Received: 30 November 2019 | Accepted: 2 March 2020 | Published: 27 March 2020
DOI:10.20870/oeno-one.2020.54.1.2952

Effect of the combined application of heat treatment and proteases on protein stability and volatile composition of Greek white wines

Piergiorgio Comuzzo[1], Sabrina Voce[1], Jacopo Fabris[1], Angelo Cavallaro[1], Gianmaria Zanella[2], Michael Karpusas[3] and Stamatina Kallithraka[4]*

[1]Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, via Sondrio 2/A, 33100 Udine, Italy
[2]Enologica Vason S.p.A., via Nassar 37, 37029 San Pietro in Cariano, Verona, Italy
[3]Department of Biotechnology, Physics Laboratory, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece
[4]Department of Food Science and Human Nutrition, Enology Laboratory, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece

*Corresponding author: stamatina@aua.gr

ABSTRACT

Aim: This work evaluates the effects of the combined use of heat treatment (HT, 75 °C, 2 min) and proteases (P) on the protein stability and volatile composition of two white wines, obtained from the Greek cv. Assyrtiko and Moschofilero.

Methods and results: Wine protein stabilization was assessed by heat test, using RP-HPLC determination of pathogenesis-related proteins (PRP) and by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The impact of bentonite and P + HT treatment on wine aroma profile was evaluated by GC-MS with liquid–liquid extraction. According to the heat test, in Assyrtiko wine the level of stability achieved with P + HT was comparable with that obtained by bentonite fining and for Moschofilero wine – where protein instability was higher – bentonite was more efficient. RP-HPLC profiles showed that, in general, higher percentages of chitinases (CH) than thaumatin-like proteins (TLP) were removed by both bentonite and P + HT, with a similar efficiency for the two treatments and sometimes better performances for the latter. Conversely, TLP were removed more efficiently by bentonite, even if some fractions were eliminated to a slightly higher extent by proteases. In the conditions of the experiment, bentonite resulted in minor changes to the wine aroma profile. However, heating during protease treatment modified wine volatile composition, reducing the concentration of esters produced during fermentation while simultaneously increasing the contents of certain esters characteristic of aging such as ethyl lactate.

Conclusions: The combination of proteases and heat treatment may be a promising technique for protein stabilization of wines. However, further investigations are needed to optimize the time:temperature ratio of the heat treatment in order to obtain the maximum protease activity and the minimum thermal deterioration of the wine quality.

Significance and impact of the study: The results of this study have a practical interest for both the scientific community and wine sector, contributing to knowledge of the efficacy and limitations of the use of protease enzymes for wine stabilization.

KEYWORDS

Assyrtiko, bentonite fining, Moschofilero, protease, protein stability, wine aroma

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/2952

INTRODUCTION

During storage of bottled white, rosé and sparkling wines, grape proteins that were initially stable can aggregate into light-dispersing particles, leading to the appearance of turbidity (Waters *et al.*, 1998). Although protein aggregation does not constitute a health risk, turbidity in wines is an important aesthetic issue and represents a quality defect (Bayly and Berg 1967; Hsu and Heatherbell, 1978). Therefore, prevention of this phenomenon, known as protein haze formation, is routine practice in commercial winemaking.

Protein haze formation is caused by the denaturation of proteins and in some cases can be accompanied by precipitate. However, protein instability does not correlate with total protein concentration because individual proteins behave differently (Bayly and Berg, 1967; Hsu and Heatherbell, 1978). In addition, the chemical composition of the wine (for example, metal ions, ionic strength, pH, alcohol content, polysaccharides and phenolic content) may also play an important role in protein haze formation as these parameters could affect protein denaturation (Waters *et al.*, 1995; Muhlack *et al.*, 2016; Toledo *et al.*, 2017).

The proteins most susceptible to denaturation are those with the lowest isoelectric points (4.1–5.8) and relatively low molecular mass (20 45 KDa) (Bayly and Berg, 1967; Hsu and Heatherbell, 1987; Esteruelas *et al.*, 2009). In more detail, some pathogenesis-related proteins (PRP), such as thaumatin-like proteins (TLP) and chitinases (CH), were identified as the cause of haze formation in wine. As both are resistant to proteolysis and low pH, they are able to remain in wine after fermentation (Waters *et al.*, 1991, 1998; Tabilo-Munizaga *et al.*, 2014). Their synthesis in healthy grapes appears to be triggered by veraison. However, stress, wounding and pathogenic attacks could further increase the content of these proteins (Monteiro *et al.*, 2003).

CH, possessing an elliptical secondary structure, are more sensitive to changes in temperature and pH, while TLP are characterized by thermostability, pH resistance and globular secondary structure (Falconer *et al.*, 2010). For this reason, the unfolding temperature of the two protein groups is slightly different, at approximately 55 °C for CH and 62 °C for TLP. In addition to temperature, they are also characterized by different unfolding properties after heating: upon cooling, CH remain unfolded and TLP refold (Falconer *et al.*, 2010). Due to the differences in their structure and physicochemical properties, these proteins are expected to behave differently in the wine matrix. Indeed, Marangon *et al.* reported that grape CH have a key role in wine haze formation (Marangon *et al.*, 2011).

As protein haze formation is a major problem, winemakers commonly treat wine with bentonite, which is a low cost and effective fining agent, in order to remove unstable proteins (Hsu and Heatherbell, 1987; Maragon *et al.*, 2009). Bentonite, which is negatively charged at wine pH (3.0–3.5), interacts electrostatically with positively charged proteins, inducing their flocculation (Hsu and Heatherbell, 1987). However, it has been demonstrated that this treatment has a negative effect on wine organoleptic properties, because bentonite is a non-selective agent, which may also remove various molecules involved as aroma and flavor compounds as well as proteins (Lubbers *et al.*, 1996). In addition, due to bentonite's significant swelling and poor settling properties, 3–10 % of the wine volume is lost and a significant amount of waste is formed (Marangon *et al.*, 2009; Tabilo-Munizaga *et al.*, 2014). For these reasons, there is significant focus on developing alternative economical practices to stabilize wine proteins without compromising wine quality. These practices include the use of other adsorbents (De Bruijn *et al.*, 2009), ultrafiltration (Hsu *et al.*, 1987; De Bruijn *et al.*, 2011), flash pasteurization (Pocock *et al.*, 2003), zirconia (Pashova *et al.*, 2004), high hydrostatic pressure (Tabilo-Munizaga *et al.*, 2014) and proteases (Marangon *et al.*, 2012; Benucci *et al.*, 2016).

Several authors have studied the effects of various proteases of microbial origin (Theron and Divol, 2014) such as those from *Aspergillus niger* (Bakalinsky and Boulton, 1985), *Saccharomyces cerevisae* (Younes *et al.*, 2011) and *Botrytis cinerea* (Cilindre *et al.*, 2007) on wine protein degradation. However, these attempts were not always successful due to the high resistance of wine proteins to proteolysis. Moreover, several studies evaluated the efficacy of either free or immobilized cysteine proteases of plant origin in decreasing wine hazing potential (Benucci *et al.*, 2011; Benucci *et al.*,

2014; Benucci *et al.*, 2016), indicating that these might be an alternative to bentonite treatment.

As unfolded proteins are more easily cleaved by enzymes, the next step was the evaluation of the combined effects of protease addition and heat treatment (Pocock *et al.*, 2003; Marangon *et al.*, 2012) on wine protein haze formation. According to Marangon *et al.* (2012) the results obtained were encouraging. They reported that the use of aspergillopepsins combined with flash pasteurization of the juice is a promising alternative to bentonite addition as the proteases were active toward wine proteins and heat treatment did not affect wine sensory properties.

It was thus of interest to apply the combination of protease addition and heat treatment (P+HT) on wines, in particular those from local grape cultivars, which are generally less studied and are often characterized by higher protein instability. The aim of this work was to compare the efficacy of P+HT with that of bentonite in decreasing the hazing potential of white wines, while preserving their aromatic quality. For this purpose, the presence of CH and TLP proteins in wines made by native Greek varieties were recorded before and after each treatment in relation to their aromatic profile.

MATERIALS AND METHODS

1. Reagents and materials

Ethanol (96 % v/v), HPLC grade acetonitrile, 99 % trifluoroacetic acid (TFA), 37 % hydrochloric acid, Tris(hydroxymethyl)aminomethane (Tris), sodium dodecylsulfate (SDS), glycerol, 2-mercaptoethanol, Coomassie Brilliant Blue R-250, glacial acetic acid, HPLC grade pentane and dichloromethane, ethyl heptanoate and sodium chloride were supplied by Sigma-Aldrich (St. Louis, MO, USA). The SDS-PAGE Broad Range Molecular Weight standard proteins were from Bio-Rad Laboratories (Richmond, CA, USA).

The two proteases used in the experiments (named protease A and B) were liquid preparations containing aspergillopepsin, both obtained from *Aspergillus niger* and supplied by Enologica Vason S.p.A. (S. Pietro in Cariano, VR, Italy). The bentonite used for fining was a commercial sodium bentonite (average particle size < 200 mesh – 74 µm) provided by American Colloid Company (Illinois, USA).

2. Wines

Unstable white wines from cv. Assyrtiko and Moschofilero, typical Greek varieties, were made in the experimental winery of the Laboratory of Oenology of the Agricultural University of Athens. The grapes were harvested at technological maturity (based on indices of sugar content and acidity), destemmed, crushed and pressed. Musts were inoculated with selected yeast strains (*Saccharomyces cerevisiae*) and fermented under controlled temperature (18–20 °C) up to complete sugar depletion. Following this, the wines were bottled and stored at 15 ± 2 °C until analyses. Several analytical enological parameters were determined in the wines according to the International Organization of Wine and Vine (2016) guidelines. In more detail, reduced sugar content (g/L), alcohol content (% v/v), titratable acidity (expressed as g tartaric acid/L), volatile acidity (expressed as g acetic acid/L), free and total SO_2 content (mg/L) and pH were measured. The reduced sugar content of both wines was below 2 g/L, indicating that fermentation was complete. The alcohol strength was 10.3 % v/v for Assyrtiko wine and 11.6 % v/v for Moschofilero. Titratable acidity values were 7.3 and 7.6 g/L and pH 2.91 and 3.11 for Assyrtiko and Moschofilero, respectively. Volatile acidity values were similar for both wines (0.35 g/L) and the total and free SO_2 contents were 52 and 13 mg/L for Assyrtiko and 105 and 27 mg/L for Moschofilero wines, respectively. All parameters measured were within the ranges commonly reported for white wines.

3. Bentonite fining and protease treatments

Bentonite fining was performed to determine the minimum amount of fining agent necessary for wine stabilization: bentonite was dosed at 50, 100, 150 and 200 mg/L in the form of aqueous suspension (4 % w/v), prepared according to the supplier's instructions. Samples were then stored at room temperature for 24 h and the sediment was separated, collecting the limpid phase for analyses.

Protease treatments were performed in two concentrations: 1 and 2 mL/L of the liquid products were added to the wine, then the samples were placed in glass test tubes and placed in a water bath at 75 ± 2 °C. When the temperature of the wine reached 75 °C, the tubes were kept in the water bath for 2 min and then

were cooled in an ice bath until room temperature was reached. Heat treatment was used to provoke protein unfolding, with the aim of facilitating their enzymatic degradation, as suggested by Marangon *et al.* (2012).

For both Assyrtiko and Moschofilero, the wines obtained after bentonite and protease fining were analyzed as reported below, in comparison with a control (unstable wine without fining). All samples were filtered on 0.45 µm pore size cellulose acetate (CA) membranes (Millipore, Merck KGaA, Darmstadt, Germany) before analysis.

4. Heat test

The heat test was carried out as reported by Vincenzi *et al.* (2011). Filtered samples were heated at 80 °C for 6 h and then cooled at 0/+4 °C for 16 h. After equilibration at room temperature, the difference in turbidity before and after heating (Δ_{TurbH}) was assessed by nephelometry. According to this test, wines are generally considered unstable if Δ_{TurbH} is higher than 2.0 NTU.

5. Protein determination by RP-HPLC

PRP were isolated from wine by ethanol precipitation according to the following procedure: 5 mL of wine was mixed with 20 mL of ethanol (96 % v/v). After precipitation, the mixture was centrifuged (4000 rpm for 10 min), the supernatant was eliminated and the pellet was re-dissolved in 1 mL of distilled water. Samples were filtered through a 0.22 µm pore size CA membrane (Millipore) and 100 µL of filtered solution was injected into the HPLC system.

Reverse phase HPLC was carried out as described by Marangon *et al.* (2009). The equipment used was an AS-1555 Intelligent Sampler, PU 2089 Plus Quaternary Gradient Pump, MD-910 Multiwavelength Detector and LC-Net II/ADC, from Jasco Corporation (Tokyo, Japan). Proteins were separated on a 5 µm packed 250 × 4.6 mm C8 Vydac 208TP column (Grace Discovery Sciences, Columbia, MD, USA) conditioned at 35 °C. Elution was performed in gradient mode, at a flow rate of 1 mL/min. The mobile phase comprised 0.1 % (v/v) TFA in 8 % (v/v) acetonitrile (solvent A) and 0.1 % TFA in 80 % (v/v) acetonitrile (solvent B). The column was initially equilibrated at 17 % solvent B, then solvent B linearly increased at 49 % in the first 7 min, subsequently reaching 57 % at 15 min, 65 % at 16 min and 81 % at 30 min. This solvent ratio (81 % B) was held for 5 min before re-equilibrating the column at the starting conditions (17 % B). Detection was carried out at 210 nm.

Qualitative analysis was performed by comparison of the retention times with those published by Peng *et al.* (1997), Van Sluyter *et al.* (2009) and Marangon *et al.* (2009). Peaks with a retention time between 8 and 12 min were assigned to TLP, while those eluted from 18 to 25 min were attributed to chitinases. For semi-quantitative analysis, the absolute areas of the detected peaks were used in data elaboration.

6. SDS-PAGE

Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was performed according to the Laemmli protocol (Laemmli, 1970), as described by Vincenzi *et al.* (2011).

The pellet obtained after ethanol precipitation was re-suspended in Tris-HCl buffer (pH 6.8) containing glycerol (15 % v/v), 2-mercapto-ethanol (3 % v/v) and SDS (1.5 % w/v) and heated for 5 min at 100 °C before loading on the gel slab. After separation, the gels were stained with Coomassie Brilliant Blue R-250 and destained with glacial acetic acid (7 % v/v).

7. Determination of wine volatile compounds

Volatile compounds were analyzed in the wine before and after fining, by liquid–liquid extraction and GC-MS, using the method published by Voce *et al.* (2019). Briefly, 5 mL of wine was mixed with 5 mL of 30 % (w/v) sodium chloride solution and 200 µL of internal standard (ethyl heptanoate, 0.5000 g/L in ethanol). The mixture was extracted three times with 5 mL of pentane:dichloromethane solution (2:1 v/v). The organic phase was collected, dried with anhydrous sodium sulfate, concentrated under a nitrogen stream to a final volume of 1 mL and stored at -18 °C until GC injection.

Compounds were separated on a 30 m × 0.25 mm i.d. DB-Wax capillary column, with a 0.25-µm thick film (Alltech, State College, PA, USA). The column temperature was programmed as follows: the initial temperature of 40 °C was held for 1 min, then ramped up at 4 °C/min to 24 °C, with a final holding time of 15 min. The injector and detector were set at 250 °C and

240 °C, respectively. The injection (1-µL volume) was performed in splitless mode with a splitless time of 60 s. Helium was the carrier gas, at a linear flow rate of 35 cm/s.

The equipment used was a Shimadzu GCMS-QP-2010 system (Shimadzu, Kyoto, Japan). The MS detector was set at 1.4 kV and electron impact mass spectra were recorded at an ionization voltage of 70 eV, within a range of 35–400 m/z. Volatile compounds were identified by comparison of their mass spectra with those reported in the mass spectrum libraries Wiley 6, NIST21 and NIST107 and/or by comparison of their mass spectra and retention times with those of standard compounds. Linear retention indices were also calculated, based on the retention times of *n*-alkanes and compared with those published in literature. Semi-quantitative results were expressed in internal standard equivalents, considering a response factor equal to 1.00.

8. Statistical analysis

Results were averages of at least three measurements, obtained from three repeated treatments. Standard deviations were also calculated and significant differences among samples were assessed at $p < 0.05$, by one-way ANOVA and the Tukey honest significant difference test (HSD test). The variances were homogeneous for Levene and Brown-Forsyte Tests. For the aroma analyses, the data were also subject to principal component analysis (PCA). All analyses were carried out using the Statistica for Windows software Version 8.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

1. Heat test results

Wine protein stability before and after the different fining treatments was assessed by heat test (Figure 1a,b). The untreated wines of the two varieties examined showed a different instability. Moschofilero was less stable and the increase of turbidity measured after the test (Δ_{TurbH}) was approximately 11 NTU, compared with the 4.7 NTU average found for untreated Assyrtiko. However, in Moschofilero, stabilization was achieved with 150 mg/L of bentonite, while in Assyrtiko a higher amount (200 mg/L) of the fining agent was required for reducing Δ_{TurbH} to below the limit of 2.0 NTU. This highlights a different reactivity of the protein fraction of the two wines toward bentonite.

In the conditions of the experiment, the combination of heating and proteases was able to significantly reduce the protein instability of untreated wines, giving interesting results, particularly in Assyrtiko (Figure 1a) where protease B (2 m/L) obtained results similar to those attained by adding the stabilizing dose of bentonite (200 mg/L). Conversely, in Moschofilero (Figure 1b), protease treatments were not sufficient to provide a complete protein stabilization and after protease fining the Δ_{TurbH} remained higher than 2.0 NTU despite achieving a significant reduction of instability.

Figure 1(c,d) shows the values of turbidity measured on the wines collected after the fining treatments, before the heat test. It is interesting to note that the combination of heating and protease treatment resulted in the appearance of a sediment in both the Assyrtiko and Moschofilero wines studied, highlighting a non-negligible effect of heating on protein coagulation. All samples were filtered through CA membranes (0.45 µm) after fining (i.e. after racking, for bentonite and after cooling, for the combination of protease and heat treatment). Despite filtration, however, the wines treated with proteases showed significantly higher turbidity with respect to the other samples (untreated and bentonite-clarified). This might be related to the effects of heating on protein denaturation, which may have provoked the formation of small particles that are able to cross the filter membrane. Presumably, these fine particles might be reasonably due to the aggregation of chitinases, which is reported to occur after unfolding at a temperature of 66 °C, provoking visual haze formation (Falconer *et al.*, 2010). To the authors' knowledge, the ability of such fine haze-forming particles to cross (or to clog) membrane filters had not been reported yet. The values of turbidity achieved in this experiment for protease-treated samples are generally low, but the trend shown in Figures 1c and 1d indicates that such particles might have a potential role in reducing wine filterability, increasing membrane fouling. For this reason, this aspect shall be further investigated for optimizing protease fining practices in winemaking.

2. RP-HPLC separation of pathogenesis-related proteins

RP-HPLC allowed the separation of TLP and chitinases in the wines analyzed. Concerning the

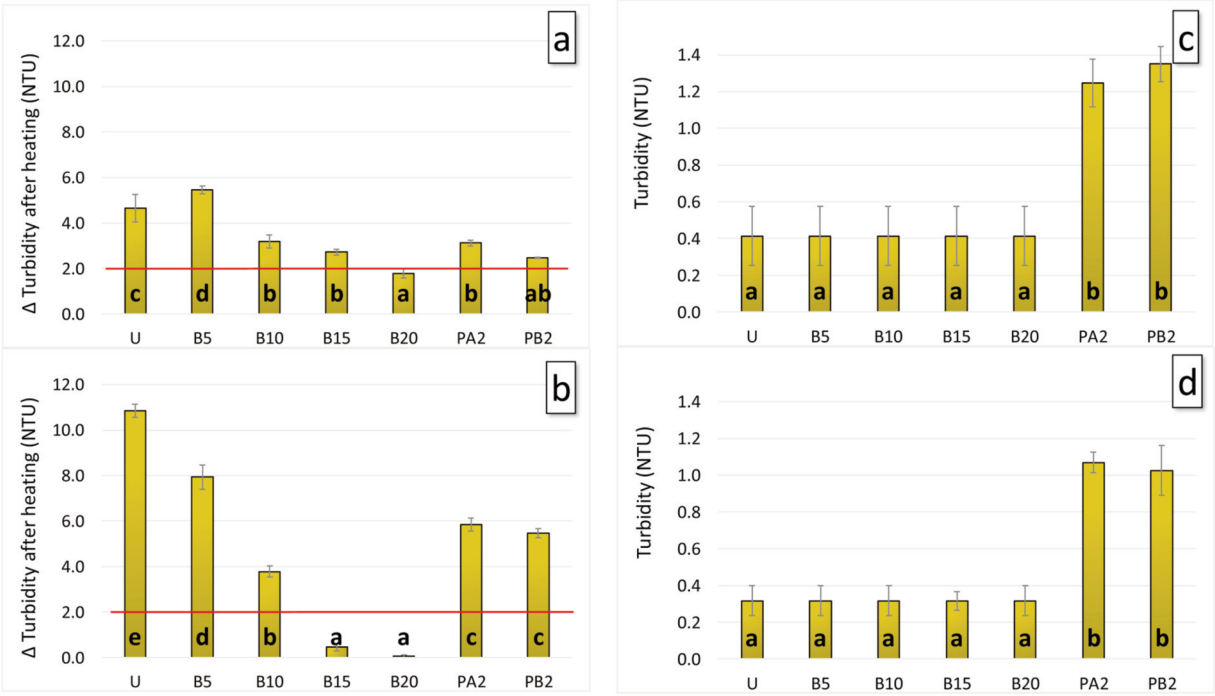

FIGURE 1. Turbidity measured after heat test in (a) Assyrtiko and (b) Moschofilero wines after bentonite and protease treatment.
The horizontal red line marks the stability threshold of 2.0 NTU. Turbidity of (c) Assyrtiko and (d) Moschofilero wines, after bentonite and protease treatment and before the heat test.
U: Untreated; B5, B10, B15, B20: bentonite, 50, 100, 150, 200 mg/L; PA2, PB2: heating + protease A and B, 2 mL/L
The letters a–e show the significant differences according to the ANOVA and Tukey HSD test at $p < 0.05$. The vertical bars represent standard deviation.

TABLE 1. PRP content (RP-HPLC absolute area / 1000) in Assyrtiko and Moschofilero untreated wines.

Wine typology	TLP1	TLP2	TLP3	Σ_TLP
Moschofilero	248	5995	244	6486
Assyrtico	945	2316	8986	12247
Wine typology	CH1	CH2	CH3	Σ_CH
Moschofilero	*n.d.*	*n.d.*	231	231
Assyrtico	1630	1689	4155	7474

former group, three main TLP fractions were quantified in untreated Assyrtiko and Moschofilero (Table 1). Surprisingly, despite the lower values of Δ_{TurbH} detected after heat test (Figure 1a,b), the total TLP content was quantitatively higher in Assyrtiko (Table 1). This might depend on the different wine chemical composition (such as acidity, pH, alcohol content) as all these parameters could affect protein denaturation and stabilization (Esteruelas *et al.*, 2009). It also possible that the differences in the protein profile of the two varieties could be the reason why Moschofilero proteins are more prone to form haze when heated. Regarding chitinases, three fractions were found in Assyrtiko, while just one (CH3) was detected in Moschofilero. For this reason, as for TLP, chitinases were also more abundant in Assyrtiko, in contrast with the greater turbidity developed by untreated Moschofilero after heat test (Figure 1 and b).

Bentonite and the combination of heating plus protease had a different effect on the protein fractions detected in the two wine typologies. In Assyrtiko (Figure 2a), bentonite showed a greater capacity for eliminating chitinases, which

were reduced to less than 9 % of their original value. TLP were reduced to a minor extent by bentonite fining and some fractions (TLP2) were poorly affected by the treatment. Heating plus the addition of proteases was less efficient than bentonite in removing TLP fractions 1 and 3, for which the residual proteins after treatment remained, on average, at 60–80 % of the original value. Contrary, regarding TLP2, proteases gave better performances, allowing a higher percent reduction. As with bentonite, the combination of proteases and heating was more efficient for chitinase removal, with an activity comparable with that of clay.

Bentonite and proteases also showed a greater capacity in removing chitinases in Moschofilero (Figure 2b). However, the CH3 fraction was less reduced by the treatments than in Assyrtiko and residual chitinases after fining in Moschofilero accounted for approximately 20 % of the original value (vs. the average of 1–9 % reported in Figure 2a). Concerning TLP, the behavior of Moschofilero was similar to that found for Assyrtiko: bentonite was more efficient than proteases in eliminating TLP1 and TLP2 fractions, but TLP3 was reduced to a greater extent by the enzymatic treatments.

However, despite certain effects and certain complementarity of action between proteases and bentonite highlighted by these results, none of the two enzyme preparations used (independently on the dosage) allowed the complete elimination of PRP fractions from wine in the experimental conditions tested. No relevant differences were found between the performances of the two preparations used (A or B), while an increase of the dosage generally reflected in a greater protein removal.

3. SDS-PAGE

The results of SDS-PAGE (Figure 3) confirm what was reported for RP-HPLC. For Moschofilero, a single band was detected in untreated wine (lane U) with a molecular weight around 22 kDa. This band may be ascribed to the presence to TLP fractions (Marangon *et al.*, 2012). Bentonite treatment (lane B15) provoked the disappearance of such a band, confirming the greater capacity of the fining agent in removing TLP fractions (Figure 2a,b). Heating plus protease treatments was also able to reduce the intensity of the band, but the increase of the amount of enzyme preparation added induced only minor results on TLP inactivation. Similar results were obtained for Assyrtiko (Supplementary Material – Figure A), even if in this case the detected bands remained well evident in the samples treated with proteases, confirming the results reported in Figure 2a (e.g. fractions TLP1 and TLP3).

Finally, lanes SA and SB represent the protein fractions detected in the sediments recovered after heating and protease treatment (PA and PB, respectively). It is interesting to observe that several bands were detected in these lanes, particularly in the molecular weight range from 21.5 to 45–60 kDa. Several bands for molecular weights between 21.5 and 6.5 kDa are present in these lanes, indicating that a part of the TLP might have been eliminated by protease treatment. The faint neighboring band, with a molecular weight close to 25 kDa, might be ascribed to chitinases, which was shown to be eliminated to a greater extent with respect to TLP, by the combined effect of heating and enzyme treatment (Figures 3 and 4) (Marangon *et al.*, 2012).

Neither the band of aspergillopepsin (AGP) nor the other protein fractions (e.g. invertase, β-glucanase) detected by Marangon *et al.* (2012) were found in the wines analyzed, in the analytical conditions used. The former (AGP), detected by the same authors at a molecular weight of 38–40 kDa, might be included (not visible) in the intense band at the top of the lanes SA and SB (38–60 kDa), confirming the observation that heat treatments may affect the stability of the enzyme in wine (Marangon *et al.*, 2012).

4. Volatile composition of wines

Thirty-six volatile compounds were tentatively identified in Assyrtiko and Moschofilero experimental wines (Table 2). The wine aroma composition only slightly changed in Assyrtiko, where a limited number of volatile compounds (seven out of 36) significantly changed after the treatments according to the ANOVA and Tukey HSD tests (Figure 4 a and b). In Moschofilero, a larger number of aromas were modified (16 out of 36), highlighting a heavier impact of fining on the aromatic profile of the wine (Figure 4c,d). This is probably the result of the higher total phenolic and flavanol content and higher antioxidant activity values of Assyrtiko wines, which might have protected the volatile

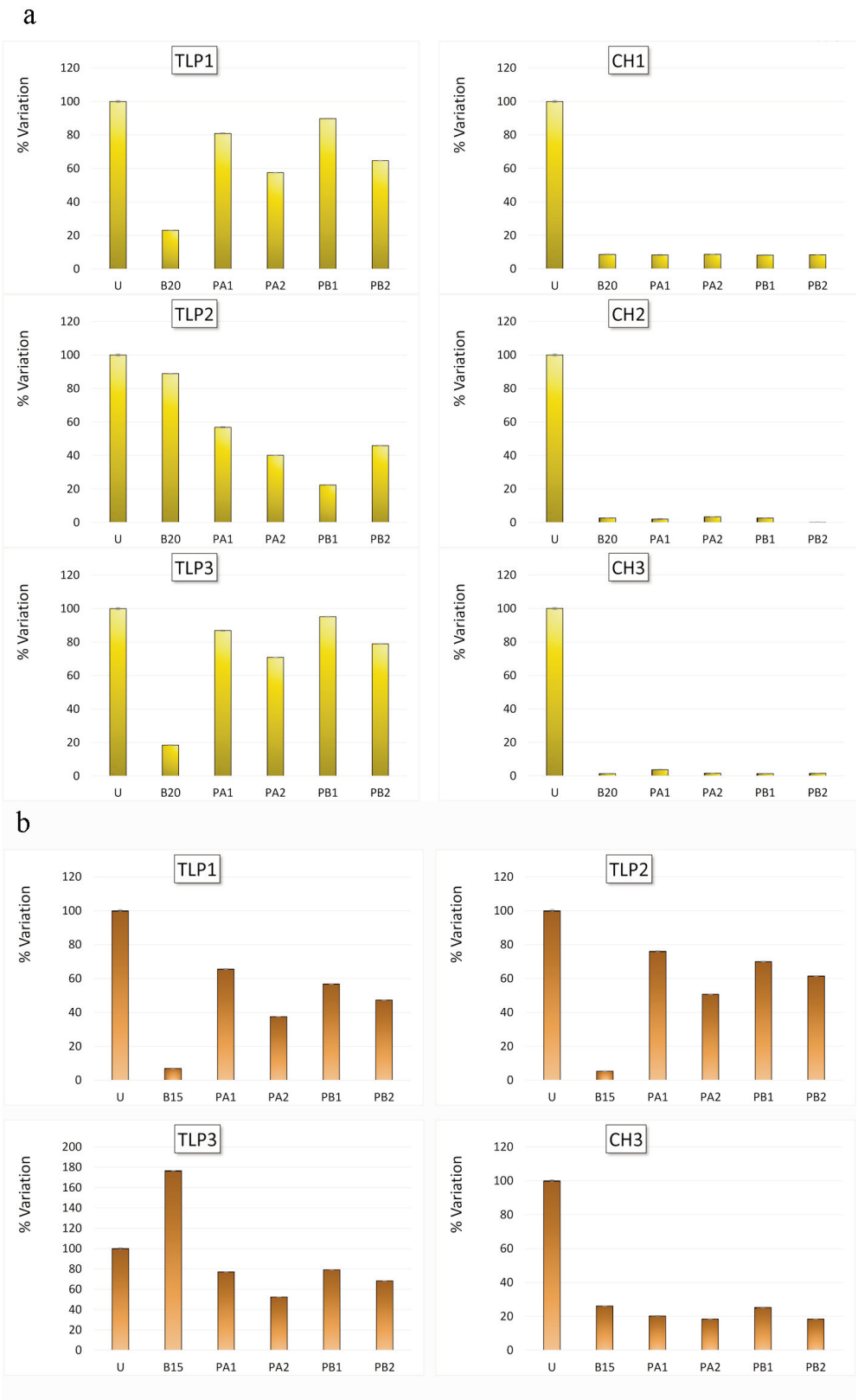

FIGURE 2. The percentage of thaumatin-like proteins (TLP) and chitinase (CH) fractions removed in (a) Assyrtiko and (b) Moschofolero wines after bentonite and protease treatment.
U: Untreated; B20: bentonite, 200 mg/L; PA1, PB1, PA2, PB2: heating + protease A and B, 1 and 2 mL/L.

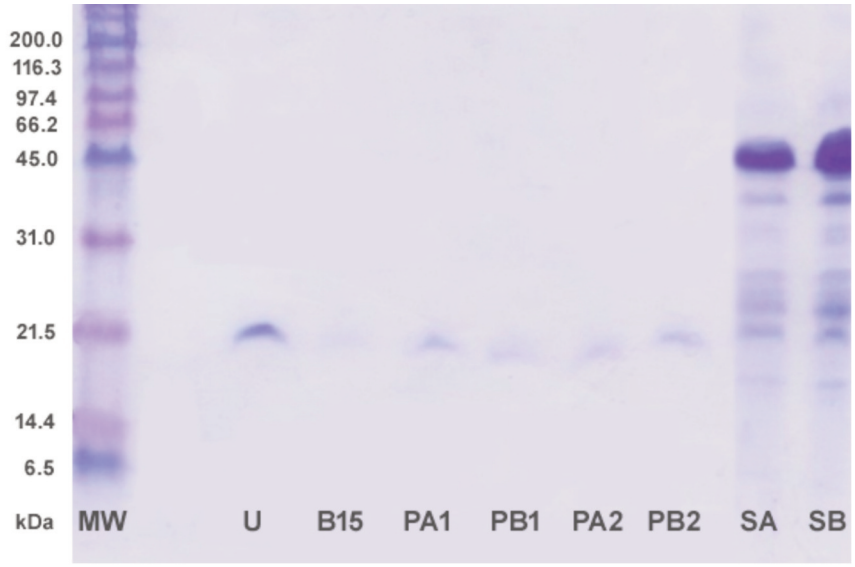

FIGURE 3. The results of SDS-PAGE separation of proteins in Moschofilero wines, before and after bentonite and protease treatment.
U: Untreated; B15: bentonite, 150 mg/L; PA1, PB1, PA2, PB2: heating + protease A and B, 1 and 2 mL/L; SA, SB: sediments collected in wine after heating + protease treatment; MW: molecular weight standard.

compounds from oxidative degradation (Salacha *et al.*, 2008).

Principal component analysis (PCA) was applied to the analytical data, which differed significantly in order to potentially categorize the wines according to the treatment. Figure 4a shows the projection of the volatile compounds of the Assyrtiko wines onto the first two principal components (PC). The first PC axis explains 71.8 % of the total variance and opposes isoamyl acetate to the rest of the volatile compounds (the aging esters – ethyl lactate, diethyl succinate and diethyl malate; the alcohols – 2-ethyl-1-hexanol and 1,2-propenediol; and the fermentation ester – hexyl acetate) while the second principle component explains 21.1 % of the total variance. The PCA made it possible to discriminate the Assyrtiko wines analyzed into two groups based on their treatment (Figure 4b); the wines treated with bentonite are situated on the right the diagram and the wines with P+H treatment are located on the left. The bentonite-treated wines were characterized by higher isoamyl acetate content and the P+H wines where characterized by higher values of esters that are characteristic of aging and higher alcohols.

Likewise, PCA was applied to the Moschofilero wines (Figure 4c,d). The first PC axis explains 53.4 % of the total variance while the second 14.2 %. The first axis opposes the fermentation esters (phenethyl acetate and ethyl-4-hydroxybutanoate), fatty acids (octanoic, 3-methylbutanoic and hexanoic acids) and alcohols (butanol, methionol) to the aging esters (ethyl lactate, diethyl succinate, ethyl hydrogen succinate, diethyl malate and diethyl tartrate), 2-3 butanediol and ethyl octanoate, Ethyl 3-hydroxybutanoate and 2-methyl-1-propanol are situated in the middle of the diagram (Figure 4c). The PCA makes it possible to discriminate Moschofilero wines into two groups based on their treatment (Figure 4d), as with Assyrtiko wines. The wines treated with bentonite are situated on the right the diagram and the wines with P+H treatment are located on the left

It is interesting to note that the grouping of the P+H treated wines is based mainly on their higher contents of esters that are characteristic of aging in comparison with the bentonite samples.

Overall, bentonite treatment had a minor impact on the volatile composition of the two wines, probably because of the, on average, small amounts of fining agent required for their stabilization (Figure 1a and b); in effect, bentonite-treated samples were found to have volatile profiles more similar to those of untreated wines (Figure 4b,d). Conversely, the combination of heating and proteases resulted in a greater modification of wine aroma compounds (probably connected to the effect of

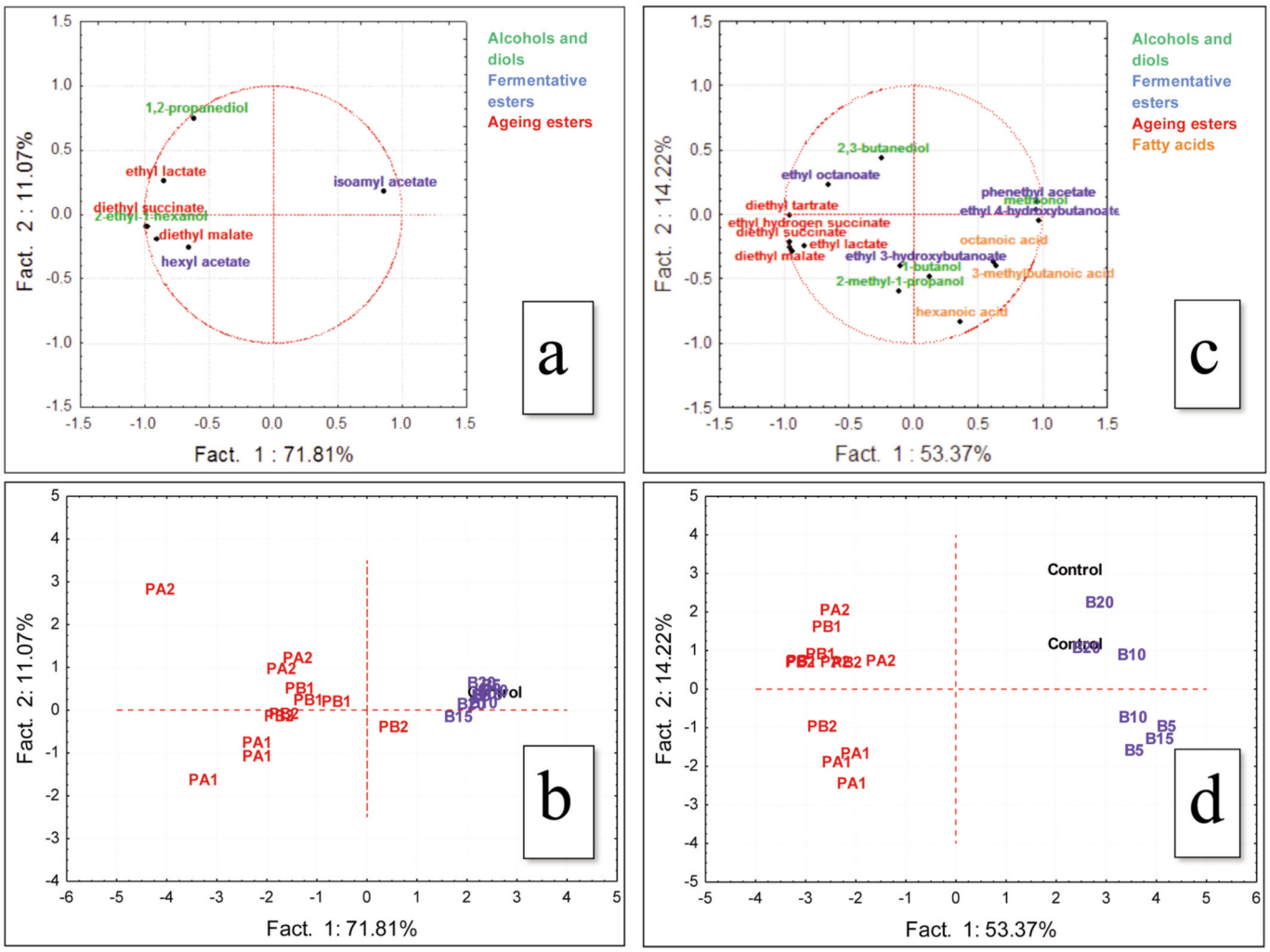

Figure 4. Results of PCA carried out on the concentrations detected for volatile compounds in (a,b) Assyrtiko and (c,d) Moschofilero wines (GC-MS analysis). The projection of variables (a and c) and cases (b and d) on the factor plane are reported.
Only the compounds that varied significantly according to ANOVA and Tukey HSD Test are considered. Control: untreated wine; B5, B10, B15, B20: bentonite, 50, 100, 150, 200 mg/L; PA1, PB1, PA2, PB2: heating + protease A and B, 1 and 2 mL/L.

heating), with a reduced concentration of certain fermentative esters and the increase of the amounts of certain esters typically found in aged wines, e.g. ethyl lactate, diethyl succinate and diethyl malate.

CONCLUSIONS

The current research investigated the behavior of two lesser-known varieties of Greek white wines with respect to their protein stabilization and low levels of instability were generally detected for both. Assyrtiko was characterized by higher amounts of TLP and chitinases, but developed a lower turbidity after heat test. Conversely, PRP were quantitively lower in Moschofilero but these seemed more prone to forming haze after heating. The combined use of heating and proteases appears to be a promising technique for wine protein stabilization; however, it resulted in a higher content of esters that are characteristic of aging.

The thermal instability of certain protein fractions (e.g. chitinases) suggests a non-negligible contribution of heating alone on protein inactivation. Further investigations are needed to elucidate the roles of protease enzymes and heating on the wine protein stabilization process. Furthermore, the optimization of the combined time–temperature parameters is necessary, for maximizing the

TABLE 2. Volatile compounds tentatively identified in Assyrtiko and Moschofilero wines.

	Compound	Ri	$Ri_{(lit)}$	Reference	Identification method
1	1-propanol	1048	1049	Tatsuka *et al.* (1990)	MS RI
2	2-methyl-1-propanol	1098	1094	Tatsuka *et al.* (1990)	MS RI S
3	3-methyl-1-butanol acetate	1129	1126	Tatsuka *et al.* (1990)	MS RI S
4	1-butanol	1152	1150	Tatsuka *et al.* (1990)	MS RI
5	2- and 3-methyl-1-butanol	1217	1211	Tatsuka *et al.* (1990)	MS RI S
6	ethyl hexanoate	1240	1241	Umano *et al.* (1994)	MS RI S
7	hexyl acetate	1273	1274	Tatsuka *et al.* (1990)	MS RI S
8	3-hydroxy-2-butanone (acetoin)	1281	1285	Umano *et al.* (1994)	MS RI S
9	ethyl lactate	1347	1340	Rezende and Fraga (2003)	MS RI S
10	1-hexanol	1363	1359	Tatsuka *et al.* (1990)	MS RI S
11	*trans*-3-hexen-1-ol	1371	1370	Tatsuka *et al.* (1990)	MS RI S
12	*cis*-3-hexen-1-ol	1389	1386	Umano *et al.* (1994)	MS RI S
13	ethyl octanoate	1441	1441	Umano *et al.* (1994)	MS RI S
14	acetic acid	1453	1452	Pozo-Bayón *et al.* (2007)	MS RI S
15	2-ethyl-1-hexanol	1491	1490	Comuzzo *et al.* (2017)	MS RI
16	ethyl 3-hydroxybutanoate	1520	1521	Pet'ka *et al.* (2001)	MS RI
17	2,3-butanediol	1548	1545	Gürbüz *et al.* (2006)	MS RI S
18	2-methylpropanoic acid	1574	1581	Botelho *et al.* (2007)	MS RI S
19	1,3-butanediol	1585	1600	Chida *et al.* (2004)	MS RI
20	1,2-propanediol	1595	1600	Pozo-Bayón *et al.* (2007)	MS RI S
21	γ-butyrolactone	1614	1611	Osorio *et al.* (2006)	MS RI S
22	butanoic acid	1633	1628	Rezende and Fraga (2003)	MS RI S
23	ethyl decanoate	1639	1643	Umano *et al.* (1994)	MS RI S
24	3-methylbutanoic acid	1677	1680	Botelho *et al.* (2007)	MS RI S
25	diethyl succinate	1680	1687	Gürbüz *et al.* (2006)	MS RI S
26	3-(methylthio)-1-propanol	1717	1715	Botelho *et al.* (2007)	MS RI S
27	ethyl 4-hydroxybutanoate	1801	1796	Wei *et al.* (2001)	MS RI
28	phenethyl acetate	1810	1822	Tatsuka *et al.* (1990)	MS RI S
29	hexanoic acid	1851	1842	Kumazawa *et al.* (2010)	MS RI S
30	2-phenylethanol	1902	1912	Comuzzo *et al.* (2017)	MS RI S
31	diethyl malate	2045	2039	Tian *et al.* (2007)	MS RI
32	octanoic acid	2070	2067	Pozo-Bayón *et al.* (2007)	MS RI S
33	ethyl hexadecanoate	2268	2229	Umano *et al.* (1994)	MS RI
34	decanoic acid	2287	2288	Shimoda *et al.* (2001)	MS RI S
35	diethyl tartrate	2331	2358	Rezende and Fraga (2003)	MS RI
36	ethyl hydrogen succinate	2389	2395	Wada and Shibamoto (1997)	MS RI

Ri: Retention index; $Ri_{(lit)}$: Retention index from bibliography. Identification method: S, comparison of mass spectra and retention time with those of standard compounds; RI, comparison of calculated retention index with those found in literature; MS, comparison of mass spectra with those reported in NIST 105s, NIST 21 and Wiley 6 MS libraries.

enzymatic activity while reducing the thermal effect on the modification of wine aroma.

REFERENCES

Bakalinsky A.T. and Boulton R., 1985. The study of an immobilized acid protease for the treatment of wine proteins. *American Journal of Enology and Viticulture*, *36*(1), 23–29.

Bayly F.C., Berg H.W., 1967. Grape and wine proteins of white wine varietals. *American Journal of Enology and Viticulture*, *18,* 18–32.

Benucci I., Esti M. and Liburdi K., 2014. Effect of free and immobilised stem bromelain on protein haze in white wine. *Australian Journal of Grape and Wine Research*, *20*(3), 347–352. doi:10.1111/ ajgw.12093

Benucci I., Liburdi K., Garzillo A.M.V and Esti M., 2011. Bromelain from pineapple stem in alcoholic-acidic buffers for wine application. *Food Chemistry*, *124*(4), 1349–1353. doi:10.1016/j.foodchem.2010.07.087

Benucci I., Lombardelli C., Liburdi K., Acciaro G., Zappino M. and Esti M., 2016. Immobilised native plant cysteine proteases: packed-bed reactor for white wine protein stabilisation. *Journal of Food Science and Technology*, *53*(2), 1130–1139. doi:10.1007/s13197-015-2125-4

Botelho G., Caldeira I., Mendes-Faia A. and Clímaco M.C., 2007. Evaluation of two quantative gas chromatography-olfactometry methods for clonal red wines differentiation. *Flavour and Fragrance Journal*, *22*(5), 414–420. doi:10.1002 /ffj.1815

Chida M., Sone Y. and Tamura H., 2004. Aroma characteristics of stored tobacco cut leaves analyzed by a high vacuum distillation and canister system. *Journal of Agricultural and Food Chemistry*, *52*(26), 7918–7924. doi:10.1021/jf049223p

Cilindre C., Castro A.J., Clément C., Jeandet P. and Marchal R., 2007. Influence of *Botrytis cinerea* infection on Champagne wine proteins (characterized by two-dimensional electrophoresis/immunodetection) and wine foaming properties. *Food Chemistry*, *103*(1), 139–149. doi:10.1016/j.foodchem.2006.07.043

Comuzzo P., Calligaris S., Iacumin L., Ginaldi F., Voce S. and Zironi R., 2017. Application of multi-pass high pressure homogenization under variable temperature regimes to induce autolysis of wine yeasts. *Food Chemistry*, *224*. doi:10 .1016/j.foodchem.2016.12.038

De Bruijn J., Loyola C., Flores A., Hevia F., Melin P. and Serra I., 2009. Protein stabilisation of Chardonnay wine using trisacryl and bentonite: a comparative study. *International Journal of Food Science and Technology*, *44*(2), 360–366. doi:10.1111/j.1365-2621.2008.01720.x

De Bruijn J., Martinez-Oyanedel J., Loyola C., Lobos F., Seiter J. and Perez R., 2011. Impact of white wine macromolecules on fractionation performance of ultrafiltration membranes. *Journal International Des Sciences de La Vigne et Du Vin*, *45*(3), 181–188. doi:10.20870/oeno-one.2011.45.3.1493

Esteruelas M., Poinsaut P., Sieczkowski N., Manteau S., Fort M.F., Canals J.M. and Zamora F., 2009. Comparison of methods for estimating protein stability in white wines. *American Journal of Enology and Viticulture*, *60*(3), 302–311.

Falconer R.J., Marangon M., Van Sluyter S.C., Neilson K.A., Chan C. and Waters E.J., 2010. Thermal stability of thaumatin-like protein, chitinase and invertse isolated from sauvignon blanc and semillon juice and their role in haze formation in wine. *Journal of Agricultural and Food Chemistry*, *58*(2), 975–980. doi:10.1021/jf902843b

Gürbüz O., Rouseff J.M. and Rouseff R.L., 2006. Comparison of aroma volatiles in commercial merlot and Cabernet-Sauvignon wines using gas chromatography-olfactometry and gas chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry*, *54*(11), 3990–3996. doi:10.1021/jf053278p

Hsu J.C., Heatherbell D.A., 1987) Isolation and characterization of soluble proteins in grapes, grape juice and wine. *American Journal of Enology and Viticulture*, *38,* 6–10.

Hsu J., Heatherbell D.A., Flores J.H. and Watson B.T., 1987. Heat-unstable proteins in grape juice and wine. II Characterization and removal by ultrafiltration. *American Journal of Enology and Viticulture*, *38*(1), 17–22.

Kumazawa K., Sakai N., Amma H., Sakamoto S., Kodama M., Wada Y. and Nishimura O., 2010. Identification and formation of volatile components responsible for the characteristic aroma of mat rush (Igusa). *Bioscience, Biotechnology and Biochemistry*, *74*(6), 1231–1236. doi:10.1271/bbb. 100053

Laemmli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685. doi:10.1038/ 27680a0

Lubbers S., Charpentier C. and Feuillat M., 1996. Study of the binding of aroma compounds by bentonite in must, wine and model systems. *Vitis, 35,* 59–62.

Marangon M., Van Sluyter S.C., Haynes P.A. and Waters E.J., 2009. Grape and wine proteins: Their fractionation by hydrophobic interaction chromatography and identification by chromatogra-phic and proteomic analysis. *Journal of Agricultural and Food Chemistry*, *57*(10), 4415–4425. doi:10.1021/jf900 0742

Marangon M., Van Sluyter S.C., Neilson K.A., Chan C., Haynes P.A., Waters E.J. and Falconer R.J., 2011. Roles of grape thaumatin-like protein and chitinase in white wine haze formation. *Journal of Agricultural and Food Chemistry*, *59*(2), 733–740. doi:10.1021/jf1038234

Marangon M., Van Sluyter S.C., Robinson E.M.C., Muhlack R.A., Holt H.E., Haynes P.A., *et al.,* 2012. Degradation of white wine haze proteins by Aspergillopepsin I and II during juice flash pasteurization. *Food Chemistry*, *135*(3), 1157–1165. doi:10.1016/j.foodchem.2012.05.042

Monteiro S., Barakat M., Piçarra-Pereira M.A., Teixeira A.R. and Ferreira R.B., 2003. Osmotin and Thaumatin from Grape: A Putative General Defense Mechanism Against Pathogenic Fungi. *Phytopathology*, *93*(12), 1505–1512. doi:10.1094/ PHYTO.2003.93.12.1505

Muhlack R.A., O'Neill B.K., Waters E.J. and Colby C.B., 2016. Optimal conditions for controling haze-forming wine protein with bentonite treatment: Investigation of matrix effects and interactions using a factorial design. *Food Bioprocess*, 9, 936-943. doi:10.1007/s11947-016-1682-5

Osorio C., Alarcon M., Moreno C., Bonilla A., Barrios J., Garzon C. and Duque C., 2006. Characterization of odor-active volatiles in champa (*Campomanesia lineatifolia* R. and P.). *Journal of Agricultural and Food Chemistry*, *54*(2), 509–516. doi:10.1021/jf052098c

Pashova V., Güell C. and López F., 2004. White Wine Continuous Protein Stabilization by Packed Column. *Journal of Agricultural and Food Chemistry*, *52*(6), 1558–1563. doi:10. 1021/jf03 4966g

Peng Z., Pocock K.F., Waters E.J., Francis I.L. and Williams P.J., 1997. Taste Properties of Grape (*Vitis vinifera*) Pathogenesis-Related Proteins Isolated from Wine. *Journal of Agricultural and Food Chemistry*, *45*(12), 4639–4643. doi:10.1021/jf970194a

Pet'ka J., Mocák J., Farkaš P., Balla B. and Kováč M., 2001. Classification of Slovak varietal white wines by volatile compounds. *Journal of the Science of Food and Agriculture*, *81*(15), 1533–1539. doi:10.1002/jsfa.979

Pocock K.F., Hoj P.B., Adams K.S., Kwiatkowski M.J. and Waters E., 2003. Combined heat and proteolytic enzyme treatment of white wines reduce haze forming protein content without detrimental effect. *Australian Journal of Grape and Wine Research*, *9*(1), 56–63. doi:10.1111 /j.1755-0238.2003.tb00232.x

Pozo-Bayón M.A., Ruíz-Rodríguez A., Pernin K. and Cayot N., 2007. Influence of eggs on the aroma composition of a sponge cake and on the aroma release in model studies on flavored sponge cakes. *Journal of Agricultural and Food Chemistry*, *55*(4), 1418–1426. doi:10.1021/jf062203y

Rezende C.M. and Fraga S.R.G., 2003. Chemical and aroma determination of the pulp and seeds of murici (*Byrsonima crassifolia* L.). *Journal of the Brazilian Chemical Society*, *14*(3), 425–428. doi:10. 1590/S0103-50532003000300014

Salacha M.I., Kallithraka S. and Tzourou E., 2008. Browning of white wines: correlation with antioxidant characteristics, total polyphenolic composition and flavanol content. *International Journal of Food Science and Technology,* 43, 1073-1077. doi:10.1111/j.1365-2621.2007.01567.x

Shimoda M., Yoshimura Y., Yoshimura T., Noda K. and Osajima Y., 2001. Volatile flavor compounds of sweetened condensed milk. *Journal of Food Science*, *66*(6), 804–807. doi:10.1111/j.1365-2621.2001. tb15176.x

Tabilo-Munizaga G., Gordon T.A., Villalobos-Carvajal R., Moreno-Osorio L., Salazar F.N., Pérez-Won M. and Acuña S., 2014. Effects of high hydrostatic pressure (HHP) on the protein structure and thermal stability of Sauvignon blanc wine. *Food Chemistry*, *155*, 214–220. doi:10.1016/ j.foodchem. 2014.01.051

Tatsuka K., Suekane S., Sakai Y. and Sumitani H., 1990. Volatile Constituents of Kiwi Fruit Flowers: Simultaneous Distillation and Extraction versus Headspace Sampling. *Journal of Agricultural and Food Chemistry*, *38*(12), 2176–2180. doi: 10.1021/ jf00102a015

Theron L.W. and Divol B., 2014. Microbial aspartic proteases: current and potential applications in industry. *Applied Microbiology and Biotechnology*, *98*(21), 8853–8868. doi:10.1007/s00253-014-6035-6

Tian Y., Zhang X., Huang T., Zou K. and Zhou J., 2007. Research advances on the essential oils from leaves of Eucalyptus. *Food and Fermentation Industries*, *33*(10), 143–147.

Toledo L.N., Salazar F.N. and Aquino A.J.A., 2017. A theoretical approach for understanding the haze phenomenon in bottled white wines at molecular level. *South African Journal of Enology and Viticulture*, *38*(1), 64–71. doi:10. 21548/38-1-837

Umano K., Hagi Y., Tamura T., Shoji A. and Shibamoto T., 1994. Identification of Volatile Compounds Isolated from Round Kumquat (*Fortunella japonica* Swingle). *Journal of Agricultural and Food Chemistry*, *42*(9), 1888–1890. doi:10.1021/jf00045a011

Van Sluyter S.C., Marangon M., Stranks S.D., Neilson K.A., Hayasaka Y., Haynes P.A. *et al.,* 2009. Two-step purification of pathogenesis-related proteins from grape juice and crystallization of thaumatin-like proteins. *Journal of Agricultural and Food Chemistry*, *57*(23), 11376–11380. doi:10.1021 /jf902365r

Vanrell G., Canals R., Estruelas M., Fort F., Canals J.M. and Zamora F., 2007. Influence of the use of bentonite as a riddling agent on foam quality and protein fraction of sparkling wines (cava). *Food Chemistry*, *104*, 148–155. doi:10.1016/j.foodchem. 2006.11.014

Vincenzi S., Marangon M., Tolin S. and Curioni A., 2011. Protein evolution during the early stages of white winemaking and its relations with wine stability. *Australian Journal of Grape and Wine Research*, *17*(1), 20–27. doi:10.1111/j.1755-0238. 2010.00113.x

Voce S., Škrab D., Vrhovsek U., Battistutta F., Comuzzo P. and Sivilotti P., 2019. Compositional characterization of commercial sparkling wines from cv. Ribolla Gialla produced in Friuli Venezia Giulia. *European Food Research and Technology*, *245*, 2279–2292. doi:10.1007/s00217-019-03334-9

Wada K. and Shibamoto T., 1997. Isolation and Identification of Volatile Compounds from a Wine Using Solid Phase Extraction, Gas Chromatography and Gas Chromatography/Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, *45*(11), 4362–4366. doi:10.1021/jf970157j

Waters E.J., Wallace W. and Williams P.J., 1991. Heat haze characteristics of fractionated wine proteins. *American Journal of Enology and Viticulture, 56*, 246–254.

Waters E.J., Hayasaka Y., Tattersal D.B., Adams K.S., Williams P.J., 1998. Sequence analysis of grape *(Vitis vinifera)* berry chitinases that cause haze formation in wines. *Journal of Agricultural and Food Chemistry*, *46*(12), 4950–4957. doi:10.1021/jf980421o

Waters E.J., Peng Z., Pocock K.F and Williams P.J., 1995. Proteins in white wine. I Procyanidin occurrence in soluble proteins and insoluble hazes and its relationship to protein instability. *Australian Journal of Grape Wine Research, 1*, 86–93. doi:10.1111/j.1755-0238.1995.tb00083.x

Wei A., Mura K. and Shibamoto T., 2001. Antioxidative activity of volatile chemicals extracted from beer. *Journal of Agricultural and Food Chemistry*, *49*(8), 4097–4101. doi:10. 1021/jf010325e

Younes B., Cilindre C., Villaume S., Parmentier M., Jeandet P. and Vasserot Y., 2011. Evidence for an extracellular acid proteolytic activity secreted by living cells of *Saccharomyces cerevisiae* PlR1: Impact on grape proteins. *Journal of Agricultural and Food Chemistry*, *59*(11), 6239–6246. doi: 10.1021/jf200348n