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Solid-Phase Extraction Followed by Gas Chromatography–Mass Spectrometry for Revealing the Effects of the Application of Bentonite, Tannins, and Their Combination during Fermentation in the Production of White Wine

Igor Lukić [1,2,*], Ivana Horvat [1], Sanja Radeka [1] and Urska Vrhovsek [3]

[1] Institute of Agriculture and Tourism, K. Huguesa 8, 52440 Poreč, Croatia; ihorvat@iptpo.hr (I.H.); sanja@iptpo.hr (S.R.)
[2] Centre of Excellence for Biodiversity and Molecular Plant Breeding, Svetošimunska 25, 10000 Zagreb, Croatia
[3] Metabolomics Unit, Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, 38098 San Michele all'Adige, TN, Italy; urska.vrhovsek@fmach.it
* Correspondence: igor@iptpo.hr; Tel.: +385-52408327

Abstract: To investigate the effects of the application of bentonite, tannins, and their combination in alcoholic fermentation, Malvazija istarska (*Vitis vinifera* L.) white grape must was treated with 95 g/L of bentonite, 25 g/L of a hydrolysable tannin preparation, while the third treatment received the aforementioned doses of both agents. Control grape must was fermented without bentonite and exogenous tannins. All of the produced wines were additionally fined after fermentation with doses of bentonite needed to achieve complete protein stability. Wines were analyzed both after fermentation and after additional bentonite fining. Standard physicochemical parameters were determined by the OIV methods, and phenols were analyzed by high-performance liquid chromatography with diode-array detection (HPLC-DAD), while the concentrations of free and bound volatile aroma compounds were obtained after solid-phase extraction (SPE) followed by gas chromatography–mass spectrometry (GC-MS). Bentonite and tannins in fermentation generally reduced the total dose of bentonite needed for complete stabilization. Treatments with bentonite slightly decreased the concentration of total dry extract, while tannins preserved total acidity. The negative effect of bentonite on flavonoids was more severe. Tannins in fermentation preserved more hydroxycinnamoyltartaric acids with respect to control wine, and this effect was additionally enhanced by bentonite. Volatile and bound aroma composition was affected by all the treatments, while the addition of tannins resulted in higher concentrations of several important odoriferous esters, such as ethyl hexanoate, ethyl decanoate, and hexyl acetate. Additional fining with bentonite to complete protein stabilization annulled some of the positive effects observed after fermentation.

Keywords: aroma; bentonite; fermentation; phenols; protein stabilization; tannins; wine

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1. Introduction

Intrinsic wine quality depends on the occurrence, quantities, and ratios of various chemical compounds that can produce a sensory response in humans after smelling and/or tasting. Besides major compounds, such as ethanol, organic acids, carbohydrates, and an array of other substances contained in wine dry extract, the major drivers of white wine quality are phenols and volatile aroma compounds. Wine phenols pertain to two major subclasses: flavonoids, which include anthocyanins, flavanols, flavonols, etc., and non-flavonoids, mostly represented by phenolic acids and stilbenes [1]. Flavonols and hydroxycinnamic acids participate in the formation of white wine color. Flavanol monomers, dimers, and oligomers are considered most responsible for wine bitterness, while flavanol polymers, also known as tannins, i.e., proanthocyanidins, followed by flavonols, have the most significant impact on astringency [1,2]. Besides being sensory active, phenols

are important as preservatives of wine oxidative stability and may also act as free radical scavengers in the human body [3]. Wine aroma compounds, numbering in the hundreds, originate from several sources. The so-called primary or varietal aromas are transferred into wine from grapes, and secondary or fermentation aromas originate from fermentation, while tertiary aroma compounds are formed during aging and maturation [4]. Wine aromas pertain to several chemical families, including terpenes, norisoprenoids, alcohols, volatile acids, esters, aldehydes, ketones, etc., that can be found in wine in wide concentration ranges from a few ng/L to several hundreds of mg/L [4]. The composition of both phenolic and volatile aroma compounds in wine is affected by many sources of variability, including cultivar, vineyard location with characteristic pedo-climatic conditions, year of harvest, grape processing and winemaking conditions, etc.

Protein stability is another important feature of white wines destined for the market [5]. The most efficient and low-cost oenological fining agent used for protein stabilization of white wines is bentonite, a clay mineral that swells in water and has a negative charge which is utilized to adsorb and remove turbidity-causing proteins [6–8]. Standard wine fining with bentonite before bottling can cause a loss of a significant portion of wine with bentonite sediment (up to 10%), as well as negatively influence wine quality because of non-selective adsorption of constituents other than proteins, mainly phenolic and volatile aroma compounds [8–11]. For these reasons, it would be of significant importance to reduce losses in both wine quantity and quality by optimizing bentonite fining procedures. Previous studies have shown positive effects of bentonite application during fermentation in contrast to standard bentonite fining after fermentation, including both the reduction in total bentonite dose and improvements in phenolic and volatile aroma composition [7,12–14]. Higher concentrations of important antioxidant hydroxycinnamoyltartrates and odoriferous esters obtained after such treatments were tentatively ascribed to the inhibition of oxidative and hydrolytic enzymes by their adsorption onto bentonite and subsequent removal by settling [7,14]. Despite such promising outcomes and in contrast to standard bentonite fining before bottling, the effects of the application of bentonite during fermentation in white winemaking were addressed only in a few studies, and the need for further investigation exists.

Oenological tannins are widely accepted as additives in modern winemaking. Tannins, in general, as well as those used in the form of commercial preparations, are divided into two main groups, condensed and hydrolysable tannins [15]. Condensed tannins originate mainly from grape skins and seeds and include oligomers and polymers composed of flavan-3-ol monomers. The units of hydrolysable tannins, gallotannin and ellagitannin, are composed of a central glucose molecule substituted by gallic (e.g., pentagalloyl glucose) and ellagic acid molecules, respectively. Gallotannins are mainly derived from exotic nuts and ellagitannins are from oak and chestnut wood [16]. Although primarily approved to facilitate clarification of musts and wines [17], tannins are today used for various other purposes, e.g., to stabilize color, control laccase activity, improve taste, eliminate reductive off-odors, and protect wine against oxidation [18–22]. Among other features, tannins are known for their interactions with proteins [23–25]. The main forces that drive hydrolysable tannin–protein interactions are considered to be essentially hydrophobic, while condensed tannins and proteins interact mostly by hydrogen bonds [26]. Although certain oenological tannin suppliers declare this feature as useful in wine protein stabilization, their effectiveness in white wine protein fining has been studied extremely rarely. Radeka et al. [27] applied different oenological tannins during pre-fermentation grape processing and achieved a reduction in total bentonite dose, as well as certain improvements in wine sensory quality. In our previous study, we observed that the application of oenological tannins in fermentation may result in a reduction in total bentonite dose, but the combined use of tannins and bentonite in fermentation did not show a synergistic effect in improving wine protein stability [28]. In general, the use and effects of oenological tannins on various white wine quality features, including phenols and volatile aroma content and composition, were studied much less compared to red wine [29].

The aim of this study was to investigate and compare the effects of the application of bentonite, tannins, and their combination during fermentation in order to improve the related knowledge and to determine whether their protective effects can act in synergy and result in further improvements in white wine composition. Standard physicochemical parameters were determined by the official OIV methods, while phenols were analyzed by high-performance liquid chromatography with diode-array detection (HPLC-DAD). The method of choice for the analysis of volatile aroma compounds was gas chromatography with mass spectrometric detection (GC-MS) because it enables successful separation of a large number of compounds and high sensitivity in their detection and identification.

2. Materials and Methods

2.1. Winemaking and Treatments

White grapes of Malvazija istarska (*Vitis vinifera* L.), the most important Croatian native white grape variety, were cultivated in the experimental vineyard of the Institute of Agriculture and Tourism in Poreč (Istria, Croatia). Grapes were harvested manually on 13 September 2016. Standard processing procedures were performed on the day of harvest with the addition of the antioxidative agent Aromax (20 g/100 kg of grapes; AEB S.p.A., Brescia, Italy). A closed-type pneumatic press of 500 L capacity (Letina Inox d.o.o., Čakovec, Croatia) was used for pressing. The juice was clarified using Endozym Rapid pectolytic enzymes (2 g/hL; AEB S.p.A.) for 36 h at 12 °C. Total acidity was adjusted with the addition of tartaric acid. The homogenized clear juice was evenly distributed into 12 stainless 80-L steel tanks and inoculated with *Saccharomyces cerevisiae* Lalvin QA 23 (30 g/hL; Lallemand Inc., Montreal, Canada), rehydrated with Go-Ferm Protect Evolution (30 g/hL; Lallemand Inc.). On the 6th and 13th days of fermentation, Fermaid E yeast supplement (20 g/hL; Lallemand Inc.) was added. Fermentations were carried out at 15 °C until the initial sugar concentration (250 g/L) dropped below 4 g/L, which lasted 21–23 days.

Four different treatments were applied in triplicates: 95 g/hL of bentonite was added near the end of fermentation when reducing sugars were at 45–55 g/L (treatment B), and 25 g/hL of tannins were added during fermentation in three portions—10 g/hL on the 1st day of fermentation, 5 g/hL in the second third of fermentation (reducing sugars at 130–160 g/L), and 10 g/hL near the end of fermentation (reducing sugars at 35–55 g/L) (treatment T); a combination of the first two treatments in which bentonite was added as in B and tannin was added as in T treatment (treatment BT), while control treatment (CO) received neither bentonite nor tannin. Bentonite was added in two stages—during (B and BT) and again after fermentation until total protein stability (all treatments). The dose applied in fermentation (95 g/hL) was chosen based on practical experience with Malvazija istarska wine and our previous study [14], with the aim not to achieve total protein stability during fermentation so that treatments could be compared later. Granular-activated sodium bentonite, montmorillonite-based (CX Bentonite Special Grain, Corimpex Service SRL, Villesse GO, Italy), was used for protein stabilization. Suspension of bentonite with tap water (70 g/L) was prepared 24 h before application. The temperature in fermentation was set to 16 °C 24 h before the addition of bentonite and lowered again to 15 °C 24 h afterwards. The oenological tannin preparation used was based on hydrolysable tannins and declared as pure gallic tannin (Tannino Etere, Enologica Vason S.p.A., Verona, Italy). The produced wines were the same as utilized in our previous study [28].

2.2. Post-Fermentation Procedures

After fermentation wines were racked and transferred into 50-L stainless steel tanks, left to spontaneously settle for two months, racked again, and then left in 34-L glass demijons. During the second racking, samples were collected for analyses (sampling after fermentation, AFerm). After that, additional doses of bentonite required to achieve complete protein stability, as determined by the standard heat stability test [7,12], were applied. After an additional two-week period of settling, samples were collected for

analyses (sampling of protein-stable wines, ProStab). The level of free SO_2 was corrected to 25–30 mg/L with potassium metabisulphite at all key stages when needed.

2.3. Protein Stability Tests

Protein stability was determined as described in our earlier study [14,28] using the standard heating test and heating test with the addition of tannins. Doses of bentonite required for protein stability were determined in our previous study [28] and are reported in Table S1.

2.4. Standard Physicochemical Analyses

Standard physicochemical analyses of wines were performed by standard methods published by the International Organization of Vine and Wine (OIV). The following parameters were analyzed: relative density, alcoholic strength by volume, total dry extract, reducing sugars, total acidity (as g/L of tartaric acid), volatile acidity (as g/L of acetic acid), and pH.

2.5. Analysis of Phenolic Compounds by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD)

Phenolic compounds were analyzed by high-performance liquid chromatography (HPLC) on an Agilent Infinity 1260 instrument with a quaternary pump, an autosampler, a column thermostat compartment, and a diode-array detector (Agilent Technologies, Palo Alto, CA, USA). The Agilent OpenLAB CDS ChemStation Edition, version 01.07.027 (Agilent Technologies) was used for identification and quantification. A method from Pati et al. [30] was modified and applied as described in Lukić et al. [31]. After filtration using 0.45 µm PTFE filters, samples were injected onto a Poroshell 120 EC-C18 column (150 mm × 4.6 mm, particle size 2.7 µm) with a guard (5 mm × 4.6 mm, particle size 2.7 µm) (Agilent Technologies). The injection volume was 10 µL. The temperature of the column was maintained at 26 °C. Gradient elution was applied with eluents A (water:formic acid, 99:1, *v*/*v*) and B (acetonitrile). Water (Honeywell, Charlotte, NC, USA) and acetonitrile (J. T. Baker, Fischer Scientific, Göteborg, Sweden) were of chromatographic purity, while formic acid was of analytical purity (VWR Chemicals, Radnor, PA, USA). Chromatograms were recorded at 280 nm and 330 nm, while spectra were recorded in the wavelength range from 200 to 600 nm. For the identification of phenolic compounds, their retention times and UV/Vis spectra were compared with those of the authentic standards. Quantification was performed using calibration curves obtained by analysis of standard solutions. For preparation of standard solutions, the standards were dissolved in synthetic wine having 12 vol.% ethanol and 5 g/L tartaric acid at pH 3.2. Qualitative standards of *trans*-coutaric and *trans*-fertaric acid were used and their *cis*-isomers were obtained by UV illumination of *trans*-isomers in methanol solution for 4 h. In cases where only qualitative standards were available, semi-quantification was performed, and concentrations of these compounds were expressed as *trans*-caftaric acid equivalents, assuming a relative response factor = 1.

Determination of total flavonoids was carried out according to Di Stefano et al. [32]. The wine sample was diluted 10 to 20 times with a mixture of ethanol:water:hydrochloric acid (37%) (70:30:1, *v*/*v*/*v*), and absorbance was read in the wavelength range from 230 to 700 nm using a Varian Cary 50 UV/Vis spectrophotometer (Varian Inc., Harbour City, CA, USA).

Determination of total phenols was carried out according to Singleton and Rossi [33]. In a 25 mL flask, 1.25 mL of Folin–Ciocalteu reagent and 15 mL of water were added to an aliquot of a sample (0.25 mL) and after 30 s, 5 mL of Na_2CO_3 and deionized water were added to the mark. After another 30 min, the absorbance was read at 765 nm using the Varian Cary 50 spectrophotometer (Varian Inc.). The concentration was determined using a calibration curve of gallic acid as a standard and expressed in mg/L of gallic acid equivalents.

2.6. Isolation of Free and Bound Volatile Aroma Compounds by Solid-Phase Extraction (SPE) and Analysis by Gas Chromatography–Mass Spectrometry (GC-MS)

Free and bound volatile aroma compounds were isolated from the wines by a protocol reported by Vrhovsek et al. [34] with slight modifications. Isolute ENV+ solid-phase extraction (SPE) cartridges (1 g sorbent, 6 mL; Biotage, Uppsala, Sweden) were activated with methanol and flushed with Milli-Q water, after which 100 mL of doubly diluted wine spiked with 100 µL of 1-heptanol (230 mg/L in ethanol) as internal standard were loaded. The sorbent was washed with Milli-Q water. Free volatiles were eluted from the cartridges by dichloromethane (30 mL), collected in a flask, and pentane (60 mL) was added followed by the addition of anhydrous sodium sulphate to eliminate water. The whole fraction was concentrated up to 200 µL using a Vigreux column prior to analysis. Glycosydically bound volatile compounds were eluted from the cartridges with methanol (30 mL), and eluate was evaporated to dryness using a rotary vacuum evaporator. The flask was then rinsed with pentane:dichloromethane (2:1, v/v) with a total volume of 10 mL. To recover the bound fraction, an aliquot of 4 mL of citrate buffer (pH 5) was added, followed by the addition of 200 µL of AR2000 enzyme (70 mg/mL). The solution with bound volatiles was incubated at 40 °C in a water bath for 24 h. The internal standard was added (25 µL) and released compounds were extracted with three 2 mL portions of pentane:dichloromethane (2:1, v/v). The extracts were united, anhydrous sodium sulphate was added to eliminate water, and after that, the solution was brought up to 200 µL using a Vigreux column before analysis.

GC-MS measurements were performed on a Trace GC Ultra gas chromatograph equipped with a PAL combi-xt autosampler (CTC, Zwingen, Switzerland) and connected to a TSQ Quantum XLS mass spectrometer (Thermo Scientific, Milan, Italy). The capillary column used was a VF-WAXms (polyethylene glycol; 30 m × 0.25 mm i.d. × 0.25 µm d.f.) from Agilent Technologies (Santa Clara, CA, USA). Injection (1 µL) was carried out in splitless mode at a temperature of 250 °C at the column inlet. Helium was used as a carrier gas at 1.2 mL/min. The GC oven was initially held at 50 °C for 1 min and then the temperature was increased at 2.5 °C/min to 250 °C with a final hold of 10 min. Positive electron ionization mode at 70 eV was applied, and mass spectra were obtained in full scan mode (40 to 350 m/z). The transfer line and source temperatures were set at 250 °C. Representative GC-MS chromatograms of the SPE extracts of free and bound volatile compounds are shown in Figure S1. Thermo XCALIBUR™ 2.2 software was used to process GC-MS data. Volatile compounds were identified by several methods (depending on the compound), including a comparison of retention times, linear retention indexes, and mass spectra to those of authentic standards and the NIST MS Search 2.0 library. Calibration curves were constructed and used to quantify a number of volatile compounds, while others were semi-quantified and their concentrations were expressed as equivalents of internal standard 1-heptanol, assuming a response factor = 1. Additional identification and quantification details are reported in Tables S2 and S3, while validation data for wine were reported in a previous paper [35].

Quantitative determination of the content of acetaldehyde, methanol, ethyl acetate, and major higher alcohols was performed using a Varian 3350 GC (Varian Inc.) with an Rtx-WAX capillary column (polyethylene glycol; 60 m × 0.25 mm i.d. × 0.25 µm d.f., Restek, Bellefonte, PA, USA). Fifty milliliters of wine were neutralized by titration with 0.1 N NaOH and distilled using an electronic distillation unit. The distillate was collected in a 100 mL flask. Before analysis, 0.25 mL of internal standard solution (1-pentanol, 31.74 mg/L) was added. Two microliters were injected in split mode (ratio 1:20). Helium was used as a carrier gas, and the pressure at the column inlet was set at 138 kPa. The temperatures of the injector and detector were 160 °C and 240 °C, respectively. The GC oven was initially held at 40 °C for 4 min, then the temperature was increased to 90 °C at 5 °C/min and then from 90 °C to 235 °C at 15 °C/min, with a final hold of 10 min. Volatile compounds were identified by comparison of their retention times with those of pure standards. Calibration curves were constructed based on the analysis of standard solutions, and the internal standard method was used for calculation.

2.7. Data Elaboration

Statistica version 13.2 software (StatSoft Inc., Tulsa, OK, USA) was used to perform statistical data elaboration by one-way analysis of variance (ANOVA) and Fischer's least significant differences (LSD) test at $p < 0.05$ to compare the mean values (n = 3). The obtained physicochemical, HPLC-DAD, GC-FID, and GC-MS analysis data were further processed by a supervised multivariate statistical analysis technique, partial least squares-discriminant analysis (PLS-DA) using MetaboAnalyst v. 5.0 software (http://www.metaboanalyst.ca, accessed on 16 July 2023). By minimizing the variance within and maximizing the variance between different categories (e.g., treatments), PLS-DA can give information about variables (parameters, compounds) with the greatest ability to differentiate investigated treatments in the form of variable importance in projection (VIP) scores. To better evaluate the effects of bentonite and tannins added during fermentation separately, PLS-DA was applied to four sets of data. Variables included in the first two sets were the analysis data obtained after fermentation (AFerm) with nine unidentified phenolic/tannin compounds (T1-T9), total flavonoids and total phenols excluded. Variables included in the third and fourth sets comprised analogous data obtained after additional bentonite fining (ProStab). In the first and third sets, the samples were divided into two groups, one containing wines fermented with the addition of bentonite (B and BT) and the other including other wines (CO and T), regardless of the presence of exogenous tannins. In the second and fourth sets, the samples were again divided into two groups, one containing wines fermented with the addition of tannins (T and BT) and the other including other wines (CO and B), regardless of the presence of bentonite.

3. Results and Discussion

3.1. Standard Physicochemical Parameters

After fermentation (AFerm), T treatment wine had higher relative density compared to B treatment wine (Table 1). The concentration of total extract without reducing sugars had the lowest value in B treatment wine, and it was significantly different from that found in T and CO wines. This was likely a result of non-selective activity of bentonite and adsorption of macromolecules other than proteins, such as high MW phenols and other positively charged species, as reported earlier [6,36,37]. On the other hand, non-fined CO treatment wine certainly retained a higher proportion of solids and macromolecules. The total dry extract was not affected by T and BT treatments. It was assumed that in BT treatment the added tannins compensated for a part of dry matter removed by bentonite. The negative effect of bentonite application on the concentration of total dry extract in wine was previously observed by Salazar et al. [38], while Lambri et al. [39] did not record a significant decrease.

Total acidity was reduced in B and T compared to CO wine, while T and BT treatment wines had the lowest pH values after fermentation (AFerm) (Table 1). The negative effect of bentonite application on wine total acidity was previously noted by several authors [4,7,40], while Salazar et al. [41] and Vela et al. [42] did not record significant effects. It is probable that bentonite adsorbed a portion of particular organic acids, as shown previously by Wu et al. [43]. It is also possible that bentonite adsorbed and removed a proportion of other chemical compounds that can act as protective colloids and provide better wine tartrate stability, such as particular proteins, glycoproteins, phenols, and polysaccharides [44,45], and thus enhanced the loss of tartaric acid by precipitation of its potassium salts [4].

After additional fining (ProStab), further effects were noted. Alcoholic strength by volume, the concentration of total extract without reducing sugars, and total acidity were reduced in wines of most treatments, so that particular significant differences observed after fermentation were not preserved, while new ones appeared (Table 1). A decrease in alcoholic strength by volume was probably caused by its partial evaporation during racking. In stable wines (ProStab), a lower concentration of total dry extract without reducing sugars was found in relation to the concentration found in the same wines after fermentation (AFerm), as a result of both spontaneous sedimentation and adsorption of various molecules

by additional bentonite doses. In several cases, the effect of additional stabilization (ProStab) was relatively more pronounced in CO treatment wine which contained a higher dry extract concentration before this procedure, eliminating the significant differences between the concentrations found in stable wines (Table 1). After additional fining (ProStab), a decrease in concentration of total acidity was observed for the majority of investigated wines (Table 1) as a consequence of precipitation of its salts [4], although additional partial adsorption of acids onto bentonite was also probable. Higher concentrations were found in wines of T and BT treatments. It was hypothesized that tannins acted as protective colloids [44] and reduced the degree of precipitation of tartaric acid salts. After additional fining (ProStab), the changes in pH were such that there were no longer significant differences among the B, T, and BT treatment wines with respect to this parameter.

Table 1. Results of standard physicochemical analyses of Malvazija istarska wines produced by different bentonite and tannins addition treatments, determined after fermentation and after final wine protein stabilization.

Code	Parameter	Stage	Treatment			
			CO	B	T	BT
SP-1	Relative density	AFerm	0.9895 ± 0.0002 ab	0.9893 ± 0.0002 b	0.9898 ± 0.0003 a	0.9895 ± 0.0001 ab
		ProStab	0.9894 ± 0.0002 b	0.9895 ± 0.0002 ab	0.9898 ± 0.0002 a	0.9896 ± 0.0002 ab
SP-2	Alcoholic strength (% vol.)	AFerm	15.04 ± 0.08 A	15.09 ± 0.07 A	14.98 ± 0.13 A	14.94 ± 0.04 A
		ProStab	14.69 ± 0.09 B	14.66 ± 0.06 B	14.61 ± 0.03 B	14.67 ± 0.04 B
SP-3	Total extract (g/L)	AFerm	22.6 ± 0.4 bA	22.4 ± 0.5 b	23.4 ± 0.4 a	22.5 ± 0.3 b
		ProStab	21.5 ± 0.4 B	21.7 ± 0.4	22.3 ± 0.8	22.0 ± 0.4
SP-4	Reducing sugars (g/L)	AFerm	3.4 ± 0.2 b	3.7 ± 0.3 b	4.2 ± 0.4 a	3.5 ± 0.2 b
		ProStab	3.4 ± 0.3 b	3.5 ± 0.2 ab	4.0 ± 0.5 a	3.6 ± 0.2 ab
SP-5	Total extract without reducing sugars (g/L)	AFerm	19.2 ± 0.2 aA	18.7 ± 0.3 bA	19.1 ± 0.2 aA	19.0 ± 0.1 abA
		ProStab	18.2 ± 0.1 B	18.2 ± 0.2 B	18.3 ± 0.3 B	18.4 ± 0.2 B
SP-6	Total acidity (as g/L tartaric acid)	AFerm	5.5 ± 0.1 aA	5.2 ± 0.1 bA	5.3 ± 0.2 b	5.4 ± 0.1 abA
		ProStab	5.0 ± 0.1 bB	5.0 ± 0.0 bB	5.1 ± 0.0 a	5.1 ± 0.0 aB
SP-7	Volatile acidity (as g/L acetic acid)	AFerm	0.67 ± 0.03	0.69 ± 0.06	0.65 ± 0.04	0.62 ± 0.06
		ProStab	0.65 ± 0.04	0.66 ± 0.04	0.63 ± 0.04	0.65 ± 0.02
SP-8	pH	AFerm	3.33 ± 0.01 bB	3.34 ± 0.01 a	3.31 ± 0.01 c	3.31 ± 0.01 c
		ProStab	3.36 ± 0.01 aA	3.31 ± 0.03 b	3.32 ± 0.01 ab	3.32 ± 0.01 ab

CO—control wine without added tannins and bentonite in fermentation; B—bentonite added near the end of fermentation; T—tannins added during fermentation without bentonite in fermentation; BT—tannins and bentonite added during fermentation in the same manner as in T and B treatments; AFerm—wine samples analyzed after fermentation; ProStab—wine samples analyzed after total protein stabilization by post-fermentation fining with bentonite. Different lowercase letters in a row represent statistically significant differences between four treatments at each production stage (AFerm, ProStab) separately, while different uppercase letters in a column represent statistically significant differences between the concentrations in AFerm and ProStab samples for each treatment (Co, B, T, BT) separately, all determined by one-way ANOVA and LSD test at $p < 0.05$.

3.2. Phenols

After fermentation (AFerm), the concentrations of most hydroxybenzoic acids in BT wine were lower than in T wine, while some concentrations were higher than in B treatment wine (Table 2). A very large increase was observed for gallic and syringic acid concentrations in T and BT compared to other wines. As the applied tannin preparation was declared as pure gallotannin, meaning its structure consisted of a central glucose molecule substituted with gallic acid units (e.g., pentagaloyl-glucose) [16], gallic acid could have been released during fermentation by gallotannin hydrolysis [46]. It is also possible that gallic acid was already a part of the tannin preparation in its free form. Neves et al. [47] also found higher concentrations of gallic acid in red wines after addition of grape tannins during and after fermentation, both in cases where gallic acid was identified and not identified as a part of the composition of the tannin extract. Most other hydroxybenzoic acids, such as protocatechuic acid, *p*-hydroxybenzoic acid, and 2,5-dihydroxybenzoic acid, were preserved in the highest concentrations in CO treatment wine (Table 2). The most pronounced decrease was observed in B and BT treatment wines, most likely due to the adsorption of the mentioned phenols onto bentonite.

Table 2. Concentrations (mg/L) of phenols in Malvazija istarska wines produced by different bentonite and tannins addition treatments, determined after fermentation and after final wine protein stabilization.

Code	Phenols	Stage	Treatment			
			CO	B	T	BT
	Hydroxybenzoic acids					
P-1	Gallic acid	AFerm	2.38 ± 0.57 c	2.90 ± 0.17 c	46.62 ± 0.39 aB	43.96 ± 1.35 b
		ProStab	2.97 ± 0.07 c	2.98 ± 0.15 c	50.51 ± 0.75 aA	47.32 ± 1.54 bA
P-2	Protocatechuic acid	AFerm	1.72 ± 0.44 a	1.05 ± 0.03 bB	1.14 ± 0.02 bB	0.96 ± 0.01 bB
		ProStab	1.20 ± 0.33	1.19 ± 0.02 A	1.38 ± 0.06 A	1.15 ± 0.03 A
P-3	*p*-Hydroxybenzoic acid	AFerm	0.54 ± 0.01 a	0.34 ± 0.02 cB	0.53 ± 0.04 a	0.45 ± 0.01 bB
		ProStab	0.52 ± 0.03 b	0.38 ± 0.01 cA	0.59 ± 0.02 a	0.50 ± 0.01 bA
P-4	2,5-Dihydroxybenzoic acid	AFerm	0.12 ± 0.02 a	0.05 ± 0.01 cB	0.09 ± 0.01 bB	0.04 ± 0.00 c
		ProStab	0.13 ± 0.01 a	0.08 ± 0.02 bA	0.12 ± 0.01 aA	0.06 ± 0.01 b
P-5	Syringic acid	AFerm	0.11 ± 0.01 cB	0.11 ± 0.00 cB	1.52 ± 0.02 bA	1.89 ± 0.02 aA
		ProStab	0.14 ± 0.00 cA	0.16 ± 0.01 cA	0.75 ± 0.03 bB	0.97 ± 0.03 aB
	Hydroxycinnamic acids					
P-6	*cis*-Caftaric acid *	AFerm	1.72 ± 0.06 abA	1.81 ± 0.05 aA	1.67 ± 0.04 bA	1.79 ± 0.04 aA
		ProStab	1.19 ± 0.08 B	1.10 ± 0.04 B	1.12 ± 0.08 B	1.11 ± 0.06 B
P-7	*trans*-Caftaric acid	AFerm	12.76 ± 1.53 d	19.13 ± 0.29 b	15.54 ± 0.29 c	21.11 ± 0.35 a
		ProStab	12.79 ± 1.37 d	19.23 ± 0.47 b	15.43 ± 0.05 c	21.35 ± 0.21 a
P-8	*cis*-Coutaric acid *	AFerm	0.82 ± 0.00 cA	0.87 ± 0.01 bA	0.93 ± 0.02 aA	0.93 ± 0.03 aA
		ProStab	0.75 ± 0.01 bB	0.77 ± 0.00 bB	0.82 ± 0.02 aB	0.82 ± 0.02 aB
P-9	*trans*-Coutaric acid *	AFerm	0.08 ± 0.01 d	0.23 ± 0.01 b	0.13 ± 0.02 c	0.39 ± 0.03 a
		ProStab	0.09 ± 0.00 d	0.23 ± 0.01 b	0.13 ± 0.01 c	0.39 ± 0.02 a
P-10	Caffeic acid	AFerm	4.88 ± 0.26 aB	3.64 ± 0.06 cB	4.45 ± 0.05 bB	2.95 ± 0.03 dB
		ProStab	5.54 ± 0.29 aA	4.04 ± 0.08 cA	5.00 ± 0.03 bA	3.13 ± 0.06 dA
P-11	*cis*-Fertaric acid *	AFerm	0.37 ± 0.01 aA	0.37 ± 0.01 aA	0.34 ± 0.02 bA	0.35 ± 0.01 abA
		ProStab	0.25 ± 0.02 aB	0.25 ± 0.01 aB	0.23 ± 0.01 abB	0.22 ± 0.01 bB
P-12	*trans*-Fertaric acid *	AFerm	1.64 ± 0.02 cB	1.77 ± 0.01 abB	1.72 ± 0.09 bcB	1.85 ± 0.01 aB
		ProStab	1.90 ± 0.03 bA	1.95 ± 0.02 aA	1.89 ± 0.01 bA	1.96 ± 0.08 aA
P-13	*p*-Coumaric acid	AFerm	0.85 ± 0.01 aB	0.56 ± 0.01 bB	0.88 ± 0.05 aB	0.55 ± 0.04 b
		ProStab	1.12 ± 0.17 aA	0.59 ± 0.01 bA	1.01 ± 0.04 aA	0.60 ± 0.01 b
P-14	Ferulic acid	AFerm	1.05 ± 0.03 a	0.88 ± 0.02 b	1.05 ± 0.03 a	0.85 ± 0.04 b
		ProStab	1.02 ± 0.03 a	0.86 ± 0.03 b	1.00 ± 0.03 a	0.83 ± 0.03 b
	Flavan-3-ols					
P-15	Catechin + tyrosol (as catechin)	AFerm	21.48 ± 0.20	20.80 ± 0.24	20.73 ± 0.20	20.67 ± 0.82
		ProStab	21.28 ± 0.30 a	20.35 ± 0.27 b	20.42 ± 0.31 b	20.45 ± 0.67 b
P-16	Epicatechin	AFerm	0.78 ± 0.22	0.79 ± 0.01 B	0.65 ± 0.05 B	0.85 ± 0.14
		ProStab	0.94 ± 0.13 b	1.17 ± 0.03 aA	0.78 ± 0.05 cA	0.74 ± 0.03 c
P-17	Procyanidin B1	AFerm	1.61 ± 0.04 b	1.70 ± 0.03 b	7.39 ± 0.09 a	7.59 ± 0.18 a
		ProStab	1.60 ± 0.13 b	1.75 ± 0.04 b	7.18 ± 0.20 a	7.28 ± 0.15 a
P-18	Procyanidin B2	AFerm	0.50 ± 0.02 bB	0.60 ± 0.03 aB	0.25 ± 0.03 dB	0.37 ± 0.01 cB
		ProStab	0.74 ± 0.02 bA	1.52 ± 0.07 aA	0.55 ± 0.08 cA	0.61 ± 0.01 cA
	Other					
P-19	Taxifolin	AFerm	0.16 ± 0.00 aB	0.11 ± 0.00 cB	0.15 ± 0.00 bB	0.11 ± 0.01 c
		ProStab	0.17 ± 0.00 aA	0.11 ± 0.00 cA	0.15 ± 0.00 bA	0.11 ± 0.00 c
P-20	*trans*-Piceid	AFerm	0.13 ± 0.01 cA	0.17 ± 0.01 a	0.16 ± 0.01 bA	0.16 ± 0.00 bB
		ProStab	0.11 ± 0.01 dB	0.17 ± 0.01 b	0.13 ± 0.01 cB	0.20 ± 0.00 aA
	Unidentified *					
P-21	T1	AFerm	n.d.	n.d.	22.93 ± 0.24 aB	22.24 ± 0.26 bB
		ProStab	n.d.	n.d.	24.17 ± 0.23 aA	23.33 ± 0.19 bA
P-22	T2	AFerm	n.d.	n.d.	15.08 ± 0.22 aB	13.49 ± 0.50 bB
		ProStab	n.d.	n.d.	18.62 ± 0.13 aA	16.49 ± 0.63 bA
P-23	T3	AFerm	n.d.	n.d.	15.64 ± 0.36 aB	13.55 ± 0.60 bB
		ProStab	n.d.	n.d.	19.84 ± 0.33 aA	17.23 ± 0.58 bA
P-24	T4	AFerm	n.d.	n.d.	79.28 ± 0.56 A	79.52 ± 2.74
		ProStab	n.d.	n.d.	76.19 ± 1.39 B	77.64 ± 2.99

Table 2. *Cont.*

Code	Phenols	Stage	Treatment			
			CO	B	T	BT
P-25	T5	AFerm	n.d.	n.d.	37.36 ± 0.20 A	38.92 ± 2.22
		ProStab	n.d.	n.d.	35.06 ± 0.78 B	36.29 ± 2.17
P-26	T6	AFerm	n.d.	n.d.	5.56 ± 0.02 B	5.93 ± 0.26 B
		ProStab	n.d.	n.d.	6.97 ± 0.12 A	6.84 ± 0.41 A
P-27	T7	AFerm	n.d.	n.d.	3.63 ± 0.05 bA	4.64 ± 0.11 aA
		ProStab	n.d.	n.d.	1.40 ± 0.08 bB	2.02 ± 0.11 aB
P-28	T8	AFerm	n.d.	n.d.	3.39 ± 0.07 bA	4.01 ± 0.29 aA
		ProStab	n.d.	n.d.	2.19 ± 0.10 B	2.42 ± 0.14 B
P-29	T9	AFerm	n.d.	n.d.	33.38 ± 0.41 B	29.01 ± 2.78
		ProStab	n.d.	n.d.	38.56 ± 0.67 aA	33.59 ± 2.89 b
P-30	*Total flavonoids*	AFerm	166.3 ± 6.8 bA	115.9 ± 0.0 cA	585.0 ± 37.1 a	558.2 ± 13.6 aA
		ProStab	86.1 ± 5.1 bB	59.4 ± 6.8 cB	540.4 ± 10.3 a	522.6 ± 13.6 aB
P-31	*Total phenols* **	AFerm	195.6 ± 1.7 bB	181.7 ± 4.5 bB	309.2 ± 21.8 a	314.9 ± 4.1 a
		ProStab	215.0 ± 2.0 bA	200.3 ± 6.0 cA	331.5 ± 8.4 a	330.8 ± 10.7 a

CO—control wine without added tannins and bentonite in fermentation; B—bentonite added near the end of fermentation; T—tannins added during fermentation, without bentonite in fermentation; BT—tannins and bentonite added during fermentation, in the same manner as in T and B treatments; AFerm—wine samples analyzed after fermentation; ProStab—wine samples analyzed after total protein stabilization by additional post-fermentation fining with bentonite; n.d.—not detected. * Semi-quantitative determination, concentrations expressed as equivalents of *trans*-caftaric acid assuming a relative response factor = 1; concentrations of T1–T9 expressed as equivalents of procyanidin B1 assuming a relative response factor = 1. ** Concentration of total phenols expressed as equivalents of gallic acid in mg/L. Different lowercase letters in a row represent statistically significant differences between four treatments at each production stage (AFerm, ProStab) separately, while different uppercase letters in a column represent statistically significant differences between the concentrations in AFerm and ProStab samples for each treatment (Co, B, T, BT) separately, all determined by one-way ANOVA and LSD test at $p < 0.05$.

After fermentation (AFerm), the highest hydroxycinnamoyltartrates concentration was found in BT and the lowest in CO treatment wine (Table 2). As in our previous study [14], a characteristic pattern of changes in the concentrations of *trans*-hydroxycinnamoyltartrates and the corresponding free hydroxycinnamic acids was observed. Wines of treatments involving the use of bentonite in fermentation, B and BT, contained higher concentrations of major hydroxycinnamoyltartrates (*trans*-caftaric, *trans*-coutaric, and *trans*-fertaric acids) compared to CO wine, which was likely due to the inhibitory activity of bentonite against hydrolytic enzymes [37,48]. A positive effect of T in relation to CO treatment on the concentrations of the mentioned phenols was also observed, although less pronounced compared to the treatment with bentonite B. Particular hydroxycinnamoyltartrates, primarily caftaric acid, are highly susceptible to oxidation and transformation into *o*-quinones and other oxidation products [49]. Tannins are known as antioxidants [50], suggesting their antioxidant activity protected hydroxycinnamoyltartrates from oxidation. The fact that both tannin and bentonite used in fermentation in separate T and B treatments, respectively, had a significant positive effect, and that the highest concentrations of the major representatives, such as *trans*-caftaric and *trans*-coutaric acids were found in wine of the combined BT treatment, proved their synergistic effect on the preservation of hydroxycinnamoyltartrates.

Significant differences after fermentation (AFerm) were also found in the case of the corresponding free hydroxycinnamic acids, such as caffeic, *p*-coumaric, and ferulic acids. The lowest concentrations were noted in bentonite-treated B and BT wines and the highest in CO wine; the latter mostly not differing from T treatment wine. Tannin in T treatment fermentation showed a protective effect on the concentrations of free hydroxycinnamic acids, although it is possible that higher concentrations compared to bentonite-treated B and BT wines were due to a higher degree of hydrolysis of the corresponding hydroxycinnamoyltartrates. It is worth noting the lower concentration of caffeic acid in T compared to CO wine and its inversely proportional relationship to the concentration of its precursor *trans*-caftaric acid, suggesting the hydrolysis *trans*-caftaric acid during T treatment was

reduced. A lower concentration of caffeic acid in wine produced with the addition of tannins compared to control wine was previously observed by Neves et al. [47], while Ghanem et al. [51] found no differences in an analog experiment.

The effect of T and BT tannin treatments on monomeric flavan-3-ol concentrations after fermentation (AFerm) was not observed (Table 2), which was consistent with the results obtained by Ghanem et al. [51], but not with the results of Neves et al. [47] who found an increase in catechin and epicatechin concentrations proportional to the concentration of the added tannin preparation. In contrast to this study, in both cited reports, condensed tannins from grapes were used [47,51]. After fermentation (AFerm), significantly higher concentrations of procyanidin B1 were found in tannin-treated T and BT wines. Free procyanidin B1 could have been an integral part of the tannin preparation and/or it was released by hydrolysis of tannin polymers [52]. Obreque-Slíer et al. [53] analyzed different commercial oenological tannins and noticed that in some cases the actual chemical content and composition differed significantly from the information stated on declaration. In addition to tannins, the analyzed oenological tannin preparations contained significant amounts of gallic acid and monomeric and dimeric flavan-3-ols [53], so it is possible that the preparation used in this study contained a certain portion of free phenolic acids and flavan-3-ols as well. The concentration of procyanidin B2 was the lowest in T and BT wines (Table 2). Such a result was in line with the findings of Ghanem et al. [51] who assumed that a negative effect of the application of a commercial condensed tannin preparation on the concentration of procyanidin B2 in red wine (−34.1%) was a result of their polymerization.

After fermentation (AFerm), the concentration of taxifolin was the highest in CO and the lowest in both bentonite-treated B and BT wines, suggesting a possibility of its adsorption onto bentonite and removal by settling. *Trans*-piceid was found in the highest concentration in B treatment wine, while it was the least abundant in CO wine. It is probable that bentonite limited the action of hydrolytic enzymes, such as glucosidases, that participate in the hydrolysis of piceid glucosides. A similar positive effect, although of a lower extent, was observed in T treatment wine, suggesting a protective effect of tannins as well. In contrast, Neves et al. [47] found a relatively weak, but statistically significant negative effect of tannin addition on *trans*-piceid concentration.

Nine unidentified phenolic compounds were detected in T and BT wines (Table 2). These compounds originated directly from the tannin preparation in unchanged form and/or were a result of tannin hydrolysis and/or resulted from the interactions of the added tannins with other wine constituents. Although tannin preparation was added in equal concentration and in the same way, the concentrations of individual unidentified compounds significantly differed between tannin-treated T and BT wines. Particular unidentified compounds were more prevalent in T (e.g., T1, T2, T3, and T9), while others were more abundant in BT wine (e.g., T6, T7, and T8). Since the presence of bentonite in fermentation was the only factor differentiating T and BT treatments, it was clear that during fermentation significant interactions between bentonite and tannins occurred, as reported previously [54,55].

In accordance with the expectations, the concentrations of total flavonoids and total phenols were the highest in tannin-treated T and BT wines (AFerm) (Table 2). Treatment B had a significant negative effect on the concentration of total flavonoids compared to CO wine (approx. −30%), which was not observed for the concentration of total phenols. This discrepancy was likely due to the characteristics of the analytical methods applied, since the Folin–Ciocalteu reagent-based method is known to be less selective and therefore gives a rough approximation of total phenolic content [33], while flavonoids were measured almost directly [32], meaning this result could be considered as a more relevant one when evaluating the investigated effects. Additionally, this result implies that bentonite showed a more significant affinity towards the adsorption of flavonoids than other groups of phenols. Main and Morris [56] noted a 21% decrease in flavonoid concentration in white wine after fining with bentonite during fermentation, while the reduction in non-flavonoid phenols was of lower extent. Smaller losses in total polyphenols, flavonoids, and non-flavonoids as

a consequence of the application of bentonite in white grape juice before fermentation of 5%, 6%, and 4%, respectively, were observed by Puig-Deu et al. [9].

After additional fining (ProStab), the concentration of gallic acid in T and BT treatment wines increased (Table 2) as a result of two phenomena with opposite signs: partial removal by additional bentonite fining and hydrolysis of tannins during a short wine maturation period. Similar processes (adsorption and chemical changes) probably affected the concentration of syringic acid. Its value was lower in stable wines (ProStab) than in wines after fermentation (AFerm), although still significantly higher in T and BT compared to CO and B treatment wines.

After additional fining (ProStab), the differences between the concentrations of most hydroxycinnamoyltartrates in wines of different treatments remained similar, with a slight increase observed for *trans*-fertaric acid in all wines. However, the concentrations of *cis*-forms, especially *cis*-caftaric acid, were significantly reduced by additional adsorption onto bentonite, although it is also likely that their hydrolysis continued after racking during a short wine maturation period, as reported earlier [57]. Correspondingly, in stable wines (ProStab), significantly higher concentrations of caffeic and *p*-coumaric acids were observed compared to the same wines after fermentation (AFerm), possibly due to hydrolysis of the *cis*-isomers of caftaric and coutaric acids.

Concentration of procyanidin B1 did not change after additional fining (ProStab). Concentration of procyanidin B2 increased significantly (Table 2), either due to hydrolysis of tannins from the added preparation or hydrolysis of previously mentioned complexes between tannins and procyanidin B2 formed during fermentation.

The concentration of taxifolin increased after additional fining (ProStab).

The concentrations of particular unidentified compounds after additional fining (ProStab) were lower (T1, T2, T3, T6, and T9), while the concentrations of others (T4, T5, T7, and T8) increased compared to those determined after fermentation (AFerm), suggesting a significant effect of additional bentonite fining.

A significant decrease in the concentration of total flavonoids after additional fining (ProStab) was observed in wines of most treatments (approx. −50% compared to AFerm), especially in CO and B wines produced without the addition of tannins. This confirmed the negative effect of bentonite fining after fermentation on phenolic compounds from this group. An increase in the concentration of total phenols, significant in the case of CO and B treatment wines (ProStab), was possibly a result of desorption of certain groups of phenols during short-term maturation period between the two sampling dates.

3.3. Free Volatile Aroma Compounds

Solid-phase extraction (SPE) was chosen for isolation of volatile compounds in this study because it provides several advantages over other extraction techniques commonly used in wine studies. By integrating sample purification and enrichment, it improves the detection sensitivity compared to traditional liquid–liquid extraction (LLE). In comparison to solid-phase microextraction (SPME) which is a non-exhaustive technique based on the partition equilibrium of volatile compounds between a sample and the solid-phase fiber, SPE is more robust regarding the matrix effects and thus more reproducible. Last but not least, with the applied SPE method, both free and bound volatile compounds can be analyzed in separate chromatographic runs [34,35], and in this way, more information can be obtained in comparison to the other mentioned extraction techniques.

After fermentation (AFerm), T and BT wines had significantly higher linalool concentrations than B treatment wine (Table 3). The added tannins could have interacted with bentonite and changed its surface structure and charge [6,36], which in a certain way could have reduced the adsorption affinity of bentonite towards linalool observed in earlier studies [9,39,40,58,59]. Bentonite and tannins acted in synergy and caused a significant decrease in concentration of free *cis*-3-hexenol in BT wine in relation to CO and B treatment wines after fermentation (AFerm). It has already been shown that fining with bentonite can reduce the concentration of C_6-alcohols by their adsorption and precipitation [7,40,58,60].

On the other hand, bentonite could also have limited the activity of enzymes responsible for formation of C_6-alcohols from long-chain fatty acids, such as lipoxygenase and alcohol dehydrogenase, as previously shown for other enzymes [48,56,61]. It was recently shown that the application of particular tannin preparations can reduce the concentration of 1-hexanol, although most of the tested tannins did not affect it [62].

Table 3. Concentrations (μg/L, except where indicated) of free volatile aroma compounds in Malvazija istarska wines produced by different bentonite and tannins addition treatments, determined after fermentation and after final wine protein stabilization.

Code	Free Aroma Compounds	Stage	Treatment			
			CO	B	T	BT
	Monoterpenes					
FV-1	Linalool	AFerm	24.41 ± 0.74 ab	22.92 ± 0.45 bB	24.78 ± 1.16 aB	24.79 ± 1.27 a
		ProStab	29.68 ± 4.92	29.24 ± 2.27 A	29.56 ± 2.52 A	28.70 ± 3.44
FV-2	α-Terpineol	AFerm	9.51 ± 0.24	10.18 ± 0.09 B	10.45 ± 0.94	9.97 ± 0.50 B
		ProStab	12.44 ± 0.43	11.92 ± 0.39 A	11.98 ± 0.34	12.23 ± 0.27 A
FV-3	α-Terpinolene *	AFerm	0.84 ± 0.73	0.97 ± 0.92	0.86 ± 0.76	1.20 ± 1.16
		ProStab	0.89 ± 0.77	1.35 ± 0.53	0.86 ± 0.75	0.85 ± 0.75
FV-4	Citronellol	AFerm	7.81 ± 0.78	8.04 ± 0.25	7.85 ± 0.22	7.73 ± 0.74
		ProStab	7.68 ± 0.31	7.59 ± 0.33	7.39 ± 0.56	7.44 ± 0.64
FV-5	Terpendiol I *	AFerm	86.78 ± 1.00 A	85.80 ± 5.46 A	83.70 ± 2.93 A	86.62 ± 5.45 A
		ProStab	77.59 ± 1.28 B	76.09 ± 2.41 B	75.70 ± 3.52 B	73.62 ± 1.03 B
	C_{13}-norisoprenoid					
FV-6	β-Damascenone	AFerm	3.10 ± 0.49	3.51 ± 0.28 A	3.20 ± 0.16 A	3.35 ± 0.27 A
		ProStab	2.29 ± 0.22	2.32 ± 0.22 B	2.30 ± 0.28 B	2.45 ± 0.19 B
	Alcohols					
FV-7	1-Propanol (mg/L)	AFerm	28.31 ± 3.18	28.25 ± 3.55	27.14 ± 1.94	26.67 ± 1.53
		ProStab	29.15 ± 0.48 a	29.37 ± 2.35 a	29.02 ± 0.99 a	25.58 ± 1.07 b
FV-8	Isobutanol (mg/L)	AFerm	18.50 ± 0.96	18.85 ± 1.58	18.80 ± 1.37	20.59 ± 4.74
		ProStab	19.63 ± 0.02	19.78 ± 0.39	19.32 ± 0.29	19.70 ± 3.74
FV-9	Isoamyl alcohol (mg/L)	AFerm	197.0 ± 9.7	199.8 ± 16.5	204.4 ± 13.6	211.4 ± 40.8
		ProStab	206.0 ± 0.5	205.5 ± 4.6	206.4 ± 3.6	205.9 ± 31.6
FV-10	1-Hexanol (mg/L)	AFerm	2.02 ± 0.07	1.97 ± 0.02 A	1.94 ± 0.06	1.99 ± 0.14
		ProStab	1.98 ± 0.06	1.91 ± 0.02 B	1.87 ± 0.04	1.96 ± 0.16
FV-11	*trans*-3-Hexenol *	AFerm	134.3 ± 1.9	133.4 ± 2.6 A	133.1 ± 5.2	132.4 ± 3.5
		ProStab	133.0 ± 4.2	127.3 ± 0.8 B	128.1 ± 4.2	129.2 ± 3.4
FV-12	*cis*-3-Hexenol *	AFerm	84.70 ± 1.56 a	83.26 ± 0.89 aA	80.92 ± 2.24 ab	74.11 ± 8.39 b
		ProStab	83.09 ± 3.01 a	78.59 ± 1.65 abB	77.74 ± 1.65 ab	72.53 ± 8.87 b
FV-13	1-Octanol *	AFerm	14.83 ± 3.13	14.46 ± 1.35	12.69 ± 1.26	15.92 ± 3.65
		ProStab	18.62 ± 4.93	15.01 ± 3.57	11.96 ± 1.34	14.02 ± 5.76
FV-14	Benzyl alcohol *	AFerm	51.59 ± 0.49 aB	28.12 ± 0.47 c	45.84 ± 0.78 bB	28.24 ± 1.20 c
		ProStab	60.79 ± 1.02 aA	28.23 ± 0.84 c	52.83 ± 1.82 bA	27.78 ± 1.52 c
FV-15	2-Phenyletanol (mg/L)	AFerm	29.90 ± 1.03 A	28.77 ± 0.79	29.64 ± 0.81 A	29.98 ± 0.84
		ProStab	27.99 ± 0.52 B	28.14 ± 0.68	27.52 ± 0.10 B	28.62 ± 1.29
	Fatty acids					
FV-16	Butyric acid (mg/L)	AFerm	1.78 ± 0.06 a	1.84 ± 0.10 a	1.79 ± 0.02 a	1.57 ± 0.13 b
		ProStab	1.75 ± 0.15	1.74 ± 0.06	1.71 ± 0.06	1.54 ± 0.15
FV-17	Hexanoic acid (mg/L)	AFerm	3.92 ± 0.16	4.11 ± 0.18	4.29 ± 0.15	3.88 ± 0.48
		ProStab	3.90 ± 0.26	3.94 ± 0.02	4.05 ± 0.24	3.67 ± 0.39
FV-18	Octanoic acid (mg/L)	AFerm	5.36 ± 0.25	5.96 ± 0.16	6.04 ± 0.33	5.51 ± 0.68
		ProStab	5.62 ± 0.56	5.68 ± 0.11	5.81 ± 0.47	5.20 ± 0.78
FV-19	Decanoic acid (mg/L)	AFerm	1.60 ± 0.08 b	1.76 ± 0.05 ab	1.91 ± 0.06 a	1.67 ± 0.16 b
		ProStab	1.84 ± 0.33	1.77 ± 0.14	1.89 ± 0.11	1.68 ± 0.23
FV-20	Dodecanoic acid *	AFerm	52.43 ± 8.08 aA	27.41 ± 13.10 b	42.71 ± 4.90 ab	27.26 ± 4.89 b
		ProStab	36.63 ± 1.93 aB	22.64 ± 5.35 b	32.21 ± 5.98 ab	24.05 ± 9.11 b
	Ethyl esters					
FV-21	Ethyl butyrate (mg/L)	AFerm	0.29 ± 0.02	0.33 ± 0.04	0.31 ± 0.01	0.27 ± 0.07
		ProStab	0.31 ± 0.04	0.32 ± 0.03	0.32 ± 0.02	0.26 ± 0.06
FV-22	Ethyl hexanoate (mg/L)	AFerm	0.49 ± 0.04 b	0.57 ± 0.03 ab	0.58 ± 0.06 a	0.51 ± 0.06 ab
		ProStab	0.60 ± 0.10	0.59 ± 0.04	0.62 ± 0.05	0.55 ± 0.07
FV-23	Ethyl octanoate (mg/L)	AFerm	0.21 ± 0.02 B	0.24 ± 0.03	0.25 ± 0.05	0.23 ± 0.05
		ProStab	0.27 ± 0.02 A	0.25 ± 0.03	0.27 ± 0.05	0.26 ± 0.07
FV-24	Ethyl decanoate (mg/L)	AFerm	0.04 ± 0.00 ab	0.03 ± 0.00 b	0.04 ± 0.01 a	0.03 ± 0.01 b
		ProStab	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01

Table 3. *Cont.*

Code	Free Aroma Compounds	Stage	Treatment			
			CO	B	T	BT
	Acetate esters					
FV-25	Ethyl acetate (mg/L)	AFerm	18.00 ± 2.79 B	20.27 ± 0.34	20.16 ± 3.62	23.53 ± 3.80
		ProStab	29.18 ± 3.28 A	21.86 ± 4.55	22.76 ± 2.81	22.17 ± 2.58
FV-26	Isoamyl acetate (mg/L)	AFerm	0.91 ± 0.03	1.14 ± 0.01 A	1.12 ± 0.14	0.89 ± 0.28
		ProStab	0.99 ± 0.20	0.99 ± 0.04 B	1.01 ± 0.09	0.79 ± 0.24
FV-27	Hexyl acetate	AFerm	67.14 ± 4.63 b	90.82 ± 3.86 ab	95.09 ± 14.65 a	70.29 ± 21.20 ab
		ProStab	82.90 ± 24.11	80.06 ± 9.95	89.11 ± 11.68	69.20 ± 24.48
FV-28	2-Phenethyl acetate (mg/L)	AFerm	0.12 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.13 ± 0.03
		ProStab	0.14 ± 0.03	0.14 ± 0.01	0.14 ± 0.01	0.12 ± 0.03
	Other esters					
FV-29	Ethyl lactate (mg/L)	AFerm	5.76 ± 0.32 B	5.51 ± 0.56 B	5.77 ± 0.47 B	5.65 ± 0.97 B
		ProStab	7.60 ± 0.45 A	7.60 ± 0.62 A	7.63 ± 0.20 A	7.91 ± 0.95 A
FV-30	Ethyl 3-hydroxybutanoate * (mg/L)	AFerm	0.25 ± 0.00	0.26 ± 0.00 A	0.25 ± 0.01	0.28 ± 0.04
		ProStab	0.25 ± 0.02	0.24 ± 0.01 B	0.24 ± 0.02	0.27 ± 0.04
FV-31	Diethyl succinate * (mg/L)	AFerm	0.44 ± 0.01 B	0.41 ± 0.05 B	0.42 ± 0.01 B	0.46 ± 0.06 B
		ProStab	0.69 ± 0.03 abA	0.67 ± 0.05 bA	0.71 ± 0.02 abA	0.78 ± 0.09 aA
FV-32	Methyl 4-hydroxybutanoate *	AFerm	22.82 ± 1.44 bA	25.06 ± 0.93 aA	22.90 ± 0.59 bA	19.62 ± 1.15 cA
		ProStab	18.08 ± 2.21 abB	19.61 ± 0.75 a	15.73 ± 0.73 bcB	14.23 ± 2.27 cB
FV-33	Ethyl 4-hydroxybutanoate * (mg/L)	AFerm	6.71 ± 0.66 ab	7.46 ± 0.40 aA	6.63 ± 0.12 abA	5.80 ± 0.83 b
		ProStab	5.44 ± 0.67 a	5.59 ± 0.30 aB	4.86 ± 0.22 abB	4.24 ± 0.72 b
FV-34	Diethyl malate * (mg/L)	AFerm	0.31 ± 0.01 B	0.29 ± 0.02 B	0.30 ± 0.02 B	0.32 ± 0.02 B
		ProStab	0.59 ± 0.01 A	0.56 ± 0.03 A	0.60 ± 0.03 A	0.60 ± 0.02 A
FV-35	Monomethyl succinate *	AFerm	80.31 ± 9.81	65.99 ± 2.87	77.14 ± 9.44	84.81 ± 15.23
		ProStab	79.26 ± 5.57 ab	72.43 ± 6.61 b	78.80 ± 4.89 ab	89.67 ± 11.04 a
FV-36	Monoethyl succinate *(mg/L)	AFerm	21.27 ± 0.95 B	19.55 ± 0.56 B	19.01 ± 0.97 B	21.06 ± 1.94 B
		ProStab	26.48 ± 1.04 A	26.30 ± 0.87 A	25.85 ± 1.57 A	26.89 ± 1.45 A
FV-37	Ethyl *p*-coumarate *(mg/L)	AFerm	0.45 ± 0.02 aB	0.40 ± 0.02 a	0.45 ± 0.06 a	0.32 ± 0.02 b
		ProStab	0.49 ± 0.02 aA	0.42 ± 0.04 b	0.51 ± 0.04 a	0.33 ± 0.03 c
	Other					
FV-38	Acetaldehyde (mg/L)	AFerm	43.04 ± 5.08	44.22 ± 6.12	42.95 ± 3.77	47.06 ± 7.42
		ProStab	44.19 ± 1.93	48.92 ± 2.81	46.78 ± 4.19	49.49 ± 7.06
FV-39	Benzaldehyde *	AFerm	9.26 ± 5.28	9.22 ± 5.24	9.03 ± 5.25	9.11 ± 4.46
		ProStab	8.52 ± 4.91	8.80 ± 5.42	8.33 ± 5.19	8.68 ± 5.16
FV-40	Methanol (mg/L)	AFerm	48.31 ± 0.80	44.42 ± 6.22	44.63 ± 4.10	51.65 ± 1.57
		ProStab	48.91 ± 2.62	52.16 ± 6.24	49.27 ± 6.32	47.12 ± 10.00
FV-41	Guaiacol *	AFerm	0.95 ± 0.04	0.97 ± 0.20	0.95 ± 0.13	0.70 ± 0.67
		ProStab	0.94 ± 0.24	1.24 ± 0.66	0.97 ± 0.12	0.96 ± 0.17
FV-42	4-Vinylguaiacol *	AFerm	366.1 ± 19.7	439.5 ± 74.6	433.1 ± 21.8 A	435.8 ± 30.5
		ProStab	382.4 ± 26.6	417.9 ± 112.9	324.5 ± 38.9 B	392.9 ± 8.2
FV-43	Acetoin *	AFerm	29.51 ± 3.44 ab	24.28 ± 1.03 b	33.48 ± 2.32 a	29.86 ± 6.12 ab
		ProStab	28.76 ± 2.30 ab	24.62 ± 1.60 b	32.06 ± 2.02 a	31.46 ± 4.22 a
FV-44	3-Hydroxy-2-pentanone *	AFerm	38.30 ± 2.73 ab	34.83 ± 4.18 b	43.73 ± 2.52 ab	48.91 ± 10.48 a
		ProStab	36.90 ± 2.17 b	35.03 ± 3.44 b	42.48 ± 4.24 ab	49.62 ± 7.68 a
FV-45	γ-Butyrolactone *	AFerm	191.0 ± 13.0	215.5 ± 25.7	201.8 ± 18.2	186.0 ± 14.0
		ProStab	232.5 ± 23.8 ab	234.6 ± 8.5 a	229.5 ± 18.4 ab	200.7 ± 15.7 b
FV-46	Pantolactone *	AFerm	45.07 ± 2.76	46.79 ± 4.45	42.51 ± 2.26	46.82 ± 4.19
		ProStab	46.88 ± 2.67	45.90 ± 1.58	44.80 ± 0.70	44.78 ± 1.61
FV-47	3-Methylthiopropanol * (mg/L)	AFerm	1.06 ± 0.05	1.05 ± 0.04	1.11 ± 0.04	1.12 ± 0.17
		ProStab	1.06 ± 0.06	1.02 ± 0.04	1.06 ± 0.03	1.08 ± 0.14
FV-48	Tryptophol * (mg/L)	AFerm	0.75 ± 0.05 b	1.07 ± 0.02 aA	1.08 ± 0.07 a	1.15 ± 0.16 a
		ProStab	0.92 ± 0.18	0.84 ± 0.06 B	0.92 ± 0.14	0.98 ± 0.03

CO—control wine without added tannins and bentonite in fermentation; B—bentonite added near the end of fermentation; T—tannins added during fermentation, without bentonite in fermentation; BT—tannins and bentonite added during fermentation, in the same manner as in T and B treatments; AFerm—wine samples analyzed after fermentation; ProStab—wine samples analyzed after total protein stabilization by additional post-fermentation fining with bentonite. * Semi-quantitative determination, concentrations expressed as equivalents of internal standard 1-heptanol, assuming a response factor = 1. Different lowercase letters in a row represent statistically significant differences between four treatments at each production stage (AFerm, ProStab) separately, while different uppercase letters in a column represent statistically significant differences between the concentrations in AFerm and ProStab samples for each treatment (Co, B, T, BT) separately, all determined by one-way ANOVA and LSD test at $p < 0.05$.

Benzyl alcohol concentration was significantly higher in CO than in other wines and was lower in B and BT than in T treatment wine. Bentonite possibly limited the action of hydrolytic enzymes, mainly β-glucosidases responsible for the cleavage of benzyl alcohol

glycosides during fermentation [63] and/or a portion of the glycosides was adsorbed. Additionally, tannins added during T and BT fermentation interacted with benzyl alcohol and reduced its concentration compared to CO wine.

Butanoic acid concentration was the lowest in BT wine, the concentration of decanoic acid was higher in T than in CO and BT treatment wines, while the concentration of dodecanoic acid was higher in CO than in B and BT treatment wines.

After fermentation (AFerm), T treatment wine contained the highest concentrations of several important volatile esters, such as ethyl hexanoate, ethyl decanoate, and hexyl acetate, although in some cases without statistically significant differences. For the concentrations of particular other esters of key importance for the fruity-floral aroma of white wine, such as ethyl octanoate and isoamyl acetate, similar tendencies towards higher concentrations in T treatment wine were observed, although without statistical significance. The preservation of ester concentrations was possibly related to the antioxidant effect of tannins [19,22,28,64]. Polyphenol oxidases trigger a series of chemical reactions in which semiquinone and quinone radicals are formed, and in the presence of transition metals, oxygen is reduced to hydrogen peroxide, which can lead to oxidation of esters and a decrease in their concentration [65]. Chen et al. [62] showed that the application of all tannin preparations included in their study increased the concentration of ethyl acetate, and some of these preparations had a positive effect on the concentration of isoamyl acetate, while 2-phenylethyl acetate was not affected. Treatment with bentonite B also showed a tendency towards increased ester concentrations, although no significant differences were determined in relation to CO wine, which was in partial disagreement with previous studies [7,14]. Horvat et al. [14] and Lira et al. [7] observed a distinct positive effect of partial fining with bentonite during fermentation on fermentation esters, presumably due to its limiting action on oxidative and hydrolytic enzymes. The observed discrepancy could have been caused by the differences in initial composition of grape juice and other conditions in this and the above-mentioned studies. Among other esters, methyl 4-hydroxybutanoate and ethyl 4-hydroxybutanoate had significantly higher concentrations in B than in BT treatment wine, while BT wine contained the lowest ethyl *p*-coumarate concentration.

The highest concentration of acetoin was found in T treatment wine, which did not coincide with the results of Chen et al. [62] who noted that the addition of tannins lowered the concentration of this compound. CO wine contained the lowest concentration of tryptophol.

In protein stable wines (ProStab), an increase in the concentration of linalool was noted, in some cases statistically significant (Table 3). It was most likely a consequence of the hydrolysis of its glycoside precursors during the short-term maturation period between the two samplings (AFerm and ProStab), as noted earlier [4,66]. Such a conclusion was partially supported by a decrease in the concentration of the bound form of linalool in the same wines (Table 4).

Table 4. Concentrations (µg/L) of bound aroma compounds in Malvazija istarska wines produced by different bentonite and tannins addition treatments, determined after fermentation and after final wine protein stabilization.

Code	Bound Aroma Compounds	Stage	Treatment			
			CO	B	T	BT
	Monoterpenes					
BV-1	*trans*-furan Linalool oxide *	AFerm	2.27 ± 0.21	2.31 ± 0.19	2.39 ± 0.51	2.42 ± 0.10
		ProStab	2.34 ± 0.12 b	2.30 ± 0.13 b	2.55 ± 0.17 ab	2.68 ± 0.15 a
BV-2	*cis*-furan Linalool oxide *	AFerm	0.77 ± 0.05 b	0.80 ± 0.07 ab	0.89 ± 0.07 a	0.87 ± 0.06 abB
		ProStab	0.84 ± 0.01 b	0.76 ± 0.04 c	0.91 ± 0.02 b	1.04 ± 0.07 aA
BV-3	Linalool	AFerm	8.61 ± 0.69 A	7.96 ± 1.40	7.93 ± 1.16 A	5.80 ± 2.36
		ProStab	6.48 ± 0.29 aB	6.03 ± 0.49 a	4.78 ± 1.07 abB	3.80 ± 1.64 b

Table 4. *Cont.*

Code	Bound Aroma Compounds	Stage	Treatment			
			CO	B	T	BT
BV-4	Hotrienol *	AFerm	1.85 ± 0.63	2.25 ± 0.35	2.48 ± 0.42	2.43 ± 0.53
		ProStab	1.81 ± 0.62	2.35 ± 0.38	2.38 ± 0.08	2.27 ± 1.02
BV-5	α-Terpineol	AFerm	1.35 ± 0.02 b	1.40 ± 0.07 b	1.86 ± 0.29 a	2.08 ± 0.13 a
		ProStab	1.33 ± 0.02 c	1.33 ± 0.02 c	1.71 ± 0.17 b	2.02 ± 0.17 a
BV-6	*trans*-pyran Linalool oxide *	AFerm	5.72 ± 0.43	5.85 ± 0.39	6.09 ± 1.13	5.89 ± 0.16
		ProStab	5.72 ± 0.49	5.77 ± 0.12	5.91 ± 0.62	6.27 ± 0.66
BV-7	Citronellol	AFerm	1.03 ± 0.04 ab	0.66 ± 0.58 ab	1.18 ± 0.26 a	0.31 ± 0.54 b
		ProStab	0.69 ± 0.60	1.04 ± 0.18	0.30 ± 0.52	0.62 ± 0.54
BV-8	Nerol	AFerm	7.45 ± 0.04	7.69 ± 0.72	7.46 ± 0.45	7.74 ± 0.87
		ProStab	7.61 ± 0.48	7.52 ± 0.35	7.25 ± 0.31	7.87 ± 1.69
BV-9	Geraniol	AFerm	43.49 ± 0.89	45.48 ± 7.90	40.04 ± 2.34	43.35 ± 7.12
		ProStab	41.76 ± 1.99	43.13 ± 2.85	39.07 ± 1.77	39.94 ± 5.41
BV-10	Terpendiol I *	AFerm	1.68 ± 0.33	1.33 ± 0.63	1.10 ± 0.27	0.94 ± 0.33
		ProStab	1.44 ± 0.41	1.32 ± 0.36	0.92 ± 0.06	1.82 ± 2.03
BV-11	*trans*-8-Hydroxy-linalool *	AFerm	29.60 ± 6.73	29.55 ± 3.55	27.04 ± 8.40	24.57 ± 1.56
		ProStab	21.40 ± 3.04	26.67 ± 4.45	19.25 ± 4.66	31.37 ± 19.12
BV-12	7-Hydroxy-geraniol *	AFerm	21.92 ± 1.83	23.23 ± 1.82	21.85 ± 3.74	22.97 ± 3.12
		ProStab	19.40 ± 4.85	23.48 ± 0.70	21.59 ± 3.13	41.95 ± 34.49
BV-13	*cis*-8-Hydroxy-linalool *	AFerm	92.78 ± 10.23	99.87 ± 9.73	91.86 ± 6.91	91.94 ± 19.18
		ProStab	83.5 ± 17.1	100.3 ± 1.3	82.2 ± 9.3	158.3 ± 134.6
BV-14	*trans*-Geranic acid *	AFerm	8.37 ± 1.53	9.37 ± 1.87	8.69 ± 1.88	9.36 ± 1.79
		ProStab	8.12 ± 0.65	9.54 ± 0.54	7.98 ± 1.33	9.88 ± 3.25
	C_{13}-norisoprenoids					
BV-15	β-Damascenone	AFerm	0.14 ± 0.02	0.10 ± 0.10	0.17 ± 0.02 A	0.17 ± 0.02
		ProStab	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01 B	0.07 ± 0.07
BV-16	3-Hydroxy-β-damascone *	AFerm	177.6 ± 8.1 A	179.8 ± 39.3	183.2 ± 4.6 A	183.4 ± 45.7
		ProStab	130.1 ± 10.3 B	135.2 ± 14.9	130.4 ± 17.0 B	129.3 ± 29.7
BV-17	3-Oxo-α-ionol *	AFerm	59.23 ± 4.77	62.71 ± 7.84	61.11 ± 12.39	58.10 ± 6.42
		ProStab	59.10 ± 1.31	60.96 ± 4.86	54.79 ± 8.24	58.88 ± 7.24
BV-18	3-Hydroxy-7,8-dihydro-β-ionol *	AFerm	50.45 ± 3.57	53.45 ± 4.93	51.14 ± 5.23	53.08 ± 4.95
		ProStab	47.42 ± 5.02	52.07 ± 2.55	48.45 ± 6.70	66.34 ± 25.02
BV-19	Vomifoliol *	AFerm	20.28 ± 4.01 ab	25.32 ± 4.41 a	22.51 ± 2.62 ab	18.84 ± 1.81 b
		ProStab	17.07 ± 5.21	20.81 ± 2.94	18.19 ± 1.65	47.68 ± 49.76
	Alcohols					
BV-20	1-Hexanol	AFerm	88.11 ± 2.15	86.98 ± 9.32	89.53 ± 5.73	90.31 ± 2.08 A
		ProStab	86.35 ± 3.27	87.49 ± 1.62	84.53 ± 2.08	86.45 ± 0.82 B
BV-21	*trans*-3-Hexenol *	AFerm	1.16 ± 0.04	1.10 ± 0.10	1.20 ± 0.09	1.13 ± 0.05
		ProStab	1.18 ± 0.05	1.16 ± 0.10	1.18 ± 0.05	1.10 ± 0.05
BV-22	*cis*-3-Hexenol *	AFerm	13.29 ± 1.09	13.46 ± 0.54	13.94 ± 0.83	14.82 ± 0.96
		ProStab	12.81 ± 0.88 b	13.17 ± 0.29 b	12.74 ± 0.24 b	14.31 ± 0.47 a
BV-23	*trans*-2-Hexenol *	AFerm	15.18 ± 0.47 b	16.37 ± 0.95 ab	15.63 ± 0.63 b	17.46 ± 0.49 a
		ProStab	15.27 ± 1.08	16.88 ± 0.22	15.51 ± 1.47	16.81 ± 0.20
BV-24	1-Octen-3-ol *	AFerm	2.49 ± 0.30 b	2.60 ± 0.05 ab	2.89 ± 0.28 a	2.62 ± 0.05 ab
		ProStab	2.53 ± 0.18	2.51 ± 0.04	2.52 ± 0.22	2.75 ± 0.54
BV-25	1-Octanol *	AFerm	200.4 ± 309.8	192.8 ± 302.5	191.8 ± 295.0	164.0 ± 252.8
		ProStab	210.6 ± 323.9	180.6 ± 280.9	199.7 ± 309.7	166.1 ± 255.7
BV-26	2-Phenylethanol *	AFerm	322.7 ± 4.6 a	266.7 ± 12.6 b	318.1 ± 36.2 a	279.6 ± 13.1 b
		ProStab	305.4 ± 32.6	273.1 ± 8.4	309.1 ± 17.7	287.0 ± 22.1
	Other					
BV-27	Benzaldehyde *	AFerm	5.09 ± 2.80 a	2.58 ± 0.75 ab	1.85 ± 0.90 b	2.82 ± 0.54 ab
		ProStab	3.12 ± 0.97 ab	2.31 ± 0.06 b	3.15 ± 0.26 ab	4.40 ± 1.69 a
BV-28	4-Vinylguaiacol *	AFerm	12.29 ± 2.94	16.20 ± 6.16	14.83 ± 1.52 A	12.43 ± 2.14
		ProStab	10.36 ± 2.64	13.41 ± 2.20	10.13 ± 1.31 B	47.28 ± 59.71
BV-29	Tryptophol *	AFerm	10.09 ± 1.90 b	13.79 ± 1.75 ab	13.10 ± 2.45 ab	14.40 ± 2.28 a
		ProStab	10.62 ± 1.27	12.64 ± 0.22	11.98 ± 2.61	15.01 ± 6.23

CO—control wine without added tannins and without bentonite in fermentation; B—bentonite added near the end of fermentation; T—tannins added during fermentation, without bentonite in fermentation; BT—tannins and bentonite added during fermentation, in the same manner as in T and B treatments; AFerm—wine samples analyzed after fermentation; ProStab—wine samples analyzed after total protein stabilization by additional post-fermentation fining with bentonite. * Semi-quantitative determination, concentrations expressed as equivalents of internal standard 1-heptanol, assuming a response factor = 1. Different lowercase letters in a row represent statistically significant differences between four treatments at each production stage (AFerm, ProStab) separately, while different uppercase letters in a column represent statistically significant differences between the concentrations in AFerm and ProStab samples for each treatment (Co, B, T, BT) separately, all determined by one-way ANOVA and LSD test at $p < 0.05$.

The concentration of α-terpineol increased in protein stable wines (ProStab) of most treatments (Table 3) as a result of conversion of other main monoterpenols, as reported earlier [66,67]. The concentration of terpenediol I decreased in all wines at least partly due to its conversion into other monoterpene forms [66] but also because of its removal by additionally applied bentonite (ProStab). A decrease in β-damascenone concentration was recorded in wines of most treatments. It is known that this may happen because of its interaction with sulfur dioxide [67], although the additional dose bentonite could have also had a negative effect. The concentration of benzyl alcohol increased in CO and T wines, possibly via hydrolysis of its glycoside precursors.

After additional fining (ProStab), there were no more significant differences between the concentrations of ethyl and acetate esters in wines of different treatments, which was at least partly caused by their stripping by additionally added bentonite, as previously shown for fining with bentonite after fermentation by many authors [11,40,60,68,69]. Among other esters, it is worth highlighting an increase in ethyl lactate and diethyl succinate concentrations in wines of all treatments, which could have primarily been a result of spontaneous esterification of their alcohol and acid precursors [4,70]. A decrease in the concentration of methyl 4-hydroxybutanoate was observed in wines of all treatments.

3.4. Bound Aroma Compounds

Treatments with tannins T and BT showed a tendency to preserve higher concentrations of glycosidically bound linalool oxides in the corresponding wines after fermentation (AFerm) (Table 4). This was even more strongly pronounced in the case of bound α-terpineol. Treatments with bentonite, B and BT, caused a decrease in the concentrations of bound citronellol and especially bound 2-phenylethanol after fermentation (AFerm), which was in line with our previous study that showed that aroma glycosides and bentonite interact during fermentation [14]. Zoecklein et al. [66] and McMahon et al. [71] pointed out that adsorption of aroma glycosides on yeast cells and solid particles from grapes, as well as precipitation, is in fact one of the causes of their decrease during fermentation.

After additional fining (ProStab), concentrations of furanoid linalool oxides were significantly higher in BT than in B treatment wine (Table 4). Lower concentrations of bound linalool were found in wines that included the application of tannins, T and BT. It is possible that in a short period between the two samplings (AFerm and ProStab), a portion of linalool glycosides interacted with the added tannins and that such complexes were better substrates for adsorption onto bentonite applied for additional final fining (ProStab). A decrease in the concentration of bound 3-hydroxy-β-damascone observed in wines of most treatments after additional fining (ProStab) was partly a consequence of its adsorption on bentonite, but also of its conversion into other C_{13}-norisoprenoid forms [4].

3.5. Partial Least Squares-Discriminant Analysis

To better evaluate the effects of bentonite and tannins added during fermentation separately, data were processed by PLS-DA, and the results are reported in Figure 1. After fermentation (AFerm), differentiation of wines by PLS-DA based on the presence/absence of bentonite during fermentation was successful (Figure 1a), suggesting bentonite produced characteristic effects in both treated wines (B and BT), regardless of the presence of exogenous tannins. The highest VIP scores (>2.0) were attributed mostly to particular phenols, such as *p*-coumaric acid (P-13), taxifolin (P-19), and ferulic acid (P-14), followed by 2,5-dihydroxybenzoic (P-4), caffeic (P-10), and *p*-hydroxybenzoic (P-3), all found in lower concentration in bentonite-treated B and BT wines, as well as *trans*-caftaric (P-7) and *trans*-coutaric acids (P-9) found in higher concentrations in these wines (Figure 1b). Such results mostly confirmed the results of ANOVA (Table 2) and the fact that bentonite exhibits ambiguous activity towards minor phenols by both reducing and preserving their amounts, depending on the compound. Among other variables, it is worth to mention a high contribution of free benzyl alcohol (FV-14) to the differentiation.

PLS-DA differentiation of wines (AFerm) based on the presence/absence of exogenous tannins during fermentation was also successful (Figure 1c), meaning tannins also produced characteristic effects in both treated wines (T and BT), regardless of the presence of bentonite. Again, the highest VIP scores (>2.0) were attributed to particular phenols, although other than those most strongly affected by bentonite. These included procyanidin B1 (P-17) and gallic (P-1), syringic (P-5), and *cis*-coutaric acid (P-8), which were positively affected by the addition of tannins in T and BT wines, as well as procyanidin B2 (P-18) whose concentration was reduced. Besides bound α-terpineol (BV-5), bound *cis*-furan linalool oxide (BV-2), and pH (SP-8), a series of free volatile compounds (FV) were also responsible for the differentiation (Figure 1d).

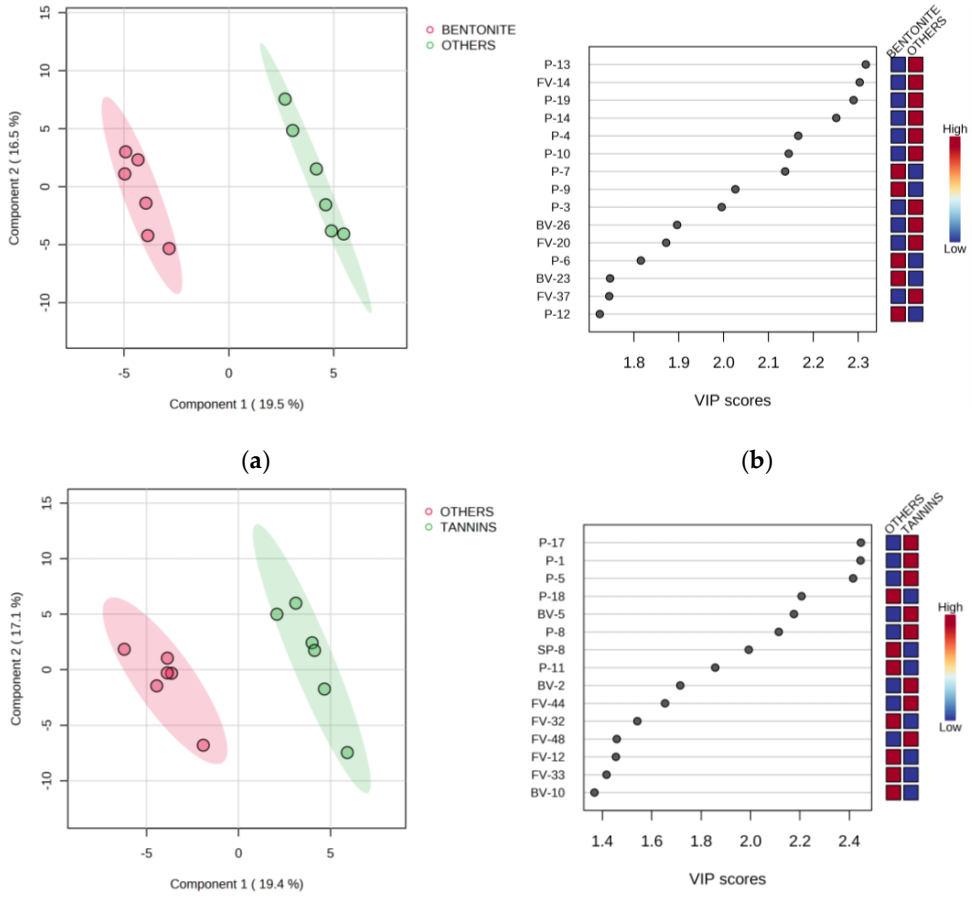

Figure 1. (**a**) Differentiation of Malvazija istarska wines produced with bentonite in fermentation (BENTONITE, B and BT) from other wines produced without bentonite in fermentation (OTHERS, CO and T) by partial least squares-discriminant analysis (PLS-DA); (**b**) variable importance in projection (VIP) scores of the variables most useful for the differentiation based on the addition of bentonite; (**c**) differentiation of Malvazija istarska wines produced with the addition of tannins in fermentation (TANNINS, T and BT) from other wines produced without tannins in fermentation (OTHERS, CO and B) by PLS-DA; (**d**) variable importance in projection (VIP) scores of the variables most useful for the differentiation based on the addition of tannins. Abbreviations: CO—control wine without added tannins and bentonite in fermentation; B—bentonite added near the end of fermentation; T—tannins added during fermentation, without bentonite in fermentation; BT—tannins and bentonite added during fermentation, in the same manner as in T and B treatments; SP—standard physicochemical parameters; P—phenols; FV—free volatile aroma compounds; BV—bound volatile aroma compounds—the codes correspond to those reported in Table 3 for wines analyzed after fermentation (AFerm).

The differentiation of wines based on the same criteria remained successful when PLS-DA was applied on wines obtained after additional bentonite fining (ProStab, Figure 2). Many variables, mostly phenols, that showed a significant response to the treatments after fermentation (AFerm, Figure 1) retained their high VIP scores in protein stable wines (ProStab, Figure 2), showing the persistence of the effects through these wine finalization steps.

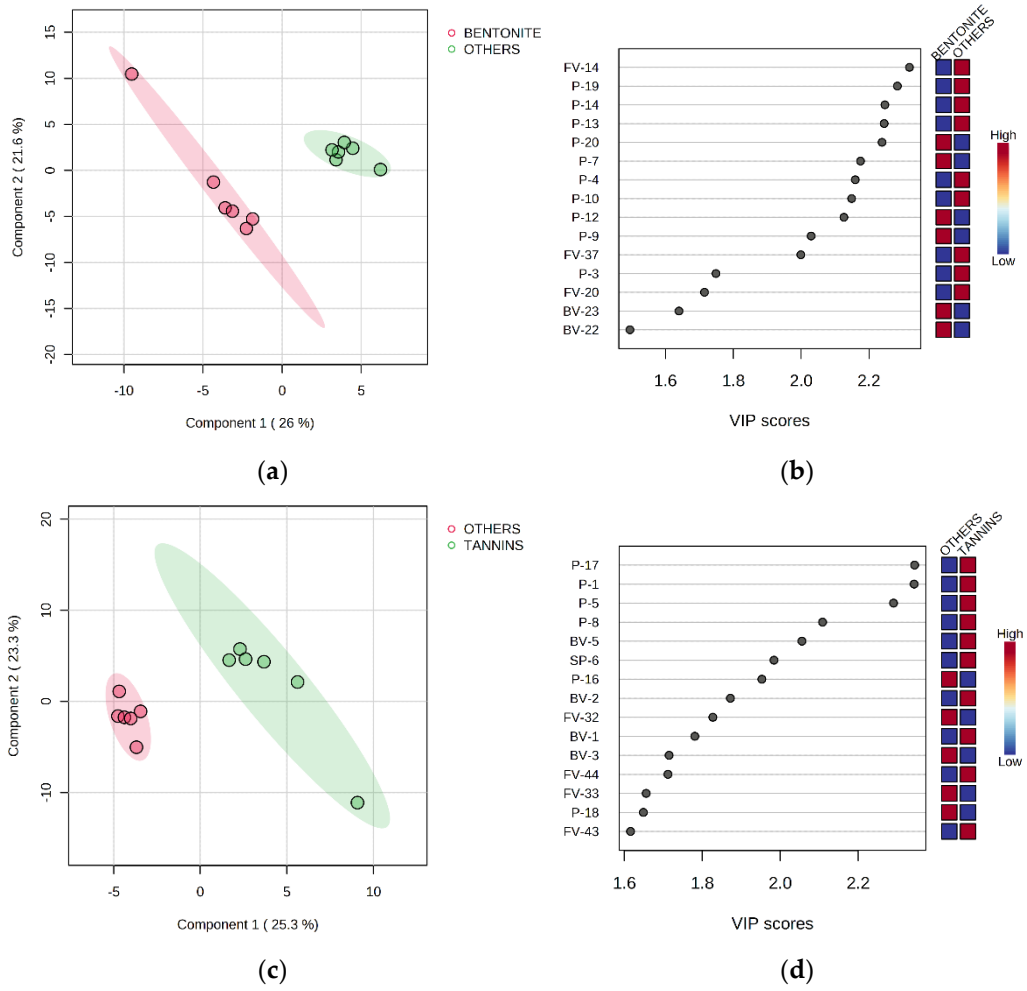

Figure 2. (**a**) Differentiation of Malvazija istarska wines produced with bentonite in fermentation (BENTONITE, B and BT) from other wines produced without bentonite in fermentation (OTHERS, CO and T) by partial least squares-discriminant analysis (PLS-DA); (**b**) variable importance in projection (VIP) scores of the variables most useful for the differentiation based on the addition of bentonite; (**c**) differentiation of Malvazija istarska wines produced with the addition of tannins in fermentation (TANNINS, T and BT) from other wines produced without tannins in fermentation (OTHERS, CO and B) by PLS-DA; (**d**) variable importance in projection (VIP) scores of the variables most useful for the differentiation based on the addition of tannins. Abbreviations: CO—control wine without added tannins and without bentonite in fermentation; B—bentonite added near the end of fermentation; T—tannins added during fermentation, without bentonite in fermentation; BT—tannins and bentonite added during fermentation, in the same manner as in T and B treatments; SP—standard physicochemical parameters; P—phenols; FV—free volatile aroma compounds; BV—bound volatile aroma compounds—the codes correspond to those reported in Table 4 for wines analyzed after additional bentonite fining (ProStab).

4. Conclusions

Treatments with the application of bentonite and tannins in fermentation, alone or in combination, significantly affected basic physicochemical parameters and composition of

phenols and free and bound volatile aroma compounds in produced wines. Treatments with bentonite decreased the concentration of total dry extract, while tannins preserved total acidity. Besides being enriched in tannins, tannin-treated wines had higher concentrations of particular phenols of lower molecular weight, including hydroxycinnamoyl tartrates and free hydroxycinnamic acids. Bentonite in fermentation not only reduced the levels of particular phenols and total flavonoids but also produced a stronger positive effect on hydroxycinnamoyl tartrates than tannins, while bentonite and tannins in combination clearly acted in synergy and provided the highest level of hydroxycinnamoyl tartrates preservation among the treatments. Treatment with tannins alone resulted in higher concentrations of several important odoriferous esters, such as ethyl hexanoate, ethyl decanoate, and hexyl acetate, while effects on the composition of glycosidically bound volatile compounds were also observed. The results of this study showed that the application of bentonite, tannins, and their combination may provide particular benefits in white winemaking and be useful for preserving or improving particular white wine features. Additional fining with bentonite during completion of wine protein stabilization annulled some of the positive effects observed after fermentation, suggesting the need to further investigate possible solutions to minimize such outcomes.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/chemosensors11100545/s1, Table S1: Doses of bentonite applied for protein stabilization of Malvazija istarska wines produced by different bentonite and tannins addition treatments. Table S2. Details of identification and quantification of free volatile aroma compounds in Malvazija istarska wines produced by different bentonite and tannins addition treatments, determined after fermentation and after final wine protein stabilization. Table S3. Details of identification and quantification of bound aroma compounds in Malvazija istarska wines produced by different bentonite and tannins addition treatments, determined after fermentation and after final wine protein stabilization. Figure S1. (a) Representative GC-MS chromatograms of an SPE extract of free volatile aroma compounds from Malvazija istarska white wine; (b) representative GC-MS chromatograms of an SPE extract of bound aroma compounds from Malvazija istarska white wine.

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