Wine Thermosensitive Proteins Adsorb First and Better on Bentonite during Fining: Practical Implications and Proposition of Alternative Heat Tests

Aude Vernhet, Eric Meistermann, Frederic Charrier, Patrick Chemardin, Celine Poncet-Legrand

Wine thermo-sensitive proteins adsorb first and better on bentonite during fining: practical implications and proposition of alternative heat-tests

Aude Vernhet[1], Eric Meistermann[2], Philippe Cottereau[2], Frederic Charrier[2], Patrick Chemardin[1], Céline Poncet-Legrand[1*]

1 – *SPO, INRAE, Montpellier SupAgro, Univ Montpellier, 34060, Montpellier, France*

2 –Institut Français de la Vigne et du Vin, F-30240 Le Grau du Roi

*Corresponding author :

Bat 28 – 2, place Pierre Viala – 34060 Montpellier Cedex 2- France

Phone : 33 (0)4 99 61 27 58

Fax : 33 (0)4 99 61 28 57

Email address : celine.poncet-legrand@inrae.fr

Abstract

Bentonite fining is the most popular treatment used to remove proteins in white and rosé wines. The usual heat test used to adjust the bentonite dose consists in heating the wine during 30 minutes at 80 °C. At this temperature, all the proteins are unfolded and this can lead to an overestimation of the dose. We have shown that proteins adsorb on bentonite in a specific order, and more importantly that the proteins responsible for haze formation adsorb first. Fluorescence spectroscopy showed that this is due to the structural properties of proteins, which can be classified in hard and soft proteins. Alternative heat-tests were performed at lower temperature (40 °C) and showed a better correlation with accelerated ageing. These tests were also less dependent on the wine pH.

Keywords: wine proteins, haze formation, hard and soft proteins, bentonite fining, alternative heat tests

Introduction

Even if they are present in rather small amounts in white and rosé wines, proteins play an important part in their colloidal stability and clarity [1,2,3]. Haze or deposit formation in bottled wines, due to protein aggregation during storage, is a common defect of commercial wines which makes them unacceptable for consumers. Over the last decades, numerous studies have led to the structural identification of the haze forming proteins, as well as to the elucidation of the factors which trigger or prevent haze formation[4]. Aggregation is usually attributed to a slow protein unfolding during storage, induced by a raise of temperatures. Chitinases, β-glucanase and some thaumatin-like proteins are the most important contributors to heat-induced protein instability[2,5,6,7] . Attempts to correlate the total wine protein contents to their sensitivity to protein haze failed, mainly because: (i) some thaumatin-like proteins as well as invertases are more resistant to heat-induced denaturation[3,8,9,10], and (ii) haze formation is strongly affected by the presence of non-protein compounds such as polyphenols, ions, acids and polysaccharides. [5,11,12,13,14,15,16,17,18]. These factors may also affect the aggregate size/structure and the eye-perception of the defect.

To prevent the formation of haze, several strategies have been proposed, which are more or less used: addition of particles onto which protein will adsorb (*i.e.* bentonite fining, addition of magnetic nanoparticles [19]), addition of stabilizing agents such as polysaccharides[20] , use of proteolytic enzymes[21,22], but the most widely used remains bentonite fining. Proteins which are positively charged at wine pH adsorb on the negatively charged clay particles. The level of bentonite addition required for stabilization is determined by heat tests. These levels have increased during the last 20 years, so that doses in the order of 100–150 $g \cdot hL^{-1}$ are often added. Though effective, bentonite fining generates different problems, especially when such high doses are needed. Indeed this treatment is not selective and aroma and anthocyanins (in Rosé wines) may also adsorb, resulting in an alteration of the organoleptic properties. Bentonite

fining also causes substantial volume losses (between 3% and 10%) and the used bentonites is an increasing source of waste.

Different tests have been proposed to assess wine stability/ instability with regards to protein haze, the most commons being based on heat-induced precipitation, leading to protein aggregation and precipitation. These tests consist in heating a wine sample to a given temperature x duration. The difference in turbidity before and after heating and cooling is used to assess whether the wine is stable or not and to determine the bentonite dose in case of instability. These tests are also used to perform fining tests with increasing doses of bentonite to determine the optimal dose in the laboratory. The use of these tests in practice requires the definition of a stability threshold below which the wine is stable. The latter is determined empirically by the experience of the wine-maker or his enology laboratory. For the test most commonly used in France, which consists of a 30-minute heating at 80 °C, the stability threshold used is 2 NTU on Sauvignon in Bordeaux, up to 20 NTU on Gewurztraminer in Alsace, and 5 NTU in other wine-growing regions. However, the results obtained with this test do not necessarily reflect changes and destabilization phenomena liable to occur in real wine storage conditions: at this temperature, all the proteins are denatured, even the most stable which unlikely unfold and aggregate in standard storage conditions. This may result in an overestimation of the bentonite dose to add, all the more important as a study showed that the most heat sensitive proteins were also the first to adsorb on bentonite[23].

The aim of this study was: i) to confirm with different wines what was already observed on one variety (i.e. the most sensitive proteins adsorb first); ii) to prove that is due to a structural feature in relation with the conformational stability of the different proteins present in wines; iii) to link these properties with the actual stability heat tests and compare them with tests performed at lower temperatures; iv) to assess the effect of pH on heat tests, especially when they are done at 80 °C.

In the present study, two different sets of wines were used. To answer the points i) and ii), bentonite fining was performed on 7 white wines (1 vintage, 4 areas, 3 varieties), through the analysis and quantification of the proteins removed by different bentonite doses. Wines were also heated at 40 °C, 60 °C and 80 °C and the residual proteins analyzed after centrifugation. To answer the points iii) and iv), various heat tests were done on a second set of white and rosé wines (55 wines, 4 vintages, 7 varieties, 5 areas): the time and temperature were varied: 30 minutes at 40 and 80 °C, 4 hours at 40 °C, and the pH was adjusted to values ranging from 2.6 to 4.2.

Materials and methods

Wines and model systems

Wine making. A first set of wines was used in the fining study. They were elaborated in 2016 in the different experimental units of the French Institute of Vine and Wine (IFV), in Rodilhan (30), Blanquefort (33), Nantes (44), and Colmar (68), France. They were made from three varieties of *Vitis Vinifera:* Chardonnay (CH), Sauvignon (SA) and Gewurztraminer (GEW). In the present study, wines will be referred to as a combination of the area code and the variety: for instance a Sauvignon wine made in Rodilhan will be noted SA30. Following fermentation, the wines were cold stabilized to prevent the crystallization of tartaric salts and clarified. No bentonite fining was performed. After a final membrane filtration, the wines were aliquoted in bottles and stored at 10 °C before use. Conventional enological parameters were analyzed according to Vine and Wine International Organisation methods, and are reported in the Table 1. A second set of wines elaborated by the IFV was used to perform alternative heat tests: it was made of 55 wines: 4 vintages from 2014 to 2017, 7 varieties (Pinot Gris, Gewurztraminer, Riesling, Sauvignon, Chardonnay, Cinsault, Caladoc), 5 sites in different

wine-growing regions: Rodilhan (Languedoc), Blanquefort (Bordeaux), Nantes (Muscadet), Colmar (Alsace) and Vidauban (Provence).

Accelerated ageing. 375 mL bottles of wines were stored at 35 °C during two weeks in order to induce the denaturation of the most thermosensitive proteins.

Bentonite fining. The bentonite treatments consisted in the addition of bentonite at doses ranging from 5 to 80 $g \cdot hL^{-1}$ (0.05 to 0.8 $g \cdot L^{-1}$). Natural activated calcium bentonite Electra® (Martin Vialatte, France) was used (same batch in all sites), following the instructions: Stock solutions of bentonite (50 $g \cdot L^{-1}$) were prepared and allowed to swell during 24 hours. The resulting gel was then mixed with wine to obtain the final concentration. Wines were kept in a room maintained at 16-18 °C during 48 hrs. After fining, wines were centrifuged (10'000 g, 10 min) heat tested and their proteins were analyzed as described below.

Heat tests. Samples of wines were submitted to different time/temperature couples: 30 minutes at 80 °C, 60 °C and 40 °C, but also 4 hours at 40 °C and 2 weeks at 35 °C. Turbidity was measured on a turbidimeter Hach TL2310 before and after heating, after the samples were cooled down to room temperature during 16-18 hours. A study on the effect of pH was also performed, and heat-tests were done after adjustment of wine pH within the range 2.6 to 4.2 with NaOH and H_2SO_4. Experiments were done in triplicate, except for the tests at 40, 60 and 80 °C in 2016 (duplicate) and the results were averaged.

Model systems. The conformational changes of proteins during adsorption on bentonite were assessed in model systems. Stock solutions of bentonite and proteins, purified and identified as described by Dufrechou et al.[24] were mixed in a synthetic wine (12% EtOH, 2 $g \cdot L^{-1}$ of tartaric acid, pH 3.5 adjusted with KOH).The protein concentration was set to a value giving reliable results and consistent with what is present in wines: 10 $mg \cdot L^{-1}$ for invertase, 20 $mg \cdot L^{-1}$ for chitinase, 35 $mg \cdot L^{-1}$ for TL19. Bentonite concentration was set to 1 $g \cdot L^{-1}$, which is both consistent with an enological use (100 $g \cdot hL^{-1}$) and a complete adsorption of proteins.

Protein analysis

Monodimensional electrophoresis (SDS PAGE). Protein analyses were performed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). Wine aliquots were mixed with the Laemmli buffer 4X (60 µL of wine, 20 µL of buffer). The resulting mixture was loaded on a 10 well polyacrylamide gel (14% acrylamide, gel length = 80 mm). A low molecular weight calibration kit (GE Healthcare), ranging from 14.4 to 97 kDa, was included in each electrophoretic run. The gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad) in 40% of ethanol and 10% of acetic acid. SDS PAGE were run on a CBS Scientific MGV-202 vertical gels system at 20 mA, until the dye front reached the bottom of the gel (approximately 2 h and 30 min). They were then destained in 10% of acetic acid during two days, with a regular renewal of the solvent. Gels were then scanned at 300 dpi with an image scanner (Biorad GS 710). Image analysis was carried out with the Phoretix 1D software and was used to calculate the proportion of proteins in each staining band taking Bovine Serum Albumine as a reference. Proteins were identified according to their molecular weight and in reference to previous results [23,10]. Electrophoresis were performed in duplicate since it was shown that the repeatability was in the order of 10% [23]. It was planned to do the triplicate if differences between duplicates were higher than 10%. Results were averaged and are presented in the Table 2.

Fluorescence spectroscopy. Measurements were performed at room temperature with an RF 5301 PC fluorescence spectrophotometer (Shimadzu, Japan) and were used to show modifications of the tertiary structure of the proteins when they adsorb on bentonite, or their absence. The excitation wavelength was 280 nm, and the emission wavelength ranged from 300 to 500 nm. Data were recorded and analyzed with the RFPC software (Shimadzu, Japan).

151 The fluorescence of bentonite at 1 $g \cdot L^{-1}$ was subtracted, and intensity was normalized to the
152 maximum intensity in order to compare the different proteins. Fluorescence experiments were
153 done in triplicate and the intensity was averaged.
154

155 ## Results and discussion

156 ### Adsorption of proteins on bentonite

157 The percentage of protein adsorbed as a function of the bentonite dose is plotted on the Figure
158 1 for each wine and each protein. In all cases, when they were present, proteins were adsorbed
159 on bentonite in this order: chitinase and β-glucanase first, lipid transfer protein (LTP),
160 thaumatin like protein (TL) 22 kDa and finally TL 19 kDa and invertase. The percentage of
161 adsorbed invertase, TL19 and TL 22 is plotted as a function of the amount of bentonite added
162 in the Figure 2. Chitinases, β-glucanases and LTP, which were present only in one wine, are
163 not represented.

164 The adsorption of a given protein was wine dependent. The GW30 was the wine needing the
165 highest bentonite dose to reach the total adsorption of wine proteins, whatever the protein
166 (invertase, TL19 or TL22). This could be due to wine pH and ionic strength (different in the
167 studied wines), which changes electrostatic interactions that drive protein adsorption onto
168 bentonite: indeed proteins can have different electrical surface charge distribution but can also
169 exhibit conformational change when the pH is modified [24]. Most of thaumatin –like proteins
170 and invertase have isoelectric points (IEPs) between 4 and 5 [23], whereas chitinases and β-
171 glucanases have IEPs between 5 and 7 [23,5]. It is worth noting that most of the studied wines had
172 a pH between 3.20 and 3.30, except SA44 (3.38) and GEW30 (3.61). Thus, all the proteins are
173 positively charged. However, their electrical charge is expected to be higher when the pH is
174 lower, which may explain why the adsorbed amount of proteins is systematically lower in

GEW30, and/or requires higher bentonite doses. On the other hand, β-glucanases and chitinases have a higher IEP, resulting in a higher positive charge, possibly explaining why, when they are present in wines, they adsorb first.

However, other differences in wine composition such as ethanol, polysaccharides, polyphenols or ions, may have an effect on protein adsorption on the bentonite surface[25,26]. GEW 30 is also the wine with the highest amount of ethanol (14.1%). But when comparing the GEW30 and the SA30 wines, ethanol does not seem to play an important part. For instance, SA30, which was the wine whose proteins had the nearest behavior to GEW30, was also the one that had the lowest ethanol content. In any case, in spite of the small differences observed, the order of adsorption was roughly the following: β-glucanases and chitinases adsorbed first, followed by LTP, then TL22, and finally TL19 and invertase (these two can be exchanged, depending on the wines). These results are in agreement with previous results from Sauvage et al., obtained on a Chardonnay wine[23].

Thermal stability

In parallel to bentonite fining, the wines were heated to 40, 60 and 80 °C during 30 minutes, then cooled and centrifuged. The remaining proteins were then analyzed. Figure 3 shows the proteins remaining in GEW30 after heating. As already observed by several authors [10,8], chitinases and β-glucanases are the most unstable proteins, unfolding and precipitating below 40 °C. The different thaumatin-like proteins are slightly stable, with melting point between 40 and 60 °C. Invertase and lipid transfer proteins are the most stable proteins: they can stand temperatures up to nearly 80 °C. These temperatures are in agreement with the temperature determined in previous studies, were chitinases were found to have a Tm of 55 °C, thaumatin-like proteins Tm's of 61 and 62 °C and invertase 81 °C.[8] The small differences observed can be explained by the effect of pH, which is different depending on proteins [10]. It also confirms

199 the partial reversibility of TL19 [10,8] and suggests that the lipid transfer proteins may have the
200 same behavior: 100% of native proteins at 60 °C, 80% at 80 °C.

201 **Protein structure, adsorption and denaturation**

202 From the two previous sections we concluded, in accordance with previous results obtained on
203 a Chardonnay wine[23], that the adsorption of wine proteins onto bentonite seems to be correlated
204 with their thermal stability. The behavior of proteins at phase boundaries has been the objective
205 of numerous studies over the last decades. In general, if a protein solution is in contact with a
206 solid surface the protein adsorbs spontaneously and, consequently, the interfacial properties are
207 modified. Arai and Norde [27] were the first to investigate the underlying principles that drive the
208 adsorption behavior of proteins on surfaces. They adapted the existing theories of the adsorption
209 of polymers to proteins by taking electrostatic interactions and the three-dimensional structures
210 of proteins into account.

211 From experimental data published [28] they concluded that the adsorption behavior of a protein
212 molecule is related to the stability of its native structure. Proteins like lysozyme and
213 ribonuclease, having a large structure stability and, therefore, a strong internal coherence,
214 behave like "hard" particles. Their interaction with an interface is governed by hydrophobic and
215 electrostatic effects. Proteins such as myoglobin or α-lactalbumin, which have a relatively low
216 structure stability, possess an additional internal factor that promotes adsorption. This factor is
217 probably related to structural rearrangements in the molecule involving an increased
218 conformational entropy. As a result, such "soft" proteins may adsorb on a hydrophilic surface
219 even under conditions of electrostatic repulsion.

220 Hard proteins have since been described as having an unfolding energy higher than soft
221 proteins: e.g. 60 $kJ \cdot mol^{-1}$ and 21 $kJ \cdot mol^{-1}$, respectively [29,30]. It has also been determined that the

energy necessary to unfold an α-helix is smaller than the one needed to unfold a β-sheet [31,32]. Thus soft proteins are expected to have a higher α-helix/ β-sheet ratio than hard proteins.

In wine, chitinases and β-glucanase are the first to adsorb on bentonite. Falconer *et al*. found that chitinases were rich in α-helices [8], which is consistent with a soft protein, but no data are available for grape β-glucanases, probably because these proteins are very unstable and thus difficult to purify. However, circular dichroism measurements on a barley β-glucanase showed that this protein is also rich in α-helices [33]. In the other hand, the *Vitis Vinifera* Thaumatin Like protein (VVTL1) is richer in β-sheets than chitinases and β-glucanases [34], and circular dichroism measurements performed on invertases purified from wines showed that these proteins are also richer in β-sheets [24]: these two proteins can thus be considered as hard proteins.

In order to assess the fact that thermosensitive proteins are soft proteins and thus change their three-dimensional conformation when they adsorb on solid surfaces, several techniques are available and have been used, e.g. FT-IR spectroscopy [35–37], fluorescence spectroscopy [38,39], and solid state NMR [40]. Fluorescence spectroscopy was chosen because it seems to be the fastest one: the fluorescence of the tryptophan residue is affected by its neighborhood [41]. Thus, a soft protein, which changes its conformation upon adsorption, is expected to exhibit a shift of its maximal emission wavelength, whereas a hard protein is not [38].

Fluorescence spectra of invertase, chitinase and TL19 were measured in a synthetic wine (EtOH 12%, tartaric acid 2 $g \cdot L^{-1}$, pH 3.5), with or without bentonite. Results are shown on the Figure 4. The fluorescence maximum emission wavelength of invertase showed no shift after adsorption on bentonite, whereas TL19 exhibited a slight shift (5 nm) and chitinase a larger one (18 nm). According to these results, invertase can be considered as a hard protein, whereas chitinase is a soft one. TL19 would have an intermediate behavior, perhaps linked to the reversibility of its temperature of denaturation and its adsorption. Another possibility is the

246 coexistence of two isoforms having two different behaviors, as observed by Marangon et al. on
247 two thaumatin like proteins, one being stable, the other unstable[34].

248 Attempts were done to perform fluorescence spectroscopy measurements on the supernatants
249 and lees of fined wines: unfortunately many other wine molecules fluoresce when they are
250 excited at 280 nm (e.g. polyphenols) and results were complex to interpret.

251 All these results show that the bentonite dose added in wine may be overestimated because
252 usual heat test performed at 80 °C unfold all the proteins in wine, even those which are stable
253 during storage. Furthermore, these proteins are the ones which require the highest bentonite
254 dose because of their low affinity for surfaces. These findings raise the question of the
255 development of alternative tests.

256 **Implication in wine-making: at which temperature should heat tests be done?**

257 The purpose of heat tests is to predict whether a wine is likely to develop a haze under normal
258 storage conditions, and to determine the bentonite dose. The question is complex for several
259 reasons: the expected stability, as well as the final protein concentration expected depend on
260 several parameters:

261 i) the channel distribution: an individual consumer may accept a slight haze
262 (sometimes developing close to the cork), whereas a wine seller will reject it;
263 ii) the other wine treatments applied during wine-making: for instance if
264 carboxymethylcellulose is added, it may form precipitates with the residual proteins;
265 iii) and the storage conditions.

266 We often consider that a normal storage temperature would be 16-18 °C in a cellar, for instance
267 Mc Rae *et al.* monitored the formation of haze after a one-year at 17° and 28 °C [42]. However,
268 wines can be exposed to much higher temperature during a limited time (60 °C in a car trunk

during the summer time), or a longer time if the wine is transported by a container ship and crosses the equator. Therefore, temperature-time conditions in usual heat-tests have been chosen to be sure that all proteins are removed from wine even those which are stable above 60 °C, a temperature which should not be reached in "normal" conditions.

To determine the conditions of a relevant accelerated ageing in normal conditions (i.e. considering that there is no problem during transport), we smeared ourselves on the paper by Falconer *et al*. which gives the theoretical half-life of chitinase (the most heat-sensitive protein with β-glucanase) as a function of the temperature: 1.3 hour at 40 °C, 14 hours at 35 °C, 4.7 days at 30 °C [8]. Working at 35 °C ensures that proteins that would never be unfolded in "normal storage" conditions, such as invertases and some thaumatin –like proteins, will not be denatured, but accelerates enough the unfolding of sensitive proteins. In order to improve the efficiency of heat tests and save time, several time-temperature couples were tested on the selected wines: 30 minutes at 80 °C, which is the standard procedure used in France [43] vs 30 minutes and 4 hours at 40 °C. The results were then compared to an accelerated ageing in 375 mL bottles at 35 °C during two weeks.

Figure 5A and B show the difference of turbidity measured after 2 weeks at 35 °C as a function of the difference of turbidity measured after 30 minutes at 80 °C.

Results showed no correlation between the heat test and the accelerated aging. It is usually admitted that a Sauvignon does not behave like a Gewurztraminer, however even for a given variety (e.g Gewurztraminer), a Δ NTU of 550 (point a of the Figure 5 A) or around 10 (points b of the Figure 5B) resulted all in a wine relatively stable with a Δ NTU of roughly 5 after 2 weeks at 35 °C.

Results obtained with the different heat tests, as well as the linear regression done with these points, are summarized in Figure 6. This confirms that the heat test at 80 °C is not correlated

293 with the accelerated ageing (R^2 = 0.05), especially for Gewurztraminer wines. On the other
294 hand results obtained when the samples are heated at 40 °C are more correlated with accelerated
295 ageing (R^2= 0.64 and 0.67). This is not surprising because below 40 °C and during the time
296 chosen, only β-glucanases and chitinases (i.e. the same proteins) will unfold, whereas all the
297 wine proteins will do at 80 °C. The small differences observed at 40 °C can be explained by the
298 predicted half-life of chitinases in synthetic wines done by Falconer *et al.* [8]: at 35 °C the
299 predicted half-life of chitinases is 14 hours (thus after 2 weeks all the chitinases are likely
300 unfolded), at 40 °C it is 1.3 hours : thus after 30 minutes much more than 50% of the chitinases
301 are still folded, whereas around 80% will be unfolded after 4 hours, resulting in a higher
302 turbidity.

303 The pH is another relevant point raised by previous results and studies[10,17,23]. An increase of the
304 pH can have different effects: for some native proteins such as chitinase, it raises the melting
305 temperature of about 10 °C between pH 2.6 and 4.0, and makes the protein more stable. On the
306 other hand, on unfolded and aggregated proteins, increasing the pH decreases the electrostatic
307 repulsions between aggregates and provokes aggregation, resulting in more turbidity[10]. It was
308 decided to perform two heat tests (30 minutes at 80 °C and 4 hours at 40 °C) at pH ranging
309 from 2.6 to 4.2. The difference of turbidity after and before heating as a function of the pH for
310 11 wines is shown in Figure 7. As expected, the results depended on the temperature. The
311 variation of turbidity is very low at 40 °C. The most important effects are found for Gew11, the
312 ΔNTU of which ranged from 15 to 30, and Mus08 (5 to 20). Different wines showed different
313 shapes of curves (bell-like, decreasing curve, increasing curve), indicating that the pH is not the
314 only factor having an effect on turbidity, as already described: polyphenols, polysaccharides,
315 ions and acids also play a part, in agreement with McRae et al [45]. At 40 °C, raising the pH may
316 increase the half-life of chitinase. As fewer proteins unfold, this results in a lower turbidity at
317 pH 4.2, counterbalancing the fact that at this pH, aggregates, if they exist, are less stable.

On the contrary, at 80 °C, all the proteins are unfolded, whatever the pH, and the turbidity is ruled by the aggregation of unfolded proteins, which is larger when the pH increases, as systematically observed with all wines, and especially on Mus08, whose ΔNTU increased from 10 to 220 when the pH increased from 2.6 to 4.2. The heat test at 80°C is more likely to overestimate the instability of a wine, especially when the pH is larger than 3.5 and its result should be examined cautiously.

To summarize, even though slight differences were observed depending on the wine matrix (pH, ethanol content, composition in other solutes), we highlighted the fact that on seven different wines, the most heat sensitive proteins are also the ones which adsorb first on the bentonite and thus are also eliminated first. Fluorescence spectra, in agreement with other structural data obtained from previous works, proved that the adsorption behavior and the thermostability are closely linked: the higher the α-helix/ β-sheet ratio is, the "softer" the protein is, leading both to its capacity to change its conformation upon solid surface adsorption, and to a lower energy to unfold. From an enological point of view, heat tests performed at 80 °C tend to overestimate both the instability of wines in relation to the conditions of conservation of the practice and the dose of bentonite required, and are more dependent on the wine pH. The tests performed at 40 °C had a better correlation with the accelerated ageing procedure and seem to be a good compromise.

Acknowledgements

This project was funded by the French Ministry of Food and Agriculture (Compte d'Affectation Spéciale Développement Agricole et Rural - CasDAR), project number 1402.

Figure captions

Figure 1: Percentage of initial proteins adsorbed as a function of the amount of added bentonite: a) CH30; b) GEW30 ; c) SA30 ; d) SA33 ; e) CH44 ; f) SA44 ; g) GEW68. Lines are a guide for the eye.

Figure 2: Percentage of initial proteins adsorbed as a function of the amount of added bentonite: a) invertase ; b) TL19; c) TL22. Lines are a guide for the eye.

Figure 3: Illustration of the thermosensitivity of the different classes of GEW30 proteins after heating at 40, 60 and 80 °C: chitinases and β-glucanases precipit below 40 °C, TL22 between 40 and 60 °C, invertases close to 80 °C, most LTP remain stable above 80 °C, as well as around 20% of TL19.

Figure 4: Normalized fluorescence emission spectra of three wine proteins in solution and adsorbed on bentonite. No λ_{max} shift is observed for invertase, a slight shift for TL19, a larger one for chitinase.

Figure 5: Relationships during the difference of turbidity (ΔNTU) measured after 2 weeks at 35 °C and 30 minutes at 80 °C (A and B), and 4 hours at 40°C (C). Figure 5B is a zoom of the black rectangle area.

Figure 6: Summary of all heat tests performed compared with an accelerated ageing. The test at 80 °C (blue circle) is not correlated with the storage at 35 °C ($R^2 = 0.05$), whereas both tests at 40°C (grey and orange) show a better correlation ($R^2 = 0.64$ and 0.67).

Figure 7: Dependence on the pH for two different heat tests: 4 hours at 40°C (A) and thirty minutes at 80 °C (B).

References

(1) Bayly, F. C.; Berg, H. W. Grape and Wine Proteins of White Wine Varietals. *Am. J. Enol. Vitic.* **1967**, *18*, 18–32.

(2) Hsu, J.-C.; Heatherbell, D. A. Heat-Unstable Proteins in Wine. I. Characterization and Removal by Bentonite Fining and Heat Treatment. *Am. J. Enol. Vitic.* **1987**, *38*, 11–16.

(3) Waters, E. J.; Wallace, W.; Williams, P. J. Heat Haze Characteristics of Fractionated Wine Proteins. *Am. J. Enol. Vitic.* **1991**, *42*, 123–127.

(4) Van Sluyter, S. C.; McRae, J. M.; Falconer, R. J.; Smith, P. A.; Bacic, A.; Waters, E. J.; Marangon, M. Wine Protein Haze: Mechanisms of Formation and Advances in Prevention. *J. Agric. Food Chem.* **2015**, *63*, 4020–4030.

(5) Dawes, H.; Boyes, S.; Keene, J.; Heatherbell, D. Protein Instability of Wines: Influence of Protein Isoelectric Point. *Am. J. Enol. Vitic.* **1994**, *45*, 319–326.

(6) Waters, E. J.; Wallace, W.; Williams, P. J. Identification of Heat-Unstable Wine Proteins and Their Resistance to Peptidases. *J. Agric. Food Chem.* **1992**, *40*, 1514–1519.

(7) Marangon, M.; Van Sluyter, S. C.; Neilson, K. A.; Chan, C.; Haynes, P. A.; Waters, E. J.; Falconer, R. J. Roles of Grape Thaumatin-like Protein and Chitinase in White Wine Haze Formation. *J. Agric. Food Chem.* **2011**, *59*, 733–740.

(8) Falconer, R. J.; Marangon, M.; Van Sluyter, S. C.; Neilson, K. a; Chan, C.; Waters, E. J. Thermal Stability of Thaumatin-like Protein, Chitinase, and Invertase Isolated from Sauvignon Blanc and Semillon Juice and Their Role in Haze Formation in Wine. *J. Agric. Food Chem.* **2010**, *58*, 975–980.

(9) Dufrechou, M.; Sauvage, F.-X.; Bach, B.; Vernhet, A. Protein Aggregation in White Wines: Influence of the Temperature on Aggregation Kinetics and Mechanisms. *J. Agric. Food Chem.* **2010**, *58*, 10209–10218.

(10) Dufrechou, M.; Poncet-Legrand, C.; Sauvage, F.-X.; Vernhet, A. Stability of White Wine Proteins: Combined Effect of PH, Ionic Strength, and Temperature on Their

Aggregation. *J. Agric. Food Chem.* **2012**, *60*, 1308–1319.

(11) Waters, E. J.; Peng, Z.; Pocock, K. F.; Williams, P. J. Proteins in White Wine, I: Procyanidin Occurrence in Soluble Proteins and Insoluble Protein Hazes and Its Relationship to Protein Instability. *Aust. J. Grape Wine Res.* **1995**, *1*, 86–93.

(12) Pocock, K. F.; Alexander, G. M.; Hayasaka, Y.; Jones, P. R.; Waters, E. J. Sulfate--a Candidate for the Missing Essential Factor That Is Required for the Formation of Protein Haze in White Wine. *J. Agric. Food Chem.* **2007**, *55*, 1799–1807.

(13) Gazzola, D.; Van Sluyter, S. C.; Curioni, A.; Waters, E. J.; Marangon, M. Roles of Proteins, Polysaccharides, and Phenolics in Haze Formation in White Wine via Reconstitution Experiments. *J. Agric. Food Chem.* **2012**, *60*, 10666–10673.

(14) Dufrechou, M.; Doco, T.; Poncet-Legrand, C.; Sauvage, F.-X.; Vernhet, A. Protein/Polysaccharide Interactions and Their Impact on Haze Formation in White Wines. *J. Agric. Food Chem.* **2015**, *63*, 10042–10053.

(15) Marangon, M.; Sauvage, F.-X.; Waters, E. J.; Vernhet, A. Effects of Ionic Strength and Sulfate upon Thermal Aggregation of Grape Chitinases and Thaumatin-like Proteins in a Model System. *J. Agric. Food Chem.* **2011**, *59*, 2652–2662.

(16) Batista, L.; Monteiro, S.; Loureiro, V. B.; Teixeira, A. R.; Ferreira, R. B. The Complexity of Protein Haze Formation in Wines. *Food Chem.* **2009**, *112*, 169–177.

(17) Lambri, M.; Dordoni, R.; Giribaldi, M.; Riva Violetta, M.; Giuffrida, M. G. Effect of PH on the Protein Profile and Heat Stability of an Italian White Wine. *Food Res. Int.* **2013**, *54*, 1781–1786.

(18) Batista, L.; Monteiro, S.; Loureiro, V. B.; Teixeira, A. R.; Ferreira, R. B. Protein Haze Formation in Wines Revisited. The Stabilising Effect of Organic Acids. *Food Chem.* **2010**, *122*, 1067–1075.

(19) Mierczynska-Vasilev, A.; Boyer, P.; Vasilev, K.; Smith, P. A. A Novel Technology for the Rapid, Selective, Magnetic Removal of Pathogenesis-Related Proteins from Wines. *Food Chem.* **2017**, *232*, 508–514.

(20) Marangon, M.; Stockdale, V. J.; Munro, P.; Trethewey, T.; Schulkin, A.; Holt, H. E.; Smith, P. A. Addition of Carrageenan at Different Stages of Winemaking for White

Wine Protein Stabilization. *J. Agric. Food Chem.* **2013**, *61*, 6516–6524.

(21) Marangon, M.; Van Sluyter, S. C.; Robinson, E. M. C.; Muhlack, R. A.; Holt, H. E.; Haynes, P. A.; Godden, P. W.; Smith, P. A.; Waters, E. J. Degradation of White Wine Haze Proteins by Aspergillopepsin i and II during Juice Flash Pasteurization. *Food Chem.* **2012**, *135*, 1157–1165.

(22) Pocock, K. F.; Høj, P. B.; Adams, K. S.; Kwiatkowski, M. J.; Waters, E. J. Combined Heat and Proteolytic Enzyme Treatment of White Wines Reduces Haze Forming Protein Content without Detrimental Effect. *Aust. J. Grape Wine Res.* **2003**, *9*, 56–63.

(23) Sauvage, F.-X.; Bach, B.; Moutounet, M.; Vernhet, A. Proteins in White Wines: Thermo-Sensitivity and Differential Adsorbtion by Bentonite. *Food Chem.* **2010**, *118*, 26–34.

(24) Dufrechou, M.; Vernhet, A.; Roblin, P.; Sauvage, F.-X.; Poncet-Legrand, C. White Wine Proteins: How Does the PH Affect Their Conformation at Room Temperature? *Langmuir* **2013**, *29*, 10475–10482.

(25) Achaerandio, I.; Pachova, V.; Güell, C.; López, F. Protein Adsorption by Bentonite in a White Wine Model Solution: Effect of Protein Molecular Weight and Ethanol Concentration. *Am J Enol Vitic.* **2001**, *52*, 122–126.

(26) Blade, W. H.; Boulton, R. Adsorption of Protein by Bentonite in a Model Wine Solution. *Am. J. Enol. Vitic.* **1988**, *39*, 193–199.

(27) Arai, T.; Norde, W. The Behavior of Some Model Proteins at Solid-Liquid Interfaces 1. Adsorption from Single Protein Solutions. *Colloids and Surfaces* **1990**, *51*, 1–15.

(28) Norde, W.; MacRitchie, F.; Nowicka, G.; Lyklema, J. Protein Adsorption at Solid-Liquid Interfaces: Reversibility and Conformation Aspects. *J. Colloid Interface Sci.* **1986**, *112*, 447–456.

(29) Haynes, C. A.; Norde, W. Structures and Stabilities of Adsorbed Proteins. *J. Colloid Interface Sci.* **1995**, *169*, 313–328.

(30) Koo, J.; Erlkamp, M.; Grobelny, S.; Steitz, R.; Czeslik, C. Pressure-Induced Protein Adsorption at Aqueous-Solid Interfaces. *Langmuir* **2013**, *29*, 8025–8030.

(31) Vijayakumar, S.; Vishveshwara, S.; Ravishanker, G.; Beveridge, D. L. Differential Stability of Beta-Sheets and Alpha-Helices in Beta-Lactamase: A High Temperature Molecular Dynamics Study of Unfolding Intermediates. *Biophys. J.* **1993**, *65*, 2304–2312.

(32) Jaber, M.; Lambert, J.-F.; Balme, S. Chapter 8: Proteins Adsorption on Clay Minerals. In *Surface and Interface Chemistry of Clay Minerals, Volume 9*; Robert Schoonheydt Cliff Johnston Faïza Bergaya, Ed.; Elsevier, 2018; p. 255.

(33) MacGregor, E. A.; Ballance, G. M. Possible Secondary Structure in Plant and Yeast β-Glucanase. *Biochem. J.* **1991**, *274*, 41–43.

(34) Marangon, M.; Van Sluyter, S. C.; Waters, E. J.; Menz, R. I. Structure of Haze Forming Proteins in White Wines: Vitis Vinifera Thaumatin-like Proteins. *PLoS One* **2014**, *9*, 1–21.

(35) Steiner, G.; Tunc, S.; Maitz, M.; Salzer, R. Conformational Changes during Protein Adsorption. FT-IR Spectroscopic Imaging of Adsorbed Fibrinogen Layers. *Anal. Chem.* **2007**, *79*, 1311–1316.

(36) Buijs, J.; Norde, W.; Lichtenbelt, J. W. T. Changes in the Secondary Structure of Adsorbed IgG and F(Ab′) 2 Studied by FTIR Spectroscopy. *Langmuir* **1996**, *12*, 1605–1613.

(37) Cheng, S. S.; Chittur, K. K.; Sukenik, C. N.; Culp, L. A.; Lewandowska, K. The Conformation of Fibronectin on Self-Assembled Monolayers with Different Surface Composition: An FTIR/ATR Study. *Journal of Colloid and Interface Science*, 1994, *162*, 135–143.

(38) Lepoitevin, M.; Jaber, M.; Guégan, R.; Janot, J. M.; Dejardin, P.; Henn, F.; Balme, S. BSA and Lysozyme Adsorption on Homoionic Montmorillonite: Influence of the Interlayer Cation. *Appl. Clay Sci.* **2014**, *95*, 396–402.

(39) Bouaziz, Z.; Soussan, L.; Janot, J. M.; Lepoitevin, M.; Bechelany, M.; Djebbi, M. A.; Amara, A. B. H.; Balme, S. Structure and Antibacterial Activity Relationships of Native and Amyloid Fibril Lysozyme Loaded on Layered Double Hydroxide. *Colloids Surfaces B Biointerfaces* **2017**, *157*, 10–17.

(40) Assifaoui, A.; Huault, L.; Maissiat, C.; Roullier-Gall, C.; Jeandet, P.; Hirschinger, J.; Raya, J.; Jaber, M.; Lambert, J.-F.; Cayot, P.; et al. Structural Studies of Adsorbed Protein (Betalactoglobulin) on Natural Clay (Montmorillonite). *RSC Adv.* **2014**, *4*, 61096–61103.

(41) Chen, Y.; Barkley, M. D. Toward Understanding Tryptophan Fluorescence in Proteins. *Biochemistry* **1998**, *37*, 9976–9982.

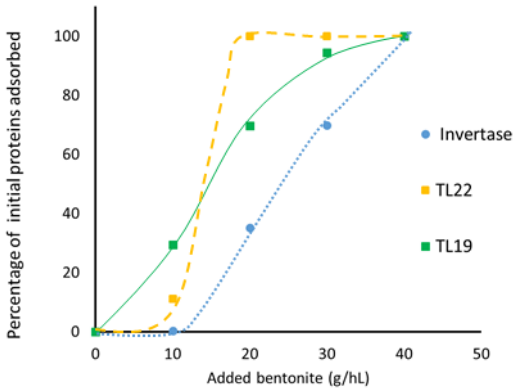
(42) McRae, J. M.; Barricklow, V.; Pocock, K. F.; Smith, P. A. Predicting Protein Haze Formation in White Wines. *Aust. J. Grape Wine Res.* **2018**, *24*, 504–511.

(43) Dubourdieu, D.; Serrano, M.; Vannier, A.-C.; Ribéreau-Gayon, P. Etude Comparée Des Tests de Stabilité Protéique. *OENO One* **1988**, *22*, 261.

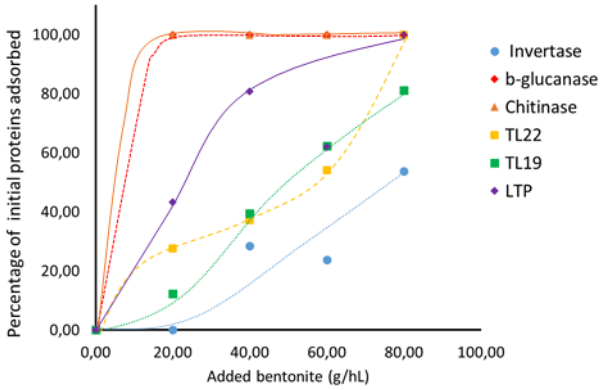
(44) Dufrechou, M.; Sauvage, F.-X.; Poncet-Legrand, C.; Vernhet, A. Combined Effects of PH and Temperature on Protein Haze Formation in White Wine. In *Oeno 2011*; Bordeaux, 2011.

(45) McRae, J. M.; Schulkin, A.; Dambergs, R. G.; Smith, P. A. Effect of White Wine Composition on Protein Haze Potential. *Aust. J. Grape Wine Res.* **2018**, *24*, 498–503.

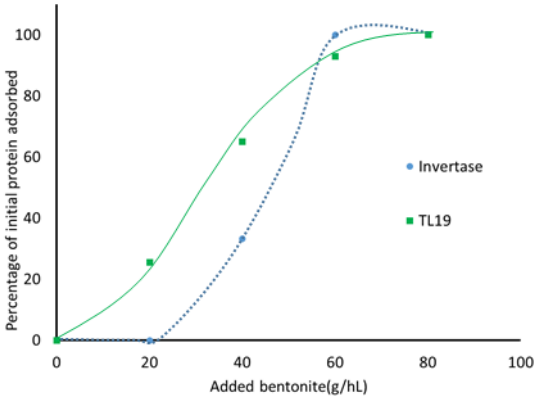
Figures



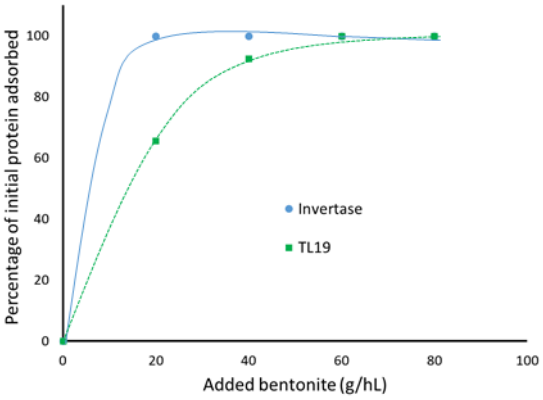
a)



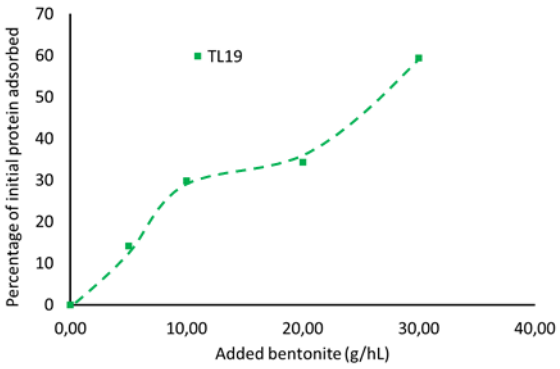
b)



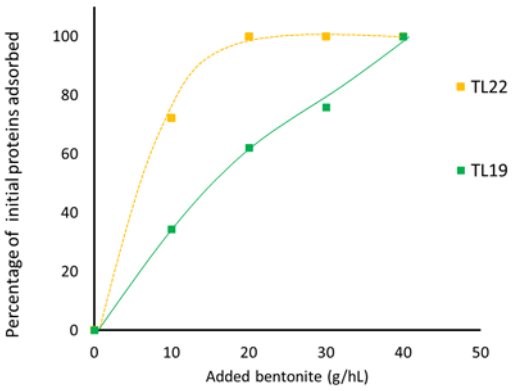
c)



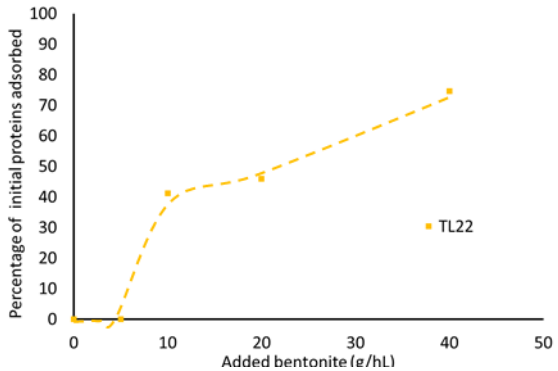
d)



e)

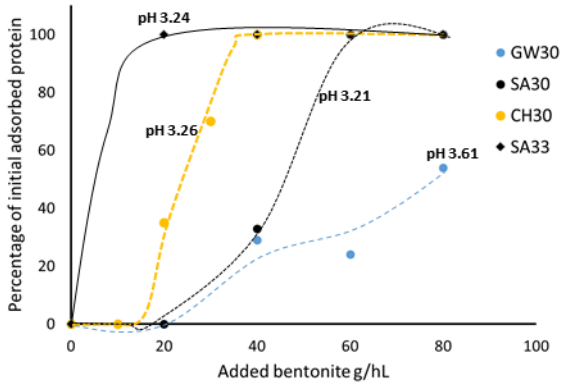


f)

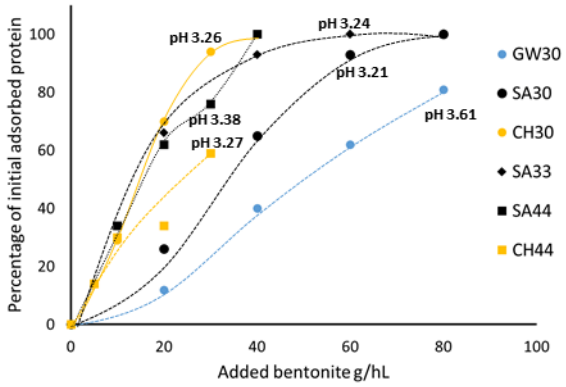


g)

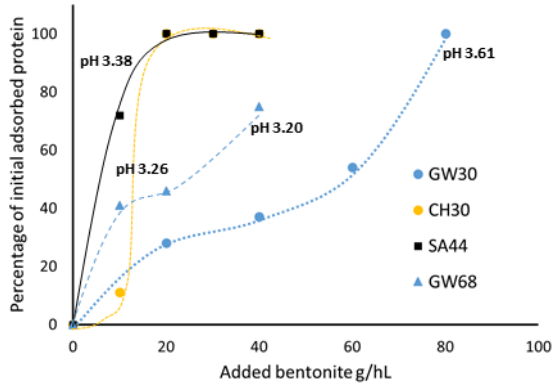
Figure 1: Percentage of initial proteins adsorbed as a function of the amount of added bentonite: a) CH30; b) GEW30 ; c) SA30 ; d) SA33 ; e) CH44 ; f) SA44 ; g) GEW68. Lines are a guide for the eye.



a)



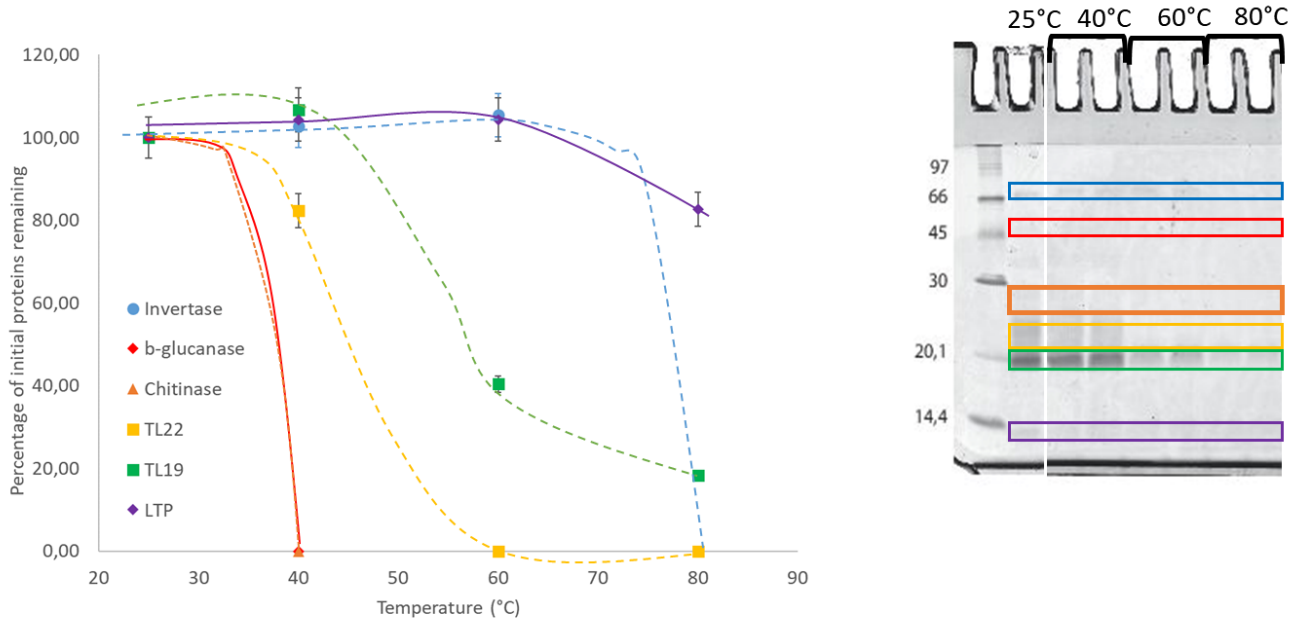
b)



c)

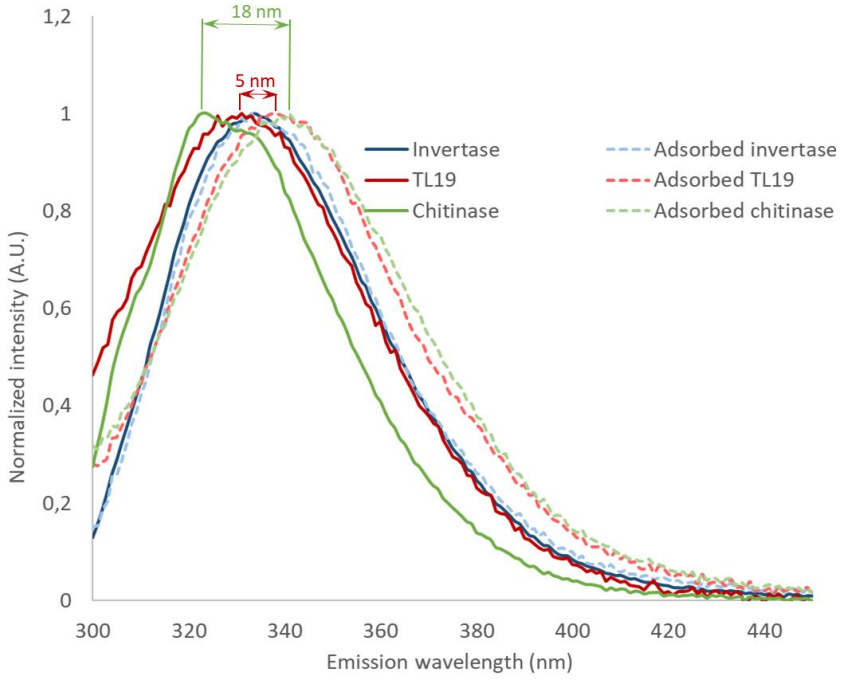
494 **Figure 2:** Percentage of initial proteins adsorbed as a function of the amount of added bentonite:
495 a) Invertase ; b) TL19; c) TL22. Lines are a guide for the eye.

496



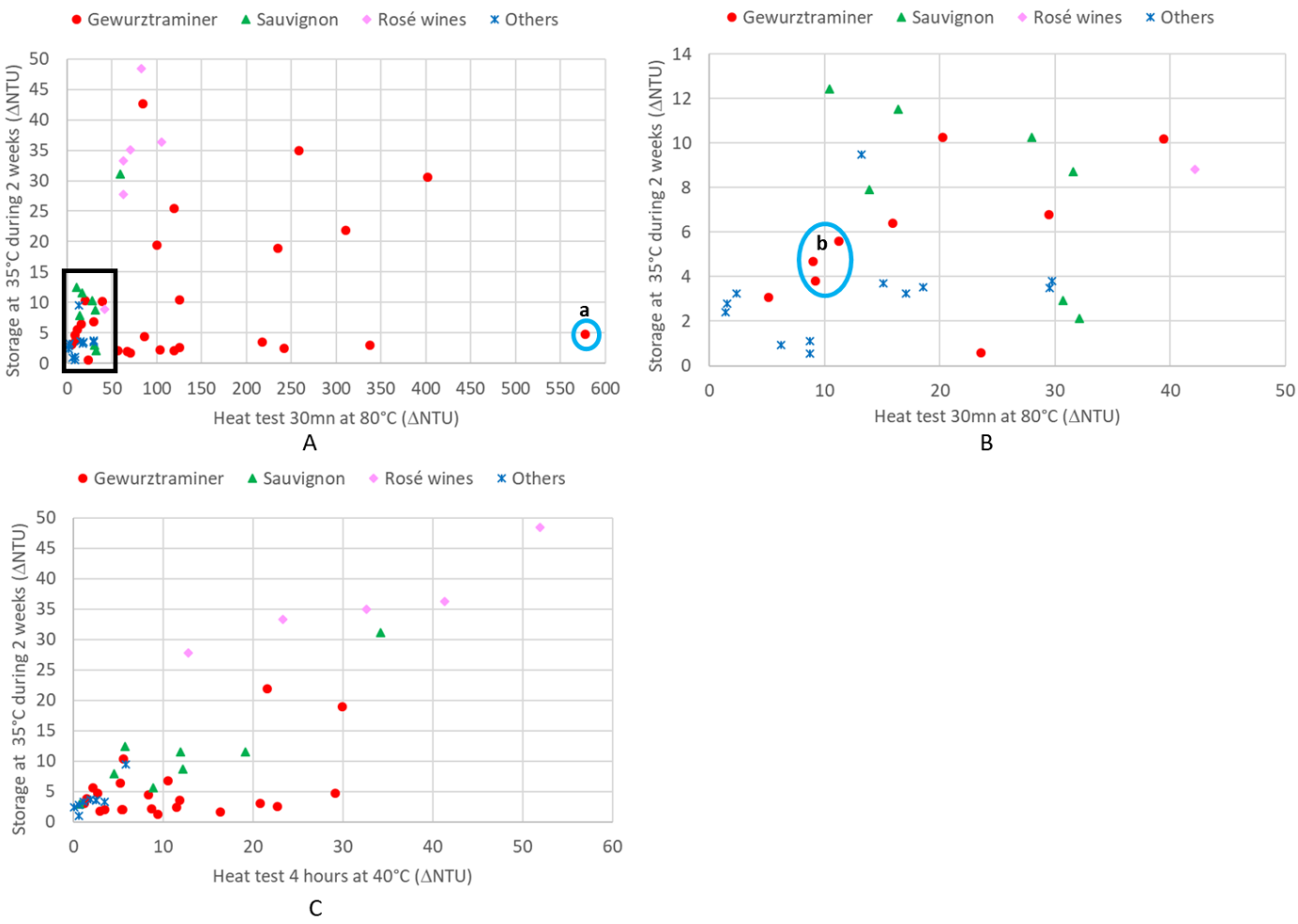
497

498 **Figure 3**: Illustration of the thermosensitivity of the different classes of GEW30 proteins after
499 heating at 40, 60 and 80 °C: chitinases and β-glucanases precipit below 40 °C, TL22 between
500 40 and 60 °C, invertases close to 80 °C, most LTP remain stable above 80 °C, as well as around
501 20% of TL19.



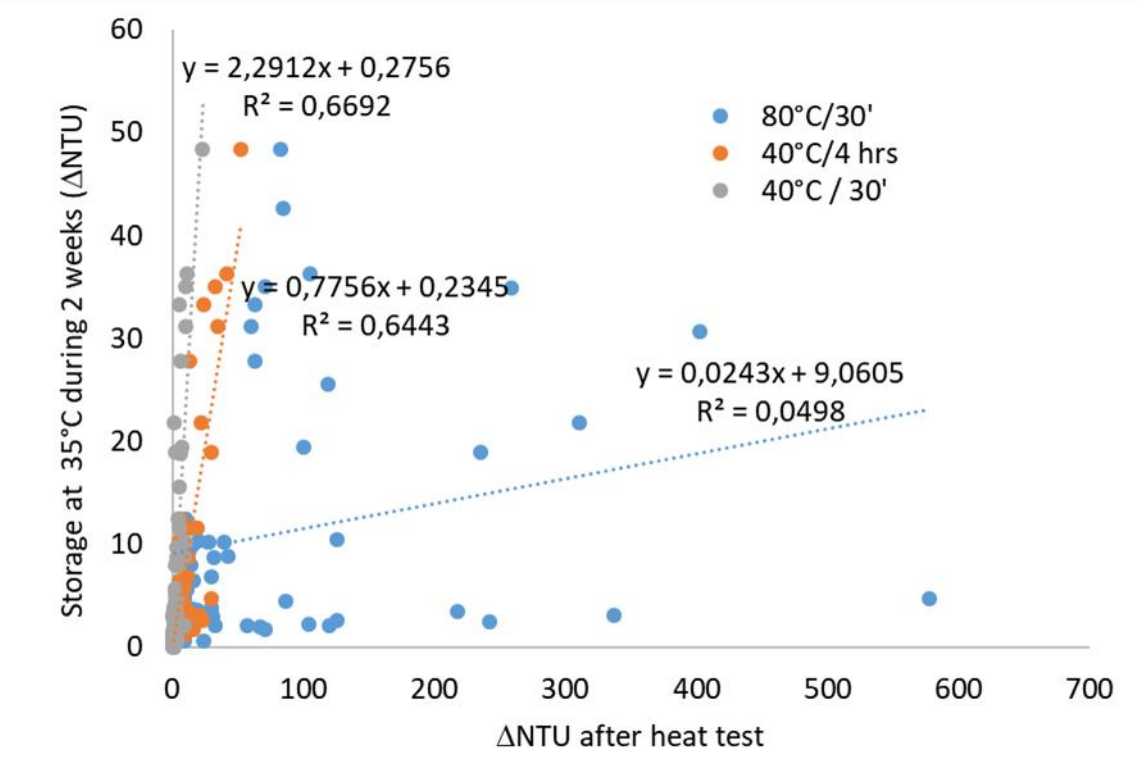
502

503 **Figure 4**: Normalized fluorescence emission spectra of three wine proteins in solution and
504 adsorbed on bentonite. No λ_{max} shift is observed for invertase, a slight shift for TL19, a larger
505 one for chitinase.



506

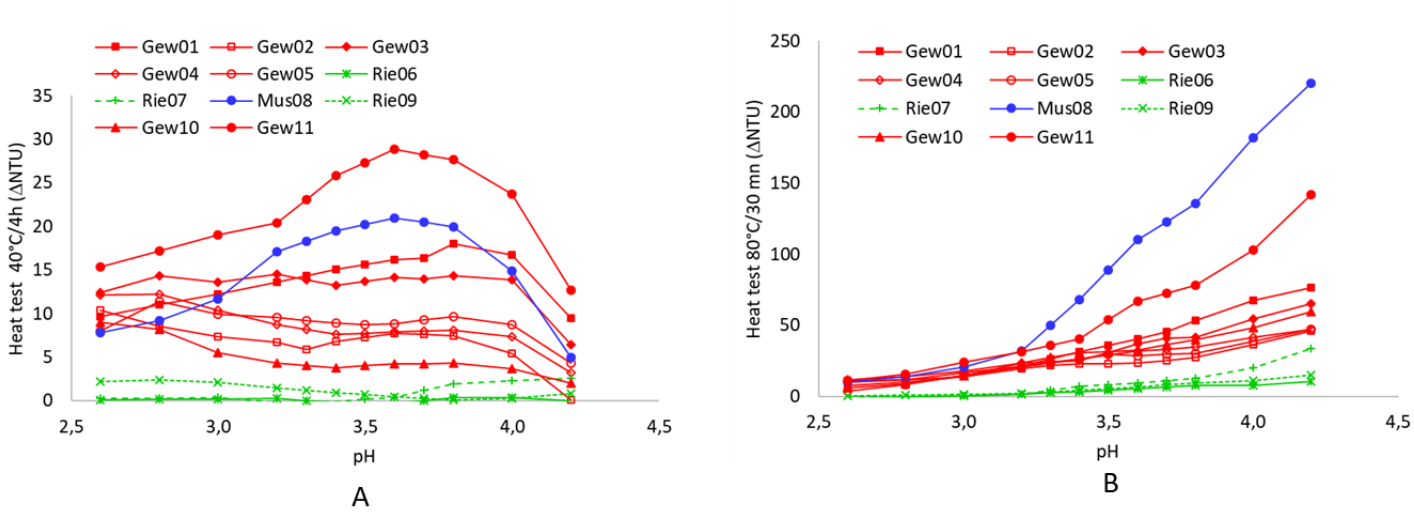
507 **Figure 5**: Relationships during the difference of turbidity (ΔNTU) measured after 2 weeks at
508 35 °C and 30 minutes at 80 °C (A and B), and 4 hours at 40 °C (C). Figure 5B is a zoom of the
509 black rectangle area.



510

511 **Figure 6:** Summary of all heat tests performed compared with an accelerated ageing. The test
512 at 80 °C (blue circle) is not correlated with the storage at 35 °C (R^2 = 0.05), whereas both tests
513 at 40 °C (grey and orange) show a better correlation (R^2 = 0.64 and 0.67).

514



515

516 **Figure 7**: Dependence on the pH for two different heat tests: 4 hours at 40 °C (A) and thirty
517 minutes at 80 °C (B).

518

519 **Tables**

520 **Table 1:** Oenological analysis of the wines at the end of the alcoholic fermentation

	pH	EtOH (% v/v)	Volatile acidity (g $H_2SO_4 \cdot L^{-1}$)	Total acidity (g $H_2SO_4 \cdot L^{-1}$)	Sugar ($g \cdot L^{-1}$)
CH30	3.26	13.3	0.36	4.55	0
GEW30	3.61	14.1	0.30	3.26	0.9
SA30	3.21	11.6	0.19	5.54	0
SA33	3.24	12.8	0.30	4.20	0
CH44	3.27	11.8	0.16	4.40	0.6
SA44	3.38	12.8	0.21	3.80	1.2
GEW68	3.20	13.9	0.27	4.51	0.9

521

522 **Table 2: Protein composition of the wines in $mg \cdot L^{-1}$**

	invertase	b-glucanase	chitinase	TL22	TL19	LTP
CH30	7.2	nd	nd	9	76	nd
GEW30	5.6	2.5	2	34	78	11
SA30	3.5	nd	nd	nd	43	nd
SA33	2.2	nd	nd	nd	67	nd
CH44	nd	nd	nd	nd	39	nd
SA44	nd	nd	nd	18	29	nd
GEW68	nd	nd	nd	63	nd	nd

523