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• • • NEW APPLICATIONS FOR PRECISION PROTEIN STABILITY • • •

Protein stability evaluation in white wines represents one of the more debated topics in the winemaking world. The market and the scientific community offer various alternatives in terms of analytical methods for the determination of the protein instability: their purpose is to determine the optimal bentonite dosage to avoid the formation of haze caused by protein precipitation on the finished wine. Perhaps, we witness a certain confusion among the users, since the methods return very different responses, with very different bentonite dosages required to reach the protein stability, at the end, precisely because the results were obtained with very different methods.

In that page, the technical-scientific articles that deal with this topic are not very helpful; in fact, besides several authors have compared the different methods, highlighting in a more or less detailed form advantages and criticisms, rarely is provided a clear and easy guideline to apply that may help to understand which test use and in what situations. Very often, this translates into an unaware decision by the technician that can be induced to use higher dosages of bentonite than what really required. Dosages that might determine a consequent "compression" of the wine's organoleptic profile.

The majority of the methods bring arbitrary alterations to the wine's composition or to its physical state up to the point haze forms; the obtained cloudiness intensity is the indication of a general protein instability. Some tests act by altering the pH, others temperature according with the principle that proteins alter their structure based on their thermolability and isoelectric point. Other tests, instead, simulate the enological conditions and so the interaction of some proteins with a specific tannin. If can be affirmed that the different tests for wine's protein stability determination rarely return the same results, the question then becomes: which is the more realistic test?

These considerations brought **VASON**GROUP R&D department to summarize in this INFOcus the results and considerations from a deep study that can provide indications on the methodology to adopt for **precision protein stability.**

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Factors responsible for protein instability

The "protein casse" can be defined as a spontaneous denaturalization followed by aggregation and flocculation processes of particular thermolabile proteins present in the wine, overall TLP (*Thaumatin-like protein*) and chitinase, proteins produced by the plant in response to pathogenic agents *(pathogenesis related proteins* or *PR proteins*). The risk of haze formation and protein precipitates, that would be visible by naked eye as suspended whitish flocs, depends of various factors, which comprehension turns essential aiming to the correct choice of the method to evaluate the protein stability. Considering the complexity and broadness of this topic, below are reported only the main factors:

- **Temperature**: heat represents the first factor responsible for the instability phenomena, with protein denaturalization kinetics strongly influenced by the temperature increase (for example during the stocking or transportation of the wine) and the different alteration temperatures of the protein fractions present in the wine (*melting temperature*), that actually turn them more or less sensible to thermal variations (Falconer et al., 2010).
- **Wine's pH**: pH variations alter the ionization states of the amino acids' side chains, modifying the charge distribution of the proteins and the possibility to form hydrogen bonds. This is therefore that pH variations (for example after MLF or blends) can alter the wine's proteins conformation stability and, consequently, cause their precipitation (Dufrechou et al., 2012 e 2013).

- **Tannins**: the destabilizing role of tannins towards proteins is well known. These phenolic compounds can derive also from releases from cork closures used for the bottling, their quality perhaps plays a fundamental role as demonstrated by Gabrielli et al., 2016.

- **Additive/adjuvant use:** the protein stability of a wine is usually evaluated in the moment of clarification. Despite this practice is justified by cellar timings that are every time shorter, the risk of forming precipitations increases exponentially after the add of additives/adjuvants shortly before bottling. In particular it was demonstrated that protein glues residuals used in the clarification (as gelatin) or the addition of lysozyme before the bottling (used as microbiological stabilizer), might cause cloudiness in the wine due to their high affinity towards tannins (Gerbaux et al., 1999; Tirelli & De Noni, 2007; Riberau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Moreover, as will be discussed in depth successively, also tartaric stabilizers as carboxymethylcellulose (CMC) and metatartaric acid can determine protein precipitations.

- **Wine Filtration:** when talking about protein stability, wine's filtration before bottling is an aspect to not ignore. Wine is an extremely complex matrix governed by balances that need to be preserve to avoid undesired problems. In this regard the protective colloid's retention, caused by a filtration done in not optimal conditions (clogging), might turn unstable a wine that was stable before the bottling.

Is interesting to highlight how often the critical issues related to protein stability are caused by a combination of two or more factors among the ones described above.

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Main test descriptions used for protein stability and comparison between the obtained results

The main test suggested by the market (Bentotest® e Proteotest®) and by the scientific community (heat test, tannin heat test) are generally considered as "guidelines" because determining in an artificial and indiscriminate way an alteration of the colloidal matrix of the wine that has, as effect, the formation of a haze. The turbidity that is created gives important indications regarding wine's predisposition to be subject to protein precipitations. In the data reported in CHART 1, is evident the extreme inhomogeneity amongst the results of the different tests that will highly influence the estimation of bentonite quantity required to bring stability to the wines.

Comparison between the results obtained on different wines

Test	White Igt Lazio	White Toscana	White Austria	Rosè Sparkling Base Austria	White Veneto	Prosecco Sparkling Base Veneto	Müller Thurgau Trentino	Nosiola Trentino	Traminer Trentino
Bentotest®	1	66	20	42	55	30	1	6	8
Proteotest®	1	62	16	49	27	29	6	12	11
Heat tannin	1	53	15	87	28	37	2	6	8
Heat	0	0	0	0	3	0	0	0	0

Limits beyond which wine is unstable	
Bentotest®	> 10 NTU
Proteotest®	> 15 NTU
Tannin-Heat Test	> 5 NTU
Heat test	> 2 NTU

CHART 1. Comparison of protein stability data of 9 wines using different analytical methods.

Observe how Bentotest® tends to overestimate the value. Observe, also, how in Nosiola and Traminer the "Heat Tannin Test" overestimates compared to the "Heat Test" to the point to make them unstable, whilst for the other test they are not. To support what the chart shows, if attention is focused on these three tests (FIG. 1), is evident how Proteotest® stays very close to the "Heat Test", the most conservative, while the "Tannin Heat Test" tends to overestimate the bentonite's quantity required for the stabilization of both the wines analyzed (Pinot Gris and Manzoni Bianco), confirming what was observed previously.

These three tests use only heat or the increase of tannin concentration (or the combination of the two) to provide to the wine real changes with respect to its future during fining, such to induce in a short time (the test's time) an indication of its protein stability. They do not bring changes to pH, an event that the wine will never face in its life-time, a destabilizing factor at the base of the excluded test.

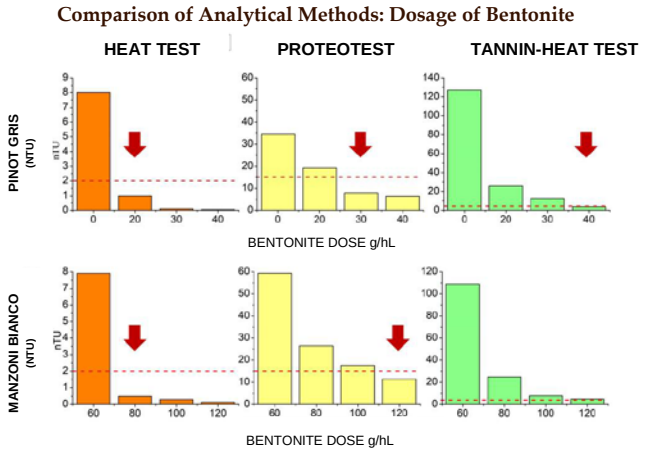

FIGURE 1. Comparison of bentonite dosage obtained using the "Heat Test", the "Cold Tannin" test (Proteotest®) and the "Tannin-Heat" test.

In the case of Pinot Gris (FIG. 1) the quantities requested of bentonite to reach protein stability in the wine are:
20 g/hL for the "Heat Test";
30 g/hL for "Proteotest®;
40 g/hL for the "Tannin-Heat Test";

In the case of Manzoni Bianco, the requested bentonite quantities are:
80 g/hL for the "Heat Test";
120 g/hL for "Proteotest®;
>120 g/hL for the "Tannin-Heat Test".

What indications emerge from these insights?

Does an ideal analytical method to adopt for evaluating the protein stability in a wine exist?

The "Heat Test" seems to be the most respectful of the wine's quality since it estimates a dosage of bentonite usually lower than the other tests, in fact this is one of the mostly used. But attention, some critiques do exist: the first one concerns the fact that different laboratories change the original methodology, this fact makes the test personalized and the results difficult to compare.

The second criticism reported is that, no matter what methodology is adopted, the test time is too long, especially if compared to different tests, like Proteotest® for example. It might be interesting to have at the ready a test that gives a similar response in forecasting terms and standardizes the obtained results, but faster.

At this point of the research, comparing the results of the "Heat Test" and the Proteotest® (very fast) takes on this precise mean, deepening the interactions between the results obtainable from the two tests.

Therefore, strict trials were prepared inducing protein instability using two macromolecules as CMC and metatartaric acid, with the aim of studying the colloidal interaction and try to avoid cloudiness following their add to the wine.

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Correlation between the results of Proteotest® and the "Heat Test"

Products such as CMC or metatartaric acid are used to avoid protein instability but can destabilize wine's balance causing unpleasant formation of haze. While the responsible mechanisms of this instability are not completely clear, it is hypothesized that interactions between the negative surface charges of CMC or metatartaric and the positive charges of wine's proteins, with the consequent formation of flocs clearly visible with naked eye, are ascribable to protein instability. In FIG. 2 it can be observed how the response given by the two tests about the three wines is very similar: when a wine is stable for the "Heat Test" it is also for Proteotest®. At this point, it turns out, it is possible to create a forecasting system that can anticipate the effects of the adds of these two adjuvants on the wine's protein stability. With these trials, in fact, the colloidal interactions between these two macromolecules are being studied, so each time you talk about wine you should keep this in consideration. In reality, either the structure of the wine and the fining techniques *sur lies* or barreled, contribute to the colloidal general stabilization system.

Subtracting this precious portion of the wine would mean a loss in overall quality; just think about the fact that an overdose of bentonite in sparkling bases could lead to the removal of mannoproteins and glycoproteins that play a fundamental role in the second fermentation (foam formation). The first results obtained from the comparison of the two methods were encouraging but for the experience's continuation, it was opportune to evaluate these behaviors on a wider base of wine's typologies.

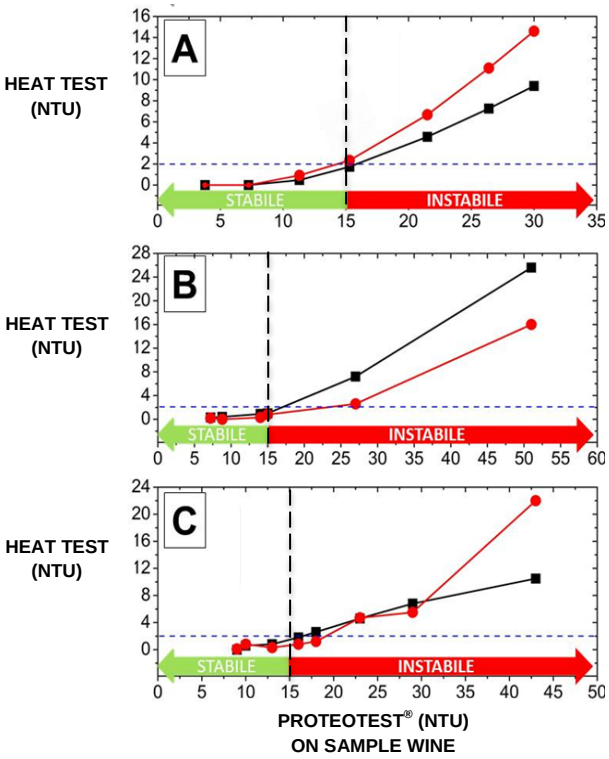

FIGURE 2. Comparison between the result of Proteotest® done in two white wines (A and B) and a rose wine (C) treated with bentonite increasing dosages and analyzed with "Heat Test", done over the dosages after the add of 10 g/hL of CMC (black) and 10 g/hL of metatartaric (red). The dashed lines show the limit values of the two tests for which a wine is considered stable (2 and 15 NTU respectively.

Proteotest® as a forecasting tool linked to the use of CMC and metatartaric acid

To better understand these aspects and those connected to the interaction with these adjuvants in the bottling phases, in the last years the R&D division of **VASON**GROUP has done numerous trials to identify the best forecasting analytical method, with the aim of preventing alterations in the clarity of the wine and/or protein precipitations after the adding of these stabilizers.

In FIG. 3 it can be observed how Proteotest® gives back important information: the test done in the wine as it is, is enough to determine if the wine will be subject to cloudiness after the add of 10g/hL of CMC or metatartaric (MA). This forecasting information is really important and something that the "Heat Test" does not highlight: observe how in the wines P1 and P2 the "Heat Test" shows the wines as stable. This is probably is true if the wines would be bottled like that, with no adds. But if an add of CMC or MA was done on P1 and P2 they would form haze. Operating with Proteotest® would save the winemaker from cloudiness or precipitations that would form only later.

As can be observed in FIG. 3A, the add of MA or CMC to stable wines according to the "Heat Test" but unstable according to Proteotest® (NTU > 15) (FIG. 3B) can cause a strong protein instability with ΔNTU's values even higher than 30 NTU (P1-P2-P3).

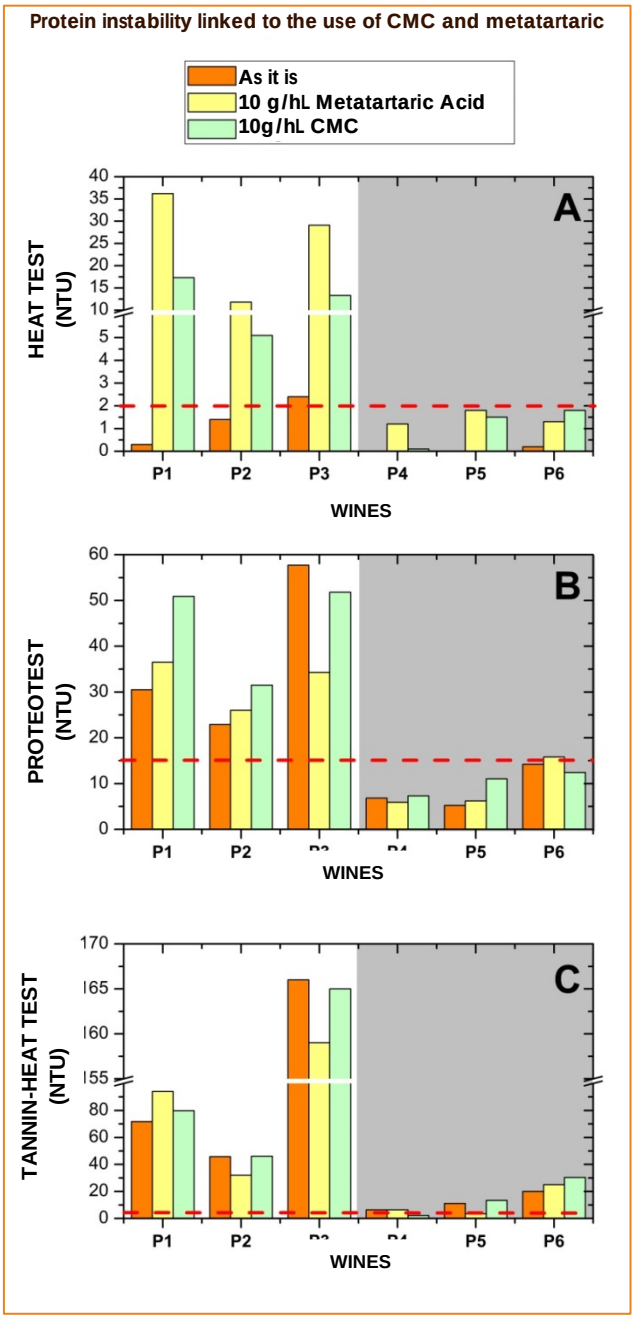

FIGURE 3. Protein stability of six wines (P1-P2-P3-P4-P5-P6) before (As it is) and after the add of 10g/hL of metatartaric acid (MA) and carboxymethylcellulose (CMC). **Method:** by heat (A), Proteotest® (**B**), Tannin-Heat (**C**). The dashed line represents the stability limit for each methodology.

Interestingly, the stable wines, according to Proteotest®, are stable also with "Heat Test" after the adds. In this case the tests agree. Instead the "Tannin-Heat Test" (FIG. 3C) also in this case, overestimates the protein instability. The three wines that showed as stable according to "Heat Test" and

Proteotest® after the add of CMC and metatartaric (P4-P5-P6) were unstable according to "Tannin-Heat Test" (ΔNTU >5), requiring a superfluous treatment with bentonite.

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Conclusions and suggestions

It is necessary to keep in mind that any test for protein stability returns a stability absolute number but rather they represent an indicative test. In this respect, the tests that forecast as result the reading of a bigger NTU range have to be considered more advantageous. Also the execution speed is surely a parameter to keep in consideration in the selection of a test.

	Heat test	Proteotest®
Execution Speed	Preparation + 90 minutes	Preparation + 5 minutes
Reading Range	from 0 to 2 NTU	from 0 to 15 NTU
Instability forecast with CMC or MA	-	Yes
Result Reading	Turbidimeter	Naked eye or Turbidimeter

Pursuant to the considerations that emerge from this technical insight, it clearly appears the choice of which test adopt to evaluate the protein stability has to be made by the winemaker depending on the wine's characteristics and its future conditions or storage. To avoid risks of undesired cloudiness and avoid, at the same time, to excessively "thin" the wines, it is highlighted how the "Heat Test" and Proteotest® generally agree in the results and require less bentonite when compared to the "Tannin-Heat Test".

As anticipated before, the "Heat Test" is considered from many laboratories as the most reliable test to evaluate the protein stability but has the big limitation of evaluating only the instability of the heat-labile proteins, without considering possible It is emphasized, therefore, that many laboratories personalize the methodology; sometimes these methodologies were historically passed on and have their significance but have made the comparability of the test against itself challenging. In this research the more classic methodology for the "Heat Test" was used: 45 minutes for incubation at 85°C + 45 minutes for cooling at room temperature.

At this point, if the wine is lately stabilized from a tartaric point of view using CMC or metatartaric, Proteotest® is able to reveal a future negative interaction, thus suggesting a dosage of bentonite adapt also to this possibility. This aspect makes Proteotest® even more useful, especially for the productive realities that use these adjuvants. Furthermore, for those that need to obtain the results in short time and can't wait the 90 minutes for the standard "Heat Test", the use of Proteotest® alone, represents the best choice both in terms of speed and reliability of the results.

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PROTEOTEST®

Proteotest® is a methodology developed by the R&D department of **VASON**GROUP that allows the user to evaluate the protein stability of the enological conditions extremely fast and reliably. The reading of the results can be done both with naked eye or a turbidimeter. Based on a specific selection of extremely reactive tannins to proteins and done at room temperature, Proteotest® does not introduce any arbitrary alterations to the system, resulting in a test that simulates the most realistic mechanisms of protein instability in the wine. The test can be done both for the evaluation of the protein stability and to

identify the correct dosage of bentonite to reach stability: in this case it would only be necessary to set in the laboratory simple clarification trials and over these run Proteotest®. The test is done at room temperature and in a few minutes the results are ready.

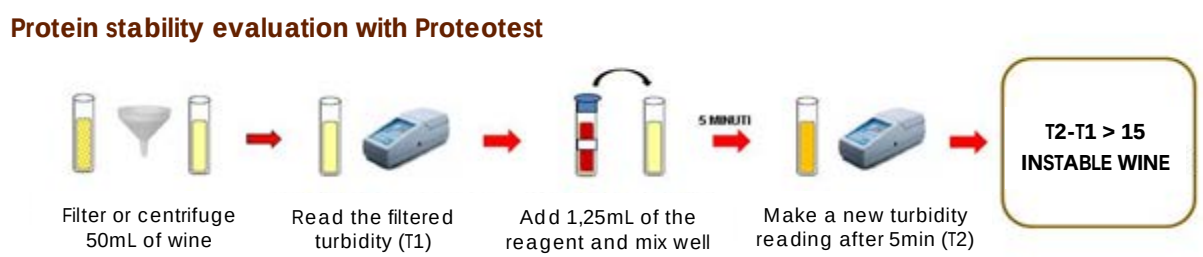

FIGURE 4. Working scheme for evaluating the protein stability using Proteotest®. The methodology requires the use of a turbidimeter but is possible to do the evaluation also by naked eye: in this case the turbidity difference will be observed comparing the sample with a filtered but untreated one.
*For further details refer to the product's TDS and the methodology given with it. In case is necessary contact the technical assistance from Enologica Vason.

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