nm at a wavelength of 1 cm [54].

6.Conclusions

The level of instability caused by the presence of protein compounds in wine is closely related to various external and internal factors, such as: exposure of wine to higher temperatures, pH of the wine sample, the presence of certain compounds: organic acids, metals, sulfur content, phenolic compounds, the level of polymerization of each type of wine, but also unknown factors that can lead to the formation of protein clusters / colloids. Technological processes for wine stability require concrete knowledge about wine-specific compounds and possible connections that take place during the precipitation process. The protein compounds that are responsible for the turbidity of the wine solution are mainly those related to pathogenesis, proteins similar to thaumatin and chitinase, which have a high resistance to the process of proteolysis during the technological processes of wine. Of all the techniques listed above for determining protein stability, the most effective is the PC test because it is very fast, safe and causes rapid neutralization of proteins without interacting with other wine compounds. The other TCA, ethanol and benthot tests are fast but not correctly estimates the required doses of stability agents.

Acknowledgments

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