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PRECISION PROTEIN STABILITY

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The protein instability is one of the most discussed issue in oenology. Even if is well known that the use of bentonite allow to drastically reduce the amount of proteins in wine and so the protein instability risk, it is not well know how to use it in the most rational and targeted way. This lack in knowledge is often reflected in an overestimation of the amount of bentonite to use, with an organoleptic depletion of the wines quality.

In this focus we are going to analyze some of the most important elements in order to dose rightly the amount of bentonite, for example the kind of bentonite to use and the protein stability test used to reveal the instability. We will also explore the destabilizing role of some coadjuvant like CMC, metatartaric acid and K-polyaspartate, giving usefull guidelines to avoid the problems related to their use.

WHAT IS PROTEIN INSTABILITY? HOW IS IT FORMED?

Wine is a complex matrix where different equilibriums coexist; these equilibriums can change due the variations of some wine compounds over time and following storage conditions leading, in some cases, to instability phenomena. This instabilities can generate visual and organoleptic alterations that can compromise the quality perceived by the consumer.

Analyzing the white wines, is possible to distinguish:

- Tartaric instability, caused by the crystallization of the tartaric salts present in wine in a oversaturated state;

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- **Microbiology instability**, due to undesired microrganisms (yeasts and/or bacteria) which alter various substrates (sugars, organic acids and alcohol);

- **Protein instability**, the most important instability to be considered in whites.

Proteins in wines come mainly from grapes and marginally from yeasts; during vinification the increase in alcohol content and the variation in solubility act as a sort of purification of the protein matrix, resulting in the extraction of chitinase and TLP (Taumatin-like protein) into the wine, the two proteins most involved in protein instability process. TLP and chitinase are part of the PR proteins (pathogenesis related proteins) produced by the plant as a result of a pathogen attack and therefore they are able to resist to proteases in acid environment.

What is common defined "protein casse" is a spontaneous denaturation followed by flocculations of the main thermolabile proteins, with consequent appearance in the wine of whitish floccules in suspension. This process is favored by particular conditions, such as low pH, high temperatures, sulfate ion in solution and high alcohol content.

Over the years, various models have been proposed by researchers to explain the onset of protein instability, based on what is called a three-phase model:

1 – Proteins insolubilization

2 – Auto-aggregation

3 – Cross aggregation between aggregates

Recently a new aggregation model has been proposed **(Fig. 1)**, *''Revised method of protein haze formation'' (Marangon et a*

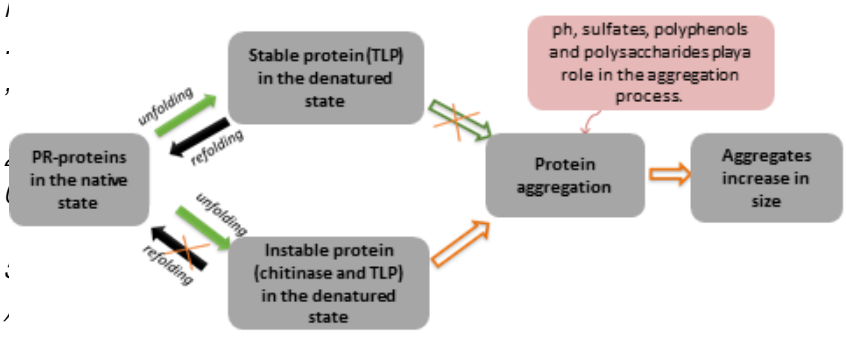

FIGURE 1. Schematization of the process of protein instability formation in wines (Marangon et al., 2015)

The model emphasizes that chitinases and some TLPs in the denatured state (high temperature, sulfate ion ...) become unstable and form aggregates (mainly due to the hydrophobic attraction), as they are unable to refolding to the stable native state.
It is therefore evident that the understanding of this phenomenon is still a process in progress, which will require years of study and application.

PROTEIN STABILIZATION: BENTONITE DOSAGES

Despite numerous research groups in recent years have looked for innovative solutions to solve the problem of protein instability, like zirconium oxyde or enzymes (from proteases family), the most common method that currently offers greater guarantees for the protein stabilization of a wine is represented by the use of bentonite. The high adsorbent surface (after hydration) and the electronegative charge of this particular type of clay in fact allows to remove the positively charged proteins at the wine pH. However, the use of bentonite is not a risk-free practice. At excessive doses, in fact, its use leads to a '' simplification '' of the organoleptic profile of wine, with indirect removal as well as proteins also of aroma (adsorption aroma-protein) and polysaccharides, with possible increases in astringency and problems related to stabilization of the foam in sparkling wines. In addition, excessive dosages of bentonite can affect the shelf-life of the wine by promoting oxidative processes responsible for premature aging, as evidenced by some tests performed by GiottoLab using the TDO - Dynamic Tests for Oxidability (Fig. 2).

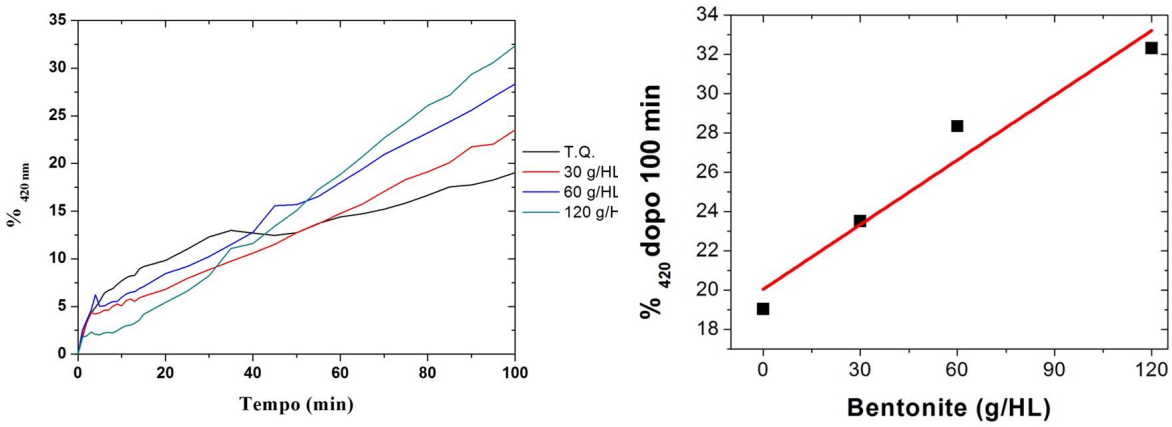

FIGURE 2. Correlation between bentonite dosage and oxidability of wine. The graph on the left shows the kinetics of the % of the increase of the 420 nm signal of a wine treated with 30, 60 and 120 g / HL of bentonite. On the right is the correlation between the % increase at 420 nm after 100 minutes of analysis and the bentonite dosage.

Currently there are no viable alternatives to the use of bentonite in terms of effectiveness, costs and regulations. A correct dosage of bentonite therefore becomes an aspect of fundamental importance for obtaining proteically stable wines but at the same time not excessively stripped of the sensory properties that distinguish them. In this cases we spoke about *precision protein stability*.

In determining the optimal dose of bentonite necessary to make a wine stable from the protein point of view the variables to be taken into account are therefore:

- the protein profile of the wine;

- the nature and the charge of the used bentonite, the swelling time but also the contact time of the clarification;

- treatments that the wine will undergo after protein stabilization, such as the addition of tannins, CMC, metatartaric acid or k-polyaspartate.

BENTONITE COMPARISON

One of the most common errors when stabilizing a wine is to choose bentonite exclusively on the basis of price, without evaluating the deproteinizing power and the ability to preserve the fruity / floral aromatic notes of the wine, properties strongly influenced by the nature of the bentonite used (ionic composition: calcium or sodium) and from the type of wine treated (young or aged wine).

The comparison of different commercial bentonites conducted by Giottolab on two wines (Pinot Grigio and Incrocio Manzoni Bianco) (Fig.3), shows how important it is to identify the best bentonite to be used in order to optimize the dosage, minimizing the organoleptic interferences of this oenological coadjuvant. The results show a remarkable variability in terms of deproteinizing power of the different bentonites compared; therefore if we wanted to stabilize Pinot Grigio with bentonite E, for example, we should probably use a dose of at least 40 g/HL, twice as much as required using bentonite C, with a consequent serious organoleptic depletion of the product.

An economic consideration deserves to be made, considering that the bentonites on the market have a unit price per kg that can vary from 2 to 22 euros/Kg; therefore, according to the cases and the useful dosages, the expense for the protein stability of a tank is very variable. In the economic evaluation of the use of a specific bentonite rather than another one, the volume of wine that is lost as a result of the protein stabilization treatment has to be taken into account. As reported in the article "*Save money and wine by choosing the right bentonite*" by Simon Kinley and Darko Obravovic in fact the loss in volume of wine can vary from 3% up to over 10% depending on the type and quantity of bentonite used.

♦ **Deproteinizing power of different bentonites**

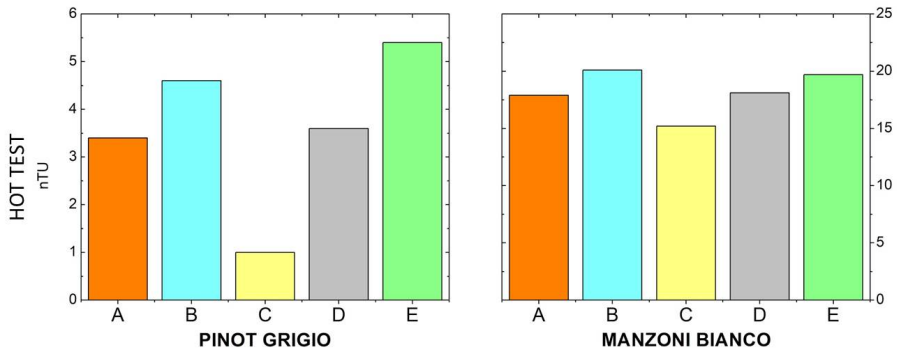

FIGURE 3. Hot test results comparison between different bentonites.
The tests were carried out by rehydrating in parallel and according to the same specifications (5 g of bentonite in 100 ml of water, for 24 hours) the 5 bentonites tested. The 2 wines tested, Pinot Grigio and Incrocio Manzoni, were used to create 5 different test, each of which dosed with 20 g/HL of the various bentonites. The stabilization of the wine was evaluated according to the *hot test*, among the most widespread tests in oenological laboratories for the evaluation of protein instability. The differences in terms of nTU between the 2 wines tested are imputable to a different protein content that characterizes the 2 varieties.

ANALYTICAL METHODS, A COMPARISON

The choice of bentonite is one of the aspects underlying a precision protein stabilization, but certainly not the most important. The key point is the identification of the analytical method able to determine the protein instability of the sample with the highest possible precision. The market offers various alternatives in terms of analytical methods for the determination of protein instability, whose role is to determine the optimal dose of bentonite to prevent wine turbidity due to protein precipitation phenomena. All the tests normally used are called '' orientative '' tests because they artificially and indiscriminately determine an alteration of the colloidal matrix of the wine leading to a protein precipitation. The main problem for the winemaker is inherent in the great variability of the results obtained using the different methods that make it difficult to evaluate the degree of protein instability of the wine. In this sense, the technical-scientific articles dealing with this subject are certainly not helpful; in fact, despite numerous authors have compared the different existing methods listing their advantages and criticalities, a clear and easily applicable guideline that can really help the enologist to understand which test to apply and in what context is rarely provided. This often results in a not very conscious choice by the oenologist who, to avoid problems of protein precipitation in the bottle, will tend to

opt for less selective methods with consequent bentonite overdoses.

A representative example is shown in Fig. 4, where it is clear how the choice of the analytical method adopted (*hot test*, *cold tannin test* and *hot tannin test*) significantly affects the amount of bentonite used and, consequently, on the organoleptic quality of the final product. So which analytical method adopt to evaluate the protein stability of a wine?

GiottoLab's ten-year experience has shown that *hot test* is the best approach to assess the stability / instability of a wine, provided the test is done on the wine in the same conditions in which it will be bottled. As will be discussed in the following paragraph, in fact, the possible addition of technological adjuvants could cause instability phenomena that can not be foreseen using only the hot method.

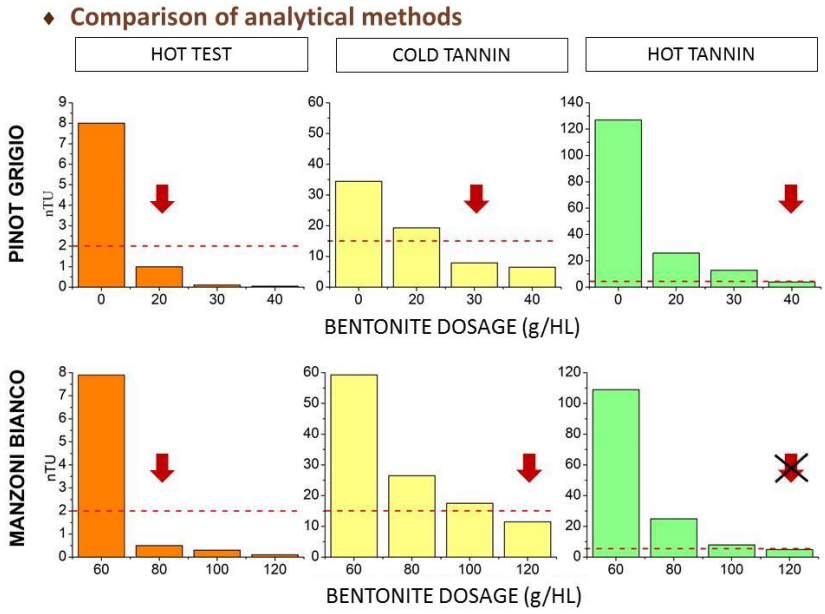

FIGURE 4. Comparison of the bentonite dosage obtained using the hot test, the cold tannin test and the hot tannin test. In the case of Pinot Grigio, the quantities of bentonite required to bring the wine to stability are equal to 20 g/HL for the hot test, 30 g/HL for the cold tannin and 40 g/HL for the hot tannin. In the case of the Manzoni white, however, the quantities of bentonite required to bring the wine to stability are equal to 80 g/HL for the hot test, 120 g/HL for the cold tannin and >120 g/HL for the hot tannin .

PROTEIN INSTABILITY DUE TO CMC AND METATARTARIC ACID ADDITION

Products like CMC or metatartaric acid, which inhibit the growth of potassium bitartrate crystals, are used to avoid tartaric instability but, even if not known to all, can destabilize the wine proteically (Fig.5A). Although the mechanisms responsible for this instability are not yet completely clear, it is possible to hypothesize interactions between the negative superficial charges of CMC and metatartaric and the positive charges of wine proteins, with consequent formation of flocs that are clearly visible to the naked eye and ascribable to the protein instability.

As already previously mentioned, the *hot test* is considered by many laboratories as the most reliable test for the determination of protein instability but has the greatest limit to evaluate only the instability of the thermolabile proteins, without considering possible interactions of the protein fraction with the metatartaric acid or with CMC.

To overcome this problem, GiottoLab has carried out numerous tests over the last few years to identify the best analytical method to avoid protein precipitation due to the addition of these technological adjuvants. As shown in Fig.5, the solution we identified is represented by the cold tannin test, a method based on the use of particular extremely reactive tannins with proteins at room temperature.

♦ **CMC and Metatartaric: protein stability**

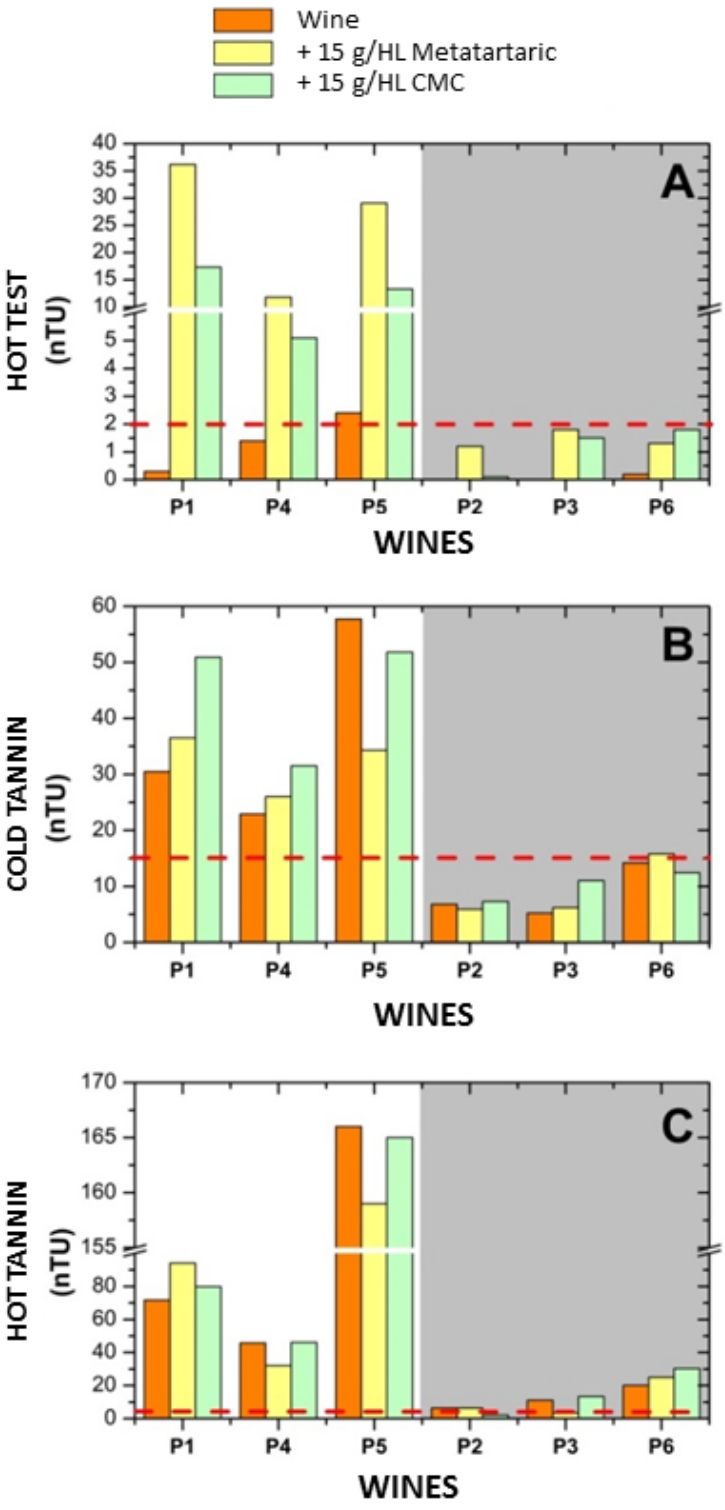

FIGURE 5. Protein stability of six wines (P1-2-3-4-5-6) before (wine) and after the addition of metatartaric and CMC.

Method:
- heat test (A),
- cold tannin test(B)
- hot tannin test (C)

The dotted red line represents the protein stability limit for each method.
As can be seen in FIG. 5A, the addition of metatartaric / CMC to stable wines according to the hot method (ΔnTU <2) but unstable (nTU> 15) by the cold tannin method (Figure 5B) can lead to a strong protein instability with ΔnTU values even higher than 30 nTU (P1-P4-P5).
Protein-stable wines according to the cold tannin method (P2-P3-P6), on the other hand, are stable even after the addition of the two technological coaudiuvants.
The hot tannin method (Figure 5C) instead overestimates protein instability.
The three wines stable after the addition of CMC and metatartaric (P2-P3-P6) were in fact unstable according to the hot tannin method (ΔnTU> 5), requiring a superfluous and, from a quality point of view, deleterious treatment with bentonite.

K-POLYASPARTATE AND PROTEIN STABILITY

Recently a new compound for the tartaric stabilization of wine has been introduced on the market: the potassium poliaspartate. This stable polymer of aspartic acid has a negative charge at the pH of wine making it capable of

sequestering potassium cations and thus interfering with the crystallization process.

Being negatively charged, however, we must pay close attention to any problems of protein destabilization in a similar way to what happens using CMC and metatartaric. Preliminary tests carried out by GiottoLab would seem to confirm problems of protein instability due to the addition of this compound (Fig.6). The use of the cold tannin test in this case therefore becomes essential to avoid problems of precipitation after bottling.

In the coming months further tests will be carried out to understand the effectiveness of the polyspartate in tartarically stabilizing the wines and to identify any problems associated with its use both in white wines (protein precipitation) and in red wines (color precipitation).

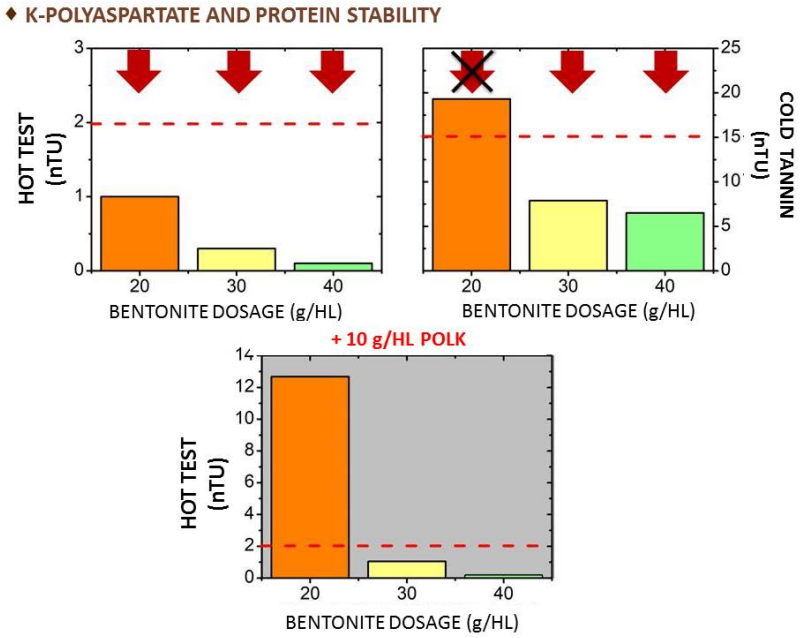

FIGURE 6. Protein instability due to the addition of potassium poliaspartate.
The tests were performed by evaluating the protein stability when hot and using the cold tannin method of a wine treated with three increasing doses of bentonite (20 g/HL - 30 g/HL - 40 g/HL). Subsequently, the three tests were added with 10 g/HL of polyaspartate to evaluate possible problems of protein instability according to the hot method. The result of this preliminary test showed a strong destabilizing effect of the polyaspartate according to the hot test for the sample treated with 20 g/HL of bentonite, while at higher dosages there were no instability problems. It should be noted that the sample treated with 20 g/HL of bentonite was the only unstable according to the cold tannin method.

GIOTTOLAB SUGGESTIONS

Taking into account the considerations made in this technical information, the choice of the test to be taken to evaluate the protein stability of a wine must be made by the winemaker according to the characteristics of the wine and its future storage conditions.

To avoid the risk of unwanted turbidity in wines, and at the same time avoid the risk of "slimming" them excessively, GiottoLab recommends to perform the *hot test* or the *cold tannin test* depending on whether the wine is stabilized or not tartarically through the use of CMC, metatartaric or poliaspartate. In the case of stabilization by metatartaric or CMC it is recommended to proceed with the addition at least 48 h before filtration. For sparkling wines it would be preferable to carry out a cold tartaric stabilization to avoid or limit the use of bentonite which would remove the protein fraction (mannoproteins and glycoproteins) capable of considerably improving the process of foaming. At the request of the customer, GiottoLab will provide for proteically unstable wines two bentonite dosages, one referring to the *hot test* (for those who do not intend to use tartaric stabilizers) and one related to the *cold tannin test* (for those who will stabilize the wine through enological adjuvants). It is fundamental to recheck protein stability following treatment with bentonite since the dosage provided by Giottolab is the result of laboratory tests carried out under extremely different conditions compared to those in the cellar.