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

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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://oenone.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, Maragón et al. reported that grape CH have a key role in wine haze formation (Maragón 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; Maragón 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 (Maragón 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 (Maragón 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 cerevisiae (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).
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 SO₂ 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 SO₂ 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 (ΔTurbH) was assessed by nephelometry. According to this test, wines are generally considered unstable if ΔTurbH 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-mercaptoethanol (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 (ΔTurbH) 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 ΔTurbH 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 ΔTurbH 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
former group, three main TLP fractions were quantified in untreated Assyrtiko and Moschofilero (Table 1). Surprisingly, despite the lower values of ΔTurbH 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

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**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  |
| Assyrtiko     | 945    | 2316   | 8986   | 12247 |
| Wine typology | CH1    | CH2    | CH3    | Σ_CH  |
| Moschofilero  | n.d.   | n.d.   | 231    | 231   |
| Assyrtiko     | 1630   | 1689   | 4155   | 7474  |

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**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.
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 4a 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.
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 were 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...
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
enzymatic activity while reducing the thermal effect on the modification of wine aroma.

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**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-butaneone (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.
and invertse isolated from sauvignon blanc and

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