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

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17 **Abstract**

18 Bentonite fining is the most popular treatment used to remove proteins in white and rosé wines.  
19 The usual heat test used to adjust the bentonite dose consists in heating the wine during 30  
20 minutes at 80 °C. At this temperature, all the proteins are unfolded and this can lead to an  
21 overestimation of the dose. We have shown that proteins adsorb on bentonite in a specific order,  
22 and more importantly that the proteins responsible for haze formation adsorb first. Fluorescence  
23 spectroscopy showed that this is due to the structural properties of proteins, which can be  
24 classified in hard and soft proteins. Alternative heat-tests were performed at lower temperature  
25 (40 °C) and showed a better correlation with accelerated ageing. These tests were also less  
26 dependent on the wine pH.

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

## 29 **Introduction**

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

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

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

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

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

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

87

## 88 **Materials and methods**

### 89 **Wines and model systems**

90 *Wine making.* A first set of wines was used in the fining study. They were elaborated in  
91 2016 in the different experimental units of the French Institute of Vine and Wine (IFV), in  
92 Rodilhan (30), Blanquefort (33), Nantes (44), and Colmar (68), France. They were made from  
93 three varieties of *Vitis Vinifera*: Chardonnay (CH), Sauvignon (SA) and Gewurztraminer  
94 (GEW). In the present study, wines will be referred to as a combination of the area code and  
95 the variety: for instance a Sauvignon wine made in Rodilhan will be noted SA30. Following  
96 fermentation, the wines were cold stabilized to prevent the crystallization of tartaric salts and  
97 clarified. No bentonite fining was performed. After a final membrane filtration, the wines were  
98 aliquoted in bottles and stored at 10 °C before use. Conventional enological parameters were  
99 analyzed according to Vine and Wine International Organisation methods, and are reported in  
100 the Table 1. A second set of wines elaborated by the IFV was used to perform alternative heat  
101 tests: it was made of 55 wines: 4 vintages from 2014 to 2017, 7 varieties (Pinot Gris,  
102 Gewurztraminer, Riesling, Sauvignon, Chardonnay, Cinsault, Caladoc), 5 sites in different

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

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

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

114 *Heat tests.* Samples of wines were submitted to different time/temperature couples: 30 minutes  
115 at 80 °C, 60 °C and 40 °C, but also 4 hours at 40 °C and 2 weeks at 35 °C. Turbidity was  
116 measured on a turbidimeter Hach TL2310 before and after heating, after the samples were  
117 cooled down to room temperature during 16-18 hours. A study on the effect of pH was also  
118 performed, and heat-tests were done after adjustment of wine pH within the range 2.6 to 4.2  
119 with NaOH and H<sub>2</sub>SO<sub>4</sub>. Experiments were done in triplicate, except for the tests at 40, 60 and  
120 80 °C in 2016 (duplicate) and the results were averaged.

121 *Model systems.* The conformational changes of proteins during adsorption on bentonite were  
122 assessed in model systems. Stock solutions of bentonite and proteins, purified and identified as  
123 described by Dufrechou et al.<sup>24</sup> were mixed in a synthetic wine (12% EtOH, 2 g·L<sup>-1</sup> of tartaric  
124 acid, pH 3.5 adjusted with KOH). The protein concentration was set to a value giving reliable  
125 results and consistent with what is present in wines: 10 mg·L<sup>-1</sup> for invertase, 20 mg·L<sup>-1</sup> for  
126 chitinase, 35 mg·L<sup>-1</sup> for TL19. Bentonite concentration was set to 1 g·L<sup>-1</sup>, which is both  
127 consistent with an enological use (100 g·hL<sup>-1</sup>) and a complete adsorption of proteins.

128 **Protein analysis**

129 *Monodimensional electrophoresis (SDS PAGE)*. Protein analyses were performed by sodium  
130 dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Wine aliquots were mixed  
131 with the Laemmli buffer 4X (60  $\mu$ L of wine, 20  $\mu$ L of buffer). The resulting mixture was loaded  
132 on a 10 well polyacrylamide gel (14% acrylamide, gel length = 80 mm). A low molecular  
133 weight calibration kit (GE Healthcare), ranging from 14.4 to 97 kDa, was included in each  
134 electrophoretic run. The gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-  
135 Rad) in 40% of ethanol and 10% of acetic acid. SDS PAGE were run on a CBS Scientific MGV-  
136 202 vertical gels system at 20 mA, until the dye front reached the bottom of the gel  
137 (approximately 2 h and 30 min). They were then destained in 10% of acetic acid during two  
138 days, with a regular renewal of the solvent. Gels were then scanned at 300 dpi with an image  
139 scanner (Biorad GS 710). Image analysis was carried out with the Phoretix 1D software and  
140 was used to calculate the proportion of proteins in each staining band taking Bovine Serum  
141 Albumine as a reference. Proteins were identified according to their molecular weight and in  
142 reference to previous results <sup>23,10</sup>. Electrophoresis were performed in duplicate since it was  
143 shown that the repeatability was in the order of 10% <sup>23</sup>. It was planned to do the triplicate if  
144 differences between duplicates were higher than 10%. Results were averaged and are presented  
145 in the Table 2.

146 *Fluorescence spectroscopy*. Measurements were performed at room temperature with an RF  
147 5301 PC fluorescence spectrophotometer (Shimadzu, Japan) and were used to show  
148 modifications of the tertiary structure of the proteins when they adsorb on bentonite, or their  
149 absence. The excitation wavelength was 280 nm, and the emission wavelength ranged from 300  
150 to 500 nm. Data were recorded and analyzed with the RFPC software (Shimadzu, Japan).

151 The fluorescence of bentonite at  $1 \text{ g}\cdot\text{L}^{-1}$  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  $\beta$ -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,  $\beta$ -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 <sup>24</sup>. Most of thaumatin –like proteins  
170 and invertase have isoelectric points (IEPs) between 4 and 5 <sup>23</sup>, whereas chitinases and  $\beta$ -  
171 glucanases have IEPs between 5 and 7 <sup>23,5</sup>. 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

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

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

### 188 **Thermal stability**

189 In parallel to bentonite fining, the wines were heated to 40, 60 and 80 °C during 30 minutes,  
190 then cooled and centrifuged. The remaining proteins were then analyzed. Figure 3 shows the  
191 proteins remaining in GEW30 after heating. As already observed by several authors<sup>10,8</sup>,  
192 chitinases and  $\beta$ -glucanases are the most unstable proteins, unfolding and precipitating below  
193 40 °C. The different thaumatin-like proteins are slightly stable, with melting point between 40  
194 and 60 °C. Invertase and lipid transfer proteins are the most stable proteins: they can stand  
195 temperatures up to nearly 80 °C. These temperatures are in agreement with the temperature  
196 determined in previous studies, where chitinases were found to have a  $T_m$  of 55 °C, thaumatin-  
197 like proteins  $T_m$ 's of 61 and 62 °C and invertase 81 °C.<sup>8</sup> The small differences observed can  
198 be explained by the effect of pH, which is different depending on proteins<sup>10</sup>. It also confirms

199 the partial reversibility of TL19<sup>10,8</sup> 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<sup>23</sup>, 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<sup>27</sup> 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<sup>28</sup> 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  $\alpha$ -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·mol<sup>-1</sup> and 21 kJ·mol<sup>-1</sup>, respectively<sup>29,30</sup>. It has also been determined that the

222 energy necessary to unfold an  $\alpha$ -helix is smaller than the one needed to unfold a  $\beta$ -sheet<sup>31,32</sup>.

223 Thus soft proteins are expected to have a higher  $\alpha$ -helix/  $\beta$ -sheet ratio than hard proteins.

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

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

239 Fluorescence spectra of invertase, chitinase and TL19 were measured in a synthetic wine (EtOH  
240 12%, tartaric acid 2 g·L<sup>-1</sup>, pH 3.5), with or without bentonite. Results are shown on the Figure  
241 4. The fluorescence maximum emission wavelength of invertase showed no shift after  
242 adsorption on bentonite, whereas TL19 exhibited a slight shift (5 nm) and chitinase a larger one  
243 (18 nm). According to these results, invertase can be considered as a hard protein, whereas  
244 chitinase is a soft one. TL19 would have an intermediate behavior, perhaps linked to the  
245 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<sup>34</sup>.

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<sup>42</sup>. However,  
268 wines can be exposed to much higher temperature during a limited time (60 °C in a car trunk

269 during the summer time), or a longer time if the wine is transported by a container ship and  
270 crosses the equator. Therefore, temperature-time conditions in usual heat-tests have been  
271 chosen to be sure that all proteins are removed from wine even those which are stable above 60  
272 °C, a temperature which should not be reached in “normal” conditions.

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

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

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

291 Results obtained with the different heat tests, as well as the linear regression done with these  
292 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  $\beta$ -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.*<sup>8</sup>: 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<sup>10,17,23</sup>. 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<sup>10</sup>. 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  $\Delta$ NTU 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.*<sup>45</sup>. 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.

318 On the contrary, at 80 °C, all the proteins are unfolded, whatever the pH, and the turbidity is  
319 ruled by the aggregation of unfolded proteins, which is larger when the pH increases, as  
320 systematically observed with all wines, and especially on Mus08, whose  $\Delta$ NTU increased from  
321 10 to 220 when the pH increased from 2.6 to 4.2. The heat test at 80°C is more likely to  
322 overestimate the instability of a wine, especially when the pH is larger than 3.5 and its result  
323 should be examined cautiously.

324 To summarize, even though slight differences were observed depending on the wine matrix  
325 (pH, ethanol content, composition in other solutes), we highlighted the fact that on seven  
326 different wines, the most heat sensitive proteins are also the ones which adsorb first on the  
327 bentonite and thus are also eliminated first. Fluorescence spectra, in agreement with other  
328 structural data obtained from previous works, proved that the adsorption behavior and the  
329 thermostability are closely linked: the higher the  $\alpha$ -helix/  $\beta$ -sheet ratio is, the “softer” the  
330 protein is, leading both to its capacity to change its conformation upon solid surface adsorption,  
331 and to a lower energy to unfold. From an enological point of view, heat tests performed at 80  
332 °C tend to overestimate both the instability of wines in relation to the conditions of conservation  
333 of the practice and the dose of bentonite required, and are more dependent on the wine pH. The  
334 tests performed at 40 °C had a better correlation with the accelerated ageing procedure and  
335 seem to be a good compromise.

### 336 **Acknowledgements**

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339 **Figure captions**

340

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349 20% of TL19.

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359 **Figure 7:** Dependence on the pH for two different heat tests: 4 hours at 40°C (A) and thirty  
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362

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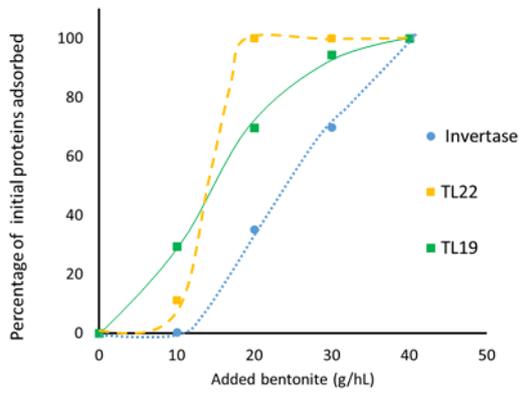
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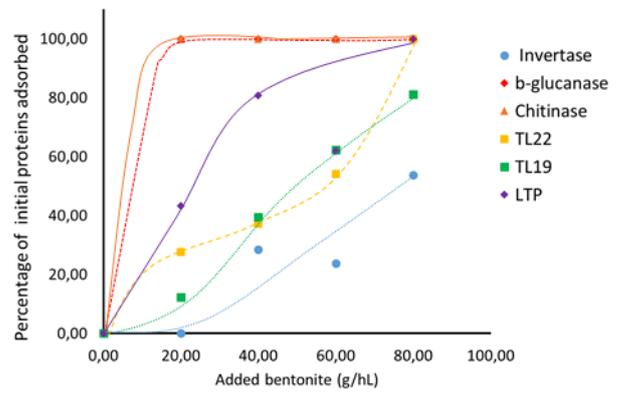
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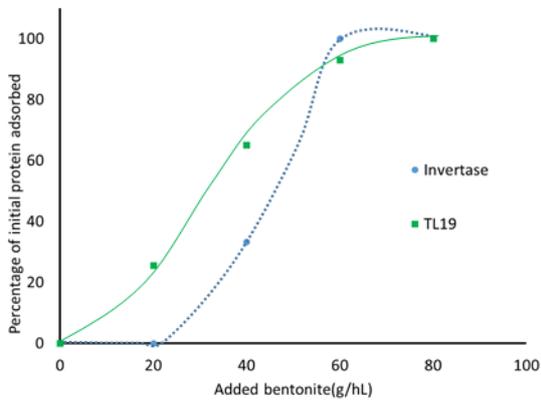
490 **Figures**



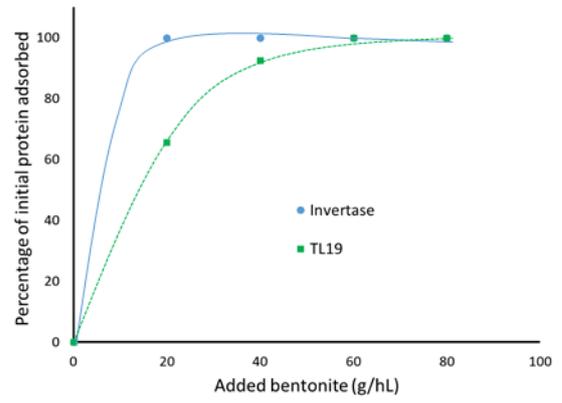
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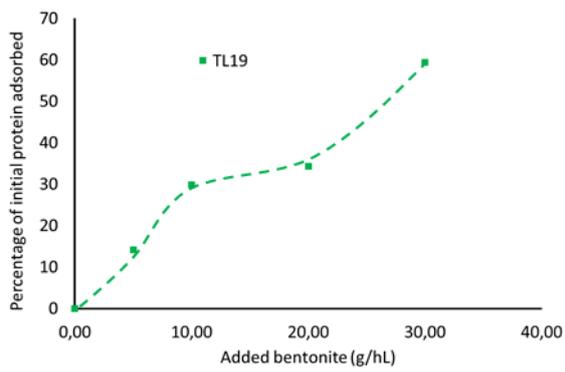
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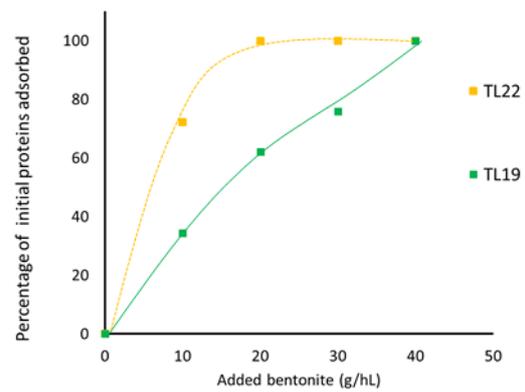
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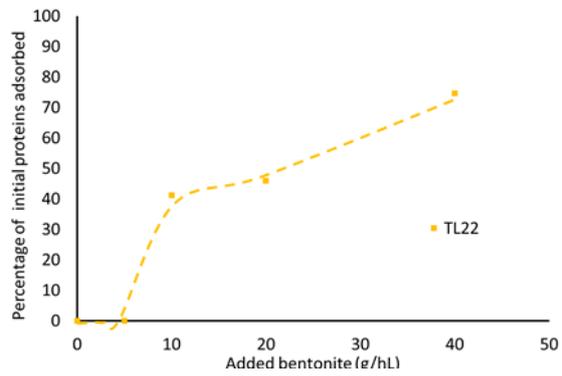
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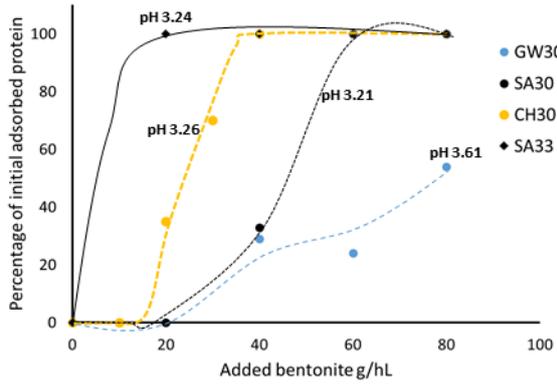


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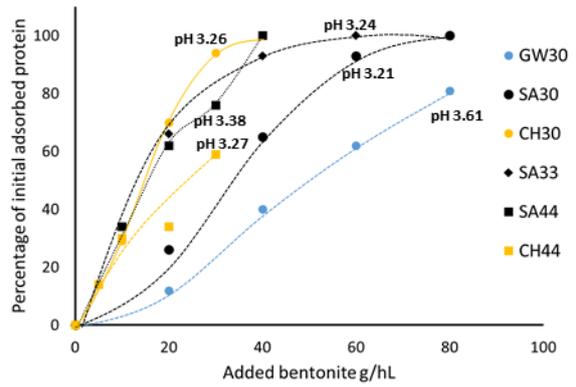


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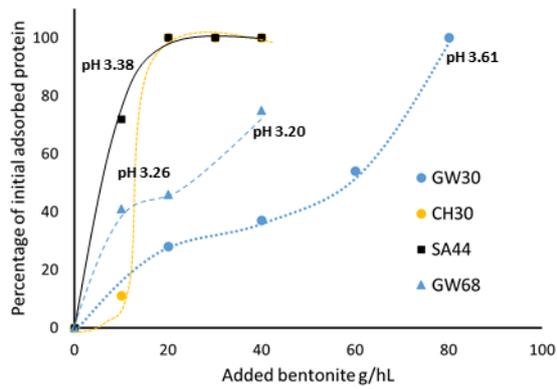
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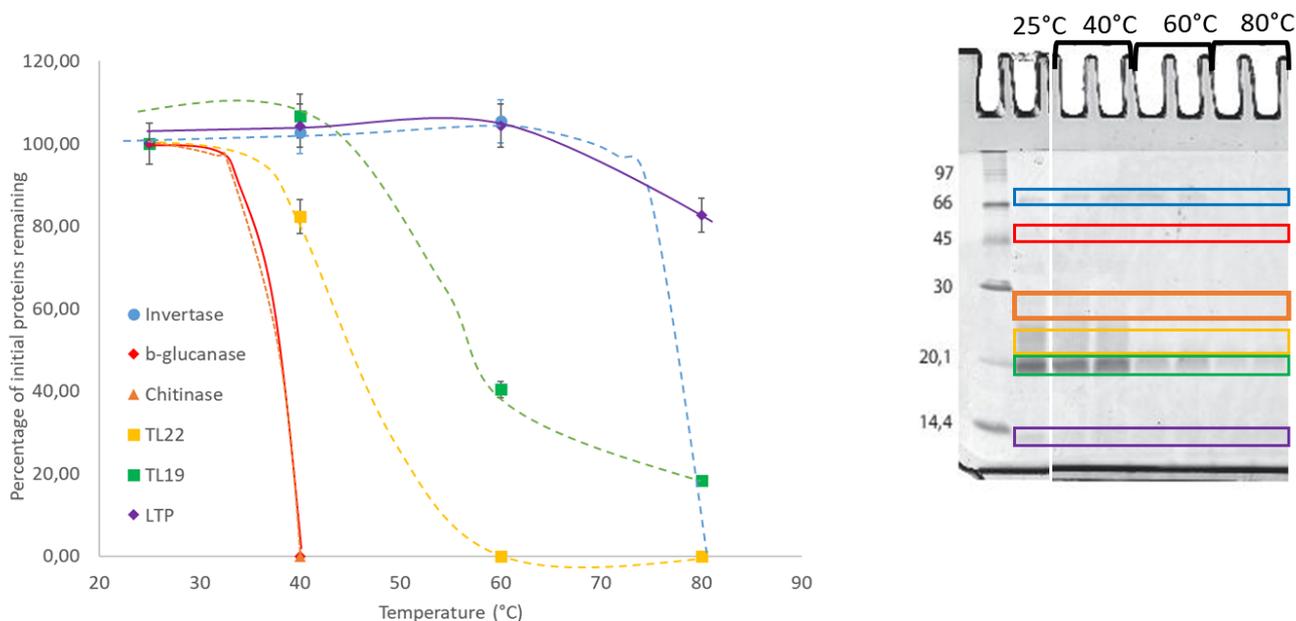
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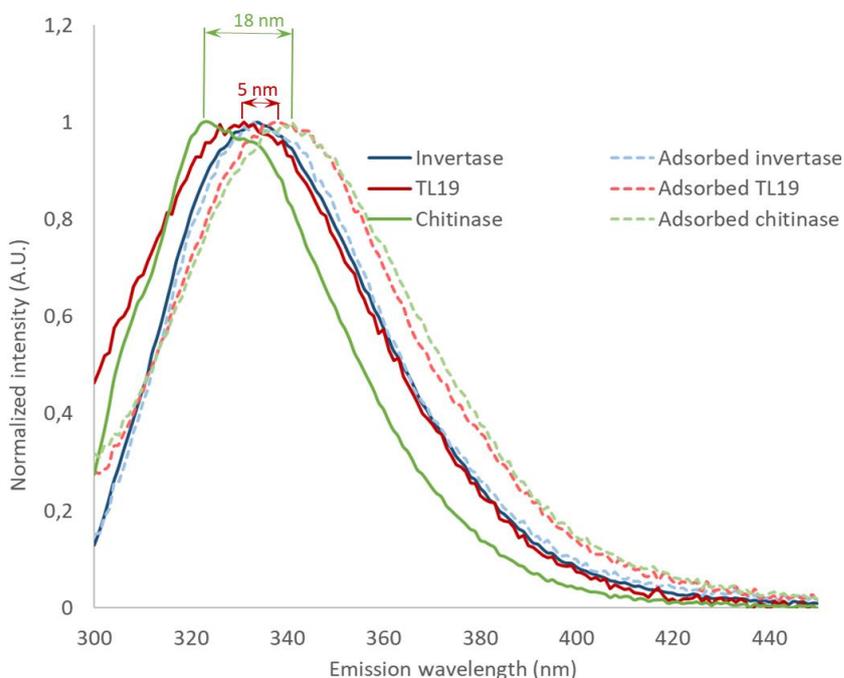
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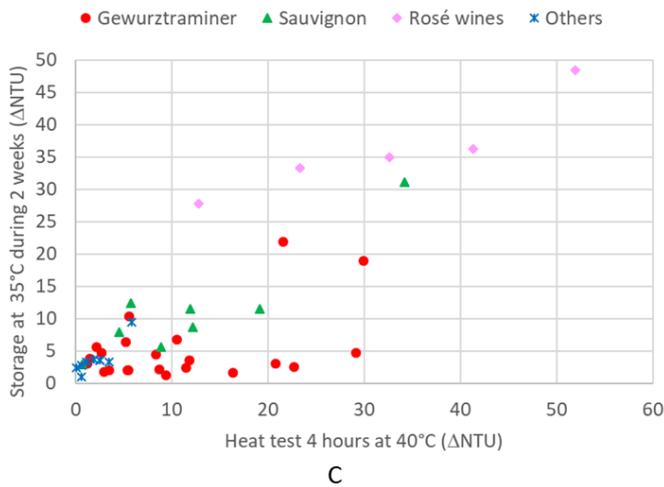
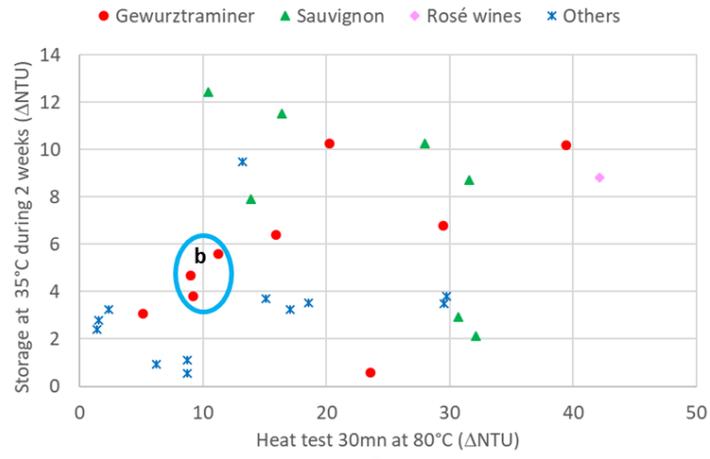
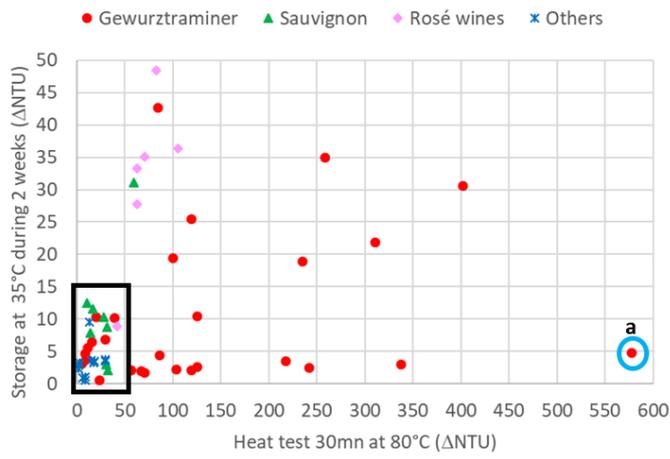
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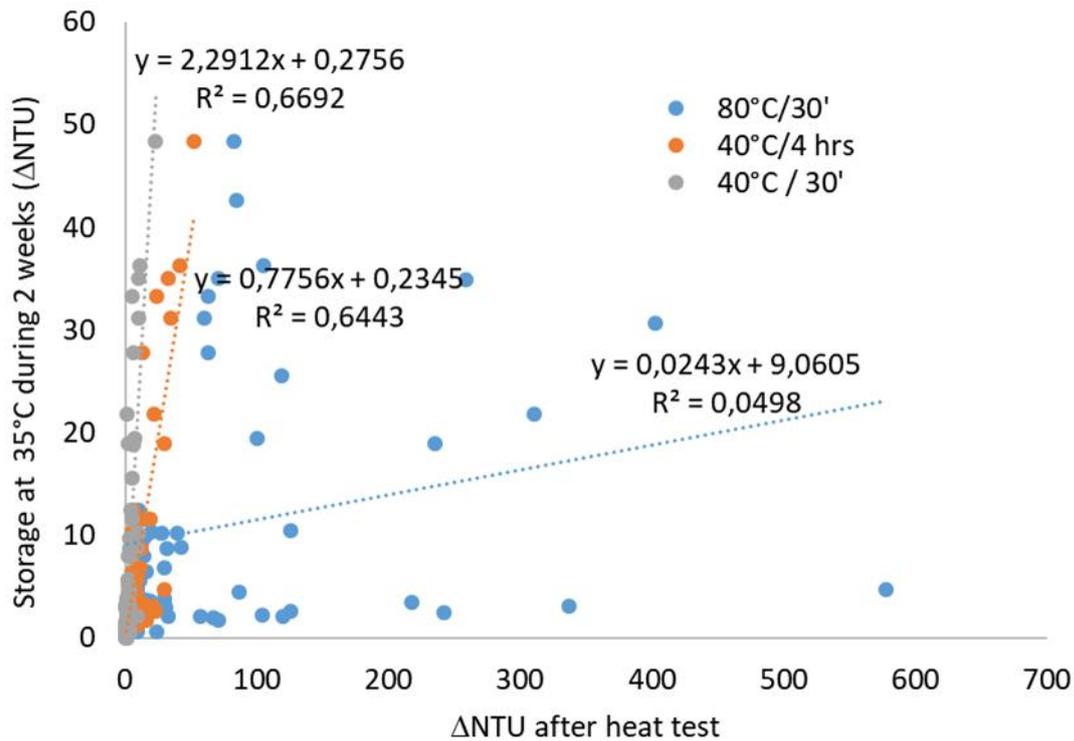
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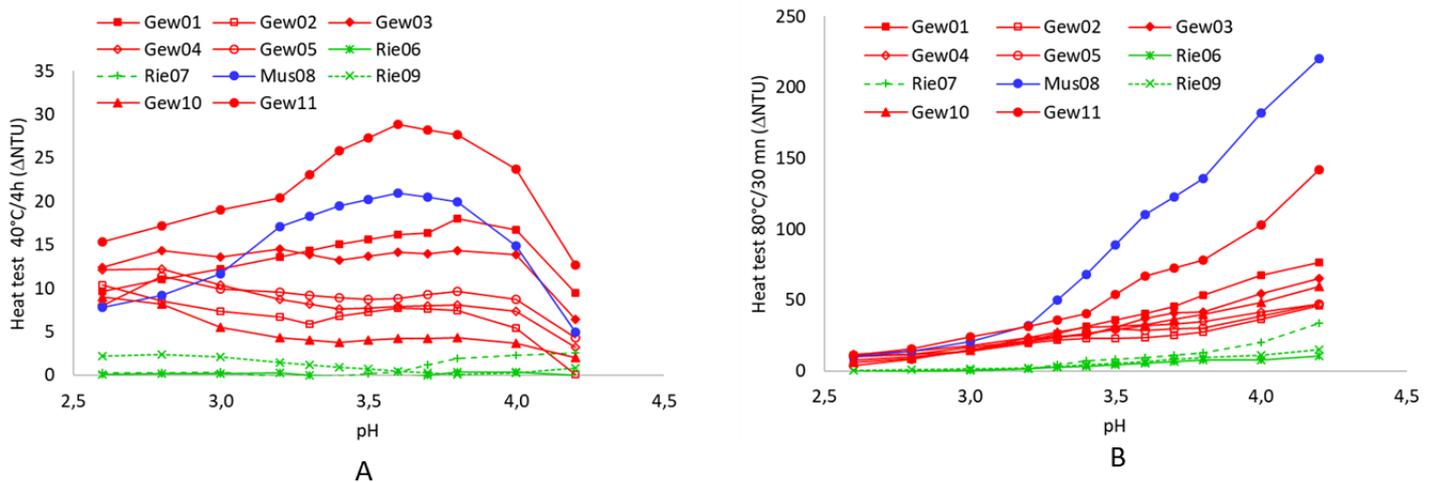
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514



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 <sub>2</sub> SO <sub>4</sub> · L <sup>-1</sup> )	Total acidity (g H <sub>2</sub> SO <sub>4</sub> · L <sup>-1</sup> )	Sugar (g · L <sup>-1</sup> )
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 · L<sup>-1</sup>

	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