New analytical approaches of protein stability evolution in wines

ROLAND Clémentine, croland@labexcell.com
NICOLATO Tommaso, tnicolato@labexcell.com
RENOUF Vincent, vrenouf@labexcell.com

In white and rosé wines, protein haze (figure 1) is a phenomenon associated with an irreversible modification of the three-dimensional structure of proteins when the wine is heated, which will make these proteins insoluble when the wine is cooled. They form a precipitate that is easily observable by bare eye and can be characterized under a microscope. During an analytical audit of wines sampled from supermarket shelves that has carried out at SARCO laboratory (now EXCELL laboratory) a few years ago, showed that 17% of the wines were potentially unstable from a protein point of view (34% white wines analyzed and 2% of rosé wines).

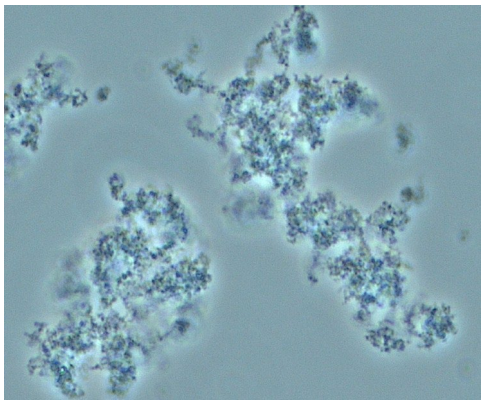

Figure 1 : Protein haze observation under the microscope (×400) (Source EXCELL Laboratory).

In recent years, several phenomena have certainly contributed to increasing the problem. Among these phenomena or these hypotheses of phenomena, let us note:

- interactions with other stabilization treatments such as the use of cellulose gum or polyaspartate for tartaric stabilization purposes which, very certainly, interact with residual proteins which would not have presented thermal instability if they had not come into contact with these products. This therefore requires anticipation work and sometimes additional stabilization efforts (higher doses of bentonites),
- the initial and intrinsic protein levels of grapes, in particular due to water and cryptogamic stress, because it is well established that many stress resistance pathways involve protein agents (Jones and Dangl 2006, Boller and Felix 2009, Cardot 2017) in plants and in berries in particular,
- - increasingly early cleaning and stabilization protocols which reduce the paths of stability acquired progressively during more traditional routes (ageing on lees, “natural” cold favoring sedimentation, wine air exposure phases management favoring the reactivity of certain constituents, etc.)
- changes in certain physico-chemical parameters of wines, such as pH (the higher the pH, the less the proteins are charged and the less effective the bentonite stabilization treatments are), copper levels (copper interacts with SH groups of sulfur amino acids present in proteins, which also contributes to reduce precipitation phenomena) …

In this global context, staying stuck behind the traditional heat test as the only analytical way to guide our partners towards the most suitable technical proposals seemed relatively restrictive to us. We therefore launched in 2020 a major study of new analytical approaches aimed at exhaustively measuring the total quantity of proteins present in grapes, musts and wines (which until now had never been done) and characterize the protein profiles in order to more accurately assess the risk of instability in all possible situations.

This article details this work and proposes different analytical approaches according to technical objectives and oenological practices. The first part is devoted to the validation of a protein assay method, the second one to the development of the method for characterizing the protein profiles of wines and finally the third part of this writing evokes concrete cases of use of these different analytical approaches. These analytical possibilities have been in place in the laboratory for several months. They already provide particularly interesting preventive management methods (especially in the context of actions anticipating the tartaric stabilization treatments to be followed).

1- Protein dosage

To measure total proteins in wine, several approaches have been considered: the KDS/Smith method (D. Gazzola, 2014) and the Bradford method. The KDS/Smith method is based on the determination of protein complexes by reduction of bicinchoninic acid while the Bradford method is based on the color change of Coomassie blue after binding with certain amino acids present in proteins. After numerous tests particularly based on proteins measured additions to different wines, the Bradford method was chosen. The method has been adapted from the thesis of P. Lui (2018).
Tests have been carried out to define a calibration range. Dans un premier temps, une gamme à partir de la protéine BSA (protéine extraite du sérum bovin) a été produite (figure 2), la protéine BSA n'étant pas présente dans le vin, une seconde gamme a été produite à partir de la protéine thaumatine (figure 3). A more precise dosage ranging from 1mg/L to 20mg/L could be achieved with the latter.

DO (nm)	BSA range (mg/L)	BSA concentration (mg/L)
0.133	5	4.04
0,231	10	10,40
0,318	15	15,40
0,383	20	20,27

Figure 2 : Calibration range made from the BSA protein

DO (nm)	Thaumatin range (mg/L)	Thaumatin concentration (mg/L)
0,02	1	0,25
0,085	5	5,25
0,159	10	10,94
0,217	15	15,4
0,268	20	19,32

Figure 3 : Calibration range made from the thaumatin protein

Getting closer to the reality of our samples, the range made from the thaumatin protein has been validated. In both cases, signal saturation was observed from 20 mg/L of thaumatin (or BSA).

Tests on the wines were then carried out (figure 4). It has been observed that for samples considered unstable, the total protein concentration varies from 11 mg/L to 17 mg/L Eq of thaumatin. It was not necessary to dilute the samples beforehand as the optical densities were in the range.

Samples	ΔDO 395nm	Thaumatine Concentration (mg/L)
1	0,237	16,9
2	0,163	11,2
3	0,197	13,9
4	0,201	14,2
5	0,222	15,8
6	0,22	15,6
7	0,229	16,3
8	0,234	16,7
9	0,232	16,6

Figure 4 : Summary table of some protein assays in wines taken in the laboratory

The results obtained with the dosage by the Bradford method and the various heat tests carried out in the laboratory in recent months have made possible to evaluate a "critical" content of 10 mg/L Eq thaumatin. This content of 10 mg/L Eq thaumatin is therefore considered as an acceptability threshold for future protein stability assessing. For example, if a white wine is naturally stable to the heat test but it contains a protein concentration at this concentration level (which means that thermostable incorporated proteins are still present at a significant concentration), we will nevertheless recommend an effort to lower this protein content with a view to possible CMC treatment for tartaric stabilisation.

2- *Protein profiles*

Once the quantification of total proteins possible by the test mentioned above, we developed an analytical way providing a description of the type of proteins present. For this we tested and compared different types of electrophoretic systems. Our choice then fell on the BioanalyZer® system offered by the Agilent company. Figure 5 illustrates the fineness potential of this analytical system over a range that was performed with the protein thaumatin.

A.

B.

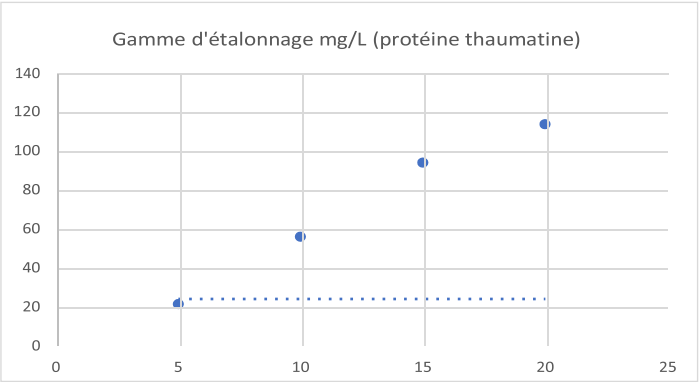

Figure 5 A : gel electrophoresis of points in the range of 5mg/L to 20mg/L of thaumatin.
5 B : representative calibration curve.

After various preparation steps, protein profiles similar to that of figure 6 can thus be established to characterize the proteins present according to their molecular weight. The literature, relatively well detailed, makes possible the identification of these proteins (Sluyter, 2015). In the vast majority of wines, the proteins mainly found in the samples are chitinases and glucanases. Figure 6 illustrates the profile of a heat-unstable wine. The number of bands and their intensity serve to overestimate the results of the heat stability test and suggest an effective dose of bentonite.

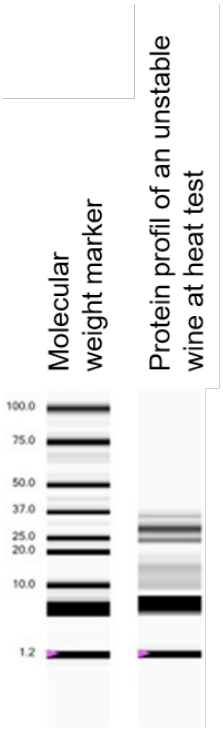

Figure 6 : Illustration of a protein profile of an unstable wine at heat test obtained with the capillar electrophoresis system available at the EXCELL laboratory.

Conclusions and analytical proposals

In order to determine the dose of bentonite necessary for the protein stabilization of a white or rosé wine, the heat test remains, in our opinion, the reference provided that certain essential elements are respected (preparation of the sample, time and temperature applied, cooling of the sample, control of the dose of bentonite determined by the first test, etc.). We frequently test and compare this test with other possible alternative paths, but we remain fixed on this position. Nevertheless, given the growing sensitivity of the issue with regard to the developments mentioned in the introduction of this article, we have sought to associate this test with elements that make possible a better anticipation of the stabilization processes as a whole. For this we have developed two analytical elements: the determination of total proteins and the characterization of wine protein profile. In the specifications for these developments, the objectives that we had set were that these analyzes must be rapid, inexpensive and easily interpretable in relation to well-established reference systems (equivalence in reference proteins, for example, etc.). The quantification of total proteins seems to us to be an essential point. At a given time t, in a wine all the proteins are not thermo-unstable or the bentonite treatment determined by the heat test only eliminates the latter. So, if initially, the protein content is high, a risk is to be anticipated if other operations can come to interact with the residual proteins.

The protein profile makes possible the characterization of present proteins. This analysis can particularly intervene in the case of recurring difficulties in the protein stabilization of a batch or a wine in order to know which proteins are responsible for it. The test can also be done at several stages of wine making in order to specify the interaction of a particular technique on the protein content. This test could also soon become a key element to objectify the treatments based on proteases which are being validated by the OIV (RESOLUTION OIV-OENO 541B-2021 and 625-2021) to be a new option in these processes of stabilization by comparing the profiles obtained before and after treatment.

Based on these developments, at the EXCELL laboratory we now offer 3 possible analytical formulas for the analysis of the protein stability of white and rosé wines (each analysis can of course be carried out alone)

- The StabProt S pack: heat test to determine the bentonite dose and check the effectiveness of this dose
- The StabProt M pack: heat test for determining the bentonite dose + total protein assay and control of the effectiveness of the dose determined by the heat test and of this increased dose in the event of significant protein quantification (> 10 mg/L in eq. Thaumatin)

The StabProt L pack: heat test for determining the bentonite dose + total protein assay and control of the effectiveness of the dose determined by the heat test and of this increased dose in the event of significant quantification of total protein (> 10 mg/L in eq. Thaumatin) + protein profiles before and after bentonite treatments.

These 3 formulas are available in two versions, the previously mentioned classic version carried out on the sample of wine as we receive it in the laboratory and a version for which the tests are carried out on the wine as it is and on the wine added with the treatment of tartaric stabilization which would be likely to be used later (CMC, polyaspartate) in order to also anticipate the possible interaction of these treatments with the protein stability which would have been acquired and to avoid having to retreat again to bentonite (and all the inconveniences associated with this kind of situation: new filtration, loss of time, loss of wine, oxygen intake, etc.).

	Pack StabProt S	Pack StabProt M	Pack StabProt L
Heat Test	X	X	X
Total Protein assay		X	X
Protein electrophoretic profile			X
On demand	With or without added CMC or polyaspartate	With or without added CMC or polyaspartate	With or without added CMC or polyaspartate