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Unconventional chitosan from mushroom by-products as an alternative fining agent for white wines stabilization

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ABSTRACT

Protein haze in white wines, conventionally managed with bentonite, poses significant drawbacks. Since bentonite fining is associated with wine losses, colour and aroma depletion, and environmental concerns, sustainable alternative are needed. This study investigated a novel and unconventional chitosan, sustainably extracted from mushroom (*Pleurotus ostreatus*) by-products using an environmentally friendly subcritical water process, as a fining agent. Chitosan was applied at 20, 60, and 100 g/hL to three unstable white wines (Graševina, Malvasia, and Traminer) to evaluate its impact on protein stability, phenolic composition, and aroma profile. The treatment significantly reduced protein instability (up to 44% in Graševina) and Pathogenesis-Related (PR) protein levels (up to 51% in Malvasia), also suggesting a variety-specific efficacy. The addition of chitosan did not significantly alter the polyphenols content or wine color and even increased an oxidizability index at the highest dosage, suggesting a preservation of wine phenolics. A dosage-dependent decrease in ester concentrations (up to 26% at 100 g/hL) was detected, while levels of alcohols, fatty acids, and varietal aroma compounds remained largely unaffected, at least above sensory thresholds, indicating a minimal impact on the wine aromatic profile. Overall, this unconventional, by-product derived chitosan offers a sustainable and promising solution for the winemaking sector, enhancing circular bioeconomy strategies.

1. Introduction

White wines are prone to several forms of instability that can compromise their appearance, flavor, and shelf life. These include protein and tartrate precipitation, oxidative browning, color degradation, and microbial spoilage - each presenting distinct challenges during production and storage. Among these, protein haze remains one of the most persistent and economically significant issues in winemaking. Although it does not affect the safety of the wine, its aesthetic impact on clarity can lead to consumer rejection and substantial financial losses (Filipe-Ribeiro et al., 2022; Van Sluyter et al., 2015).

Protein instability in white wines is primarily attributed to grape-derived pathogenesis-related (PR) proteins, notably thaumatin-like proteins (TLPs) and chitinases (Cosme, Fernandes, et al., 2020). These proteins are highly resistant to proteolytic degradation, stable under acidic conditions, and capable of unfolding and aggregating under thermal stress, leading to visible turbidity post-bottling. Their concentration and composition are influenced by grape variety, vineyard conditions (e.g., water stress, fungal infections), and harvesting methods, with mechanical harvesting often exacerbating protein accumulation due to the removal of stems that are rich of tannins. Furthermore, other pre-fermentative techniques can affect the protein amounts, as sulfite addition and grape pressing (Tian et al., 2017).

Among the various strategies employed by winemakers to prevent protein haze, bentonite fining remains the most widely adopted. Bentonite, a natural clay with a strong negative surface charge, binds unstable proteins through electrostatic interactions, forming insoluble complexes that precipitate out of solution. Despite its proven effectiveness, bentonite treatment is labor-intensive, non-selective, and often associated with undesirable side effects such as wine volume loss, aroma and phenols depletion (Horvat et al., 2019; Rakonczás et al., 2023; Ubeda et al., 2021). The addition of bentonite in winemaking also raises environmental concerns due to its interaction with proteins and other compounds, resulting in the formation of lees. These lees, classified as winery waste, possess high moisture content, which increases the energy demand for handling and disposal. Moreover, if not properly managed, they can contribute substantial organic load to wastewater systems, posing challenges for sustainable effluent treatment (Sommer &

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Tondini, 2021).

Consequently, there has been growing interest in alternative stabilization strategies that preserve wine quality while enhancing sustainability. Emerging alternatives include enzymatic hydrolysis, ultrafiltration, mannoprotein addition, and novel adsorbents such as zeolites, magnetic nanoparticles, and chitosan - each offering varying degrees of protein removal, reduced wine loss, and improved sensory preservation (Liu, Xu, et al., 2023; Silva-Barbieri et al., 2022).

Among various novel adsorbents, chitosan has garnered particular attention for its multifunctional role. Chitosan is the deacetylated derivative of chitin, the second most abundant biopolymer on earth, extracted from shellfish, insects and fungi. Chitosan has a linear structure, composed of β-1,4 linked D-glucosamine and N-acetyl-glucosamine, and it is basic and positively charged below pH 6.5. In contrast with chitin, that is highly hydrophobic, chitosan is soluble in acidic solutions according to its degree of deacetylation. Chitosan showed properties of high interest for winemaking applications: antimicrobial activity against spoilage yeasts such as *Brettanomyces bruxellensis* (Miot-Sertier et al., 2022; Tedesco et al., 2025), ability to chelate heavy metals like iron and copper (Bornet & Teissedre, 2008), ability to remove ochratoxin A (Quintela et al., 2013), and its contribution to oxidative stability through radical scavenging and browning inhibition (Castro Marín et al., 2019; Castro Marin et al., 2021; Chinnici et al., 2014).

Chitosan from fungal sources offers a biodegradable and allergen-free alternative to traditional additives. Its versatility extends to novel formats such as immobilized enzyme supports, active packaging films, and nano-structured adsorbents, positioning chitosan as a promising candidate for sustainable and quality-preserving wine production (Castro Marín et al., 2021).

Fungal chitosan from *Aspergillus niger* has been approved as a "generally recognized as safe" (GRAS) material for use in the production of alcoholic beverages without objection by the US Food and Drug Administration (US FDA) in 2011 (US Food and Drug Administration, 2011). The application in winemaking was also approved by European Commission, defining addition limits ranging from 10 g/hl to 500 g/hl according to the goal (European Union (EU), 2011); for fining purposes this limit is set at 100 g/hL (OIV (International Organization of Vine and Wine), 2009). Additionally, in 2022, the proposal of the Canadian company Chinova Bioworks Inc. to consider chitosan, specifically that obtained from white mushrooms (*Agaricus bisporus*), as a GRAS material for use as an antimicrobial in food and alcoholic beverages has been accepted (US Food and Drug Administration, 2022).

The efficiency and functionality of chitosan are strongly dependent on the processing methods and conditions employed during its extraction and modification. Variables such as temperature, reaction time, chemical concentration, and the sequence of deacetylation and depolymerization steps directly affect its molecular weight and degree of acetylation, which in turn determine key characteristics like solubility, bioactivity, and mechanical strength (Kou et al., 2021). Although the conventional chemical extraction method is widely adopted due to its shorter processing times, it presents significant drawbacks (El Knidri et al., 2018). These include the use of high temperatures and harsh reagents, high water consumption, and elevated energy demands, all of which contribute to excessive waste generation and significant environmental impact. The harsh process conditions often result in inconsistent polymer characteristics and structural integrity of chitosan, yielding materials with broad variability in polymerization and acetylation profiles (Bastiaens et al., 2019; Wattjes et al., 2020; Yang & Yan, 2018). The development of alternative strategies to replace hazardous chemicals, reduce environmental impact, and enhance control over the final properties of chitosan has gained increasing attention. Several innovative approaches have been explored, including the use of ultrasounds, microwaves, pulsed electric fields, ionic liquids, deep eutectic solvents, and subcritical water (Egorov et al., 2023; Tyliszczak et al., 2020). Subcritical water represents a promising and environmentally sustainable technology for chitosan recovery from diverse sources, offering superior efficiency and shorter processing times than conventional methods. Its inherent versatility allows process conditions to be tailored to the biomass source and target polymer properties, profoundly shaping chitosan's polymeric structure to meet specific needs. Moreover, chitosan can be successfully extracted from mushroom stipe residues - by-products of edible mushroom cultivation - thus contributing to circular bioeconomy strategies. This approach not only reduces environmental impact but also offers a viable alternative to traditional sources, expanding the potential for sustainable chitosan production (Natolino et al., 2025). Indeed, global edible mushroom production is estimated at 44–50 million tonnes per year, generating 20–30% waste/by-products, corresponding to roughly 9–15 million tonnes of potentially valorisable biomass. In contrast, total crustacean production is approximately 6–8 million tonnes annually, yielding about 2.5–3.5 million tonnes of shells (around 40% of biomass), which currently supply nearly 70% of the commercial chitosan feedstock. Beyond this quantitative advantage, mushroom stems are available year-round and are not constrained by seasonality or environmental variabilities affecting fisheries, offering a more stable and predictable resource base (Terkula Iber et al., 2022; Verardi et al., 2023).

This study investigated the use of unconventional chitosan, extracted by subcritical water from mushroom stems (*Pleurotus ostreatus*), as a fining agent in white wine production. Experimental trials were conducted by applying three dosages (20, 60, and 100 g/hL) of chitosan to three unstable white wines produced from different grape varieties—-Graševina, Malvasia, and Traminer. The impact of chitosan addition was assessed through heat tests to evaluate protein stability, and PR-proteins were quantified using high-performance liquid chromatography (HPLC). Furthermore, the study examined effects on color, phenolic composition, and aromatic profile, evaluating insights into the broader enological potential of mushroom stems-derived chitosan.

2. Material and methods

2.1. Reagents and solvents

Analytical grade ethanol (purity >99%) and acetonitrile (purity >99%) were purchased from VWR international Srl (Milan, Italy). Analytical grade chemicals including Folin-Ciocalteau reagent, sodium tartrate, sodium carbonate, sodium hydroxide, sodium potassium tartrate, copper (II) sulfate, trifluoroacetic acid, malic acid, hydrogen peroxide (30% v/v), glucose, and bovine serum albumin were also purchased from Sigma Aldrich Co. (Milan, Italy).

2.2. Chitosan production

Chitosan was recovered from mushroom (*Pleurotus ostreatus*) stems, underutilized biomass byproduct generated during the trimming process prior to packaging and commercialization of edible mushrooms. Furthermore, a novel subcritical water treatment process was performed according to the most effective operational parameters previously reported in the literature (Natolino et al., 2025). Briefly, deproteinization and purification step was performed using subcritical water at 120 °C and with the addition of 10% of malic acid. A Dionex ASE 350 Accelerated Solvent Extraction System (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used with 100 mL stainless-steel cells, loaded with the dried and grounded mushroom stems (25 g), and 250 mL collection vial. The operating pressure was set at 10.3 MPa (1500 psi), the flush volume at 50 %, the purge time at 60 s, selecting the press solvent saver mode, and the processing static time was set at 30 min. Finally, the chitin recovered as solid residue was decoloured and deacetylated following the methods reported in literature (Hao et al., 2021; Liu, Matouri, et al., 2023). The obtained chitosan was lyophilized, grounded to a fine powder, and stored in dark screw-capped vial at room temperature.

2.3. Chitosan characterization

2.3.1. Moisture, ash and protein content determination

The moisture content of chitosan was measured by a moisture analyser (PCE MB-60C, PCE Italia Srl), using the thermogravimetric method, where approximately 1 g sample was heated at 105 °C until constant weight was achieved, and moisture was calculated as the percentage weight loss. The ash content was determined by incinerating approximately 1 g of sample in a muffle furnace at 550 °C for 6 h until constant weight was reached. The ash content was calculated as the percentage of residual weight relative to the initial dry sample.

Finally, the protein content was determined by first preparing a 1% (w/v) chitosan solution by dissolving the chitosan powder in 1% (v/v) acetic acid aqueous solution. The reaction mixture was prepared by combining 1 mL of 1% (w/v) $CuSO_4$ solution, 1 mL of 2% (w/v) sodium tartrate solution, and 100 mL of 2% (w/v) Na_2CO_3 in 0.1 N NaOH. Then, 2 mL of this reagent was added to 400 μL of chitosan solution, mixed, and incubated for 10 min. Subsequently, 200 μL of Folin–Ciocalteu reagent was added, and the mixture was vigorously homogeneized. After 30 min, absorbance was measured at 750 nm in 10-mm path-length cuvettes using a UV–Vis spectrophotometer (Shimadzu), against a water blank. The protein content, expressed as milligrams per gram (mg/g), was calculated using a calibration curve created with standard solutions of bovine serum albumin in the range of 0–100 mg/L (R^2 = 0.98).

2.3.2. Dynamic (DLS) and electrophoretic (ELS) light scattering measurement

Chitosan was dissolved in a tartaric acid buffer (pH 3.2) containing 12% ethanol at a concentration of 1 g/L, then agitated for 48 h prior to analysis. Dynamic and electrophoretic light scattering measurements were carried out with a Nicomp 380 ZLS Nanoparticle Size Analyzer (Particle Sizing Systems, Santa Barbara, CA) equipped with a 10 mW He-Ne laser at a wavelength of 633 nm. The mean particle diameter distribution, reported in micrometres (μm), was obtained at a 90° scattering angle relative to the incident beam. The ζ-potential, expressed in millivolt (mV), was determined via ELS at a scattering angle of 17.5° and an electric field strength of 5 V/cm.

2.3.3. Fourier-Transform Infrared (FT-IR) spectrometer analysis

The infrared spectra of chitosan obtained from mushroom stems by subcritical water was performed using a Bruker Alpha-P ATR FT-IR spectrometer (Bruker Corporation, USA) equipped with Opus software (version 7.5). The analyses were conducted in the frequency range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} and 64 scans. The specific absorbances at 3450 cm^{-1} (A_{3450}), 2920 cm^{-1} (A_{2920}), 1655 cm^{-1} (A_{1655}) and 1379 cm^{-1} (A_{1379}) were acquired. The degree of acetylation (DA) and crystallinity index (CI) were calculated using the following equations:

$$DA = \frac{A_{1655}}{A_{3450}} \times 115$$

$$CI = \frac{A_{1379}}{A_{2920}}$$

2.3.4. Solubility measurements in wine-model solution

Chitosan was dispersed in a wine model solution consisting of a tartaric buffer (pH 3.2) containing 12% v/v ethanol, at a concentration of 1 g/L. The suspension was agitated for 48 h at room temperature and then filtered through a Büchner funnel equipped with a 0.22 μm nylon membrane. The filtrate was collected, dehydrated in a ventilated oven at 105 °C for 6 h, and weighed after cooling to room temperature in a desiccator. Chitosan solubility in the wine model solution was expressed as milligrams per liter (mg/L) and calculated with the following equation:

$$S = \frac{W_{chi} - W_{res}}{V}$$

where W_{chi} (mg) is the initial amount of chitosan dispersed in the volume V (L) of wine model solution, and W_{res} (mg) is the mass of solid residue recovered from the retentate after filtration and drying.

2.4. Wine samples

Three white wines exhibiting protein instability were selected from distinct viticultural regions. Two samples, corresponding to the Graševina and Malvasia cultivars from the 2024 vintage, were obtained from a winery located in the Istria region of Croatia. The third sample, a Traminer cultivar also from the 2024 vintage, was sourced from a winery in the Trentino-Alto Adige region of Italy. The initial physiochemical characteristics of the wines including ethanol content, pH, total titratable acidity, concentration of reducing sugars, and levels of free and total sulfur dioxide—are detailed in Table 1.

2.5. Wine treatments

Chitosan derived from mushroom stems was added to each wine sample at concentrations of 20, 60, and 100 g/hL. The treatments were conducted in 250 mL amber glass bottles. After addition, the samples were continuously agitated on an orbital shaker at 150 rpm and 16-18 °C for 48 h. Subsequently, the wine samples were filtered using syringe filters equipped with a 0.8 μm cellulose membrane. All the experiments were carried out in triplicate.

2.6. Analytical determinations

2.6.1. Heat stability test (HT)

Ten milliliters of sample were filtered through 0.45 μm filters and sealed in test tubes with screw caps. The tubes were heated at 80 °C for 6 h (Gabrielli et al., 2016; McRae et al., 2018). Afterward, the sample was left at refrigeration conditions (T = +4 °C) for 24 h to ensure the precipitation of the unstable wine proteins.

The samples turbidity was measured before and after heating and the difference was calculated with the following formula:

$$HT = NTU_H\text{-}NTU_1$$

where *HT* is the heat test, NTU_H is the value of turbidity after heat treatment, and NTU_1 is the initial turbidity value. The wine samples were considered unstable when HT values were ≥5.5 (Moreno-Arribas & Polo, 2009).

2.6.2. Protein determination by High Performance Liquid Chromatography (HPLC)

Wine proteins were recovered by mixing 4 mL of wine sample with 20 mL of 96% (v/v) ethanol. The mixture was stored at 4 °C overnight. Subsequently, the mixture was centrifuged at 3000 rpm for 10 min and the supernatant ethanol was discarded. The resulting protein precipitate

Table 1
Chemical characterization of untreated wines.

		Graševina	Malvasia	Traminer
Alcohol content	(% v/v)	12.2 ± 0.1 c[a]	14.3 ± 0.1 a	13.2 ± 0.2 b
pH	(—)	3.57 ± 0.01 a	3.17 ± 0.01 b	3.56 ± 0.01 a
Titratable acidity	(g/L)	7.24 ± 0.05 a	6.25 ± 0.01 b	5.04 ± 0.04 c
Reducing sugars	(mg/L)	6.54 ± 0.09 a	5.80 ± 0.05 b	4.34 ± 0.03 c
Free SO_2	(mg/L)	84.5 ± 2.1 a	70.0 ± 0.1 b	61.5 ± 0.7 c
Total SO_2	(mg/L)	12.5 ± 0.7 a	13.5 ± 0.7 a	10.0 ± 0.1 b

Each data represent the mean of three replicates ± standard deviation

[a] Different letter within line indicate significant differences ($p < 0.05$).

was washed twice with ethanol before being dissolved in 2 mL of Milli-Q water. HPLC analysis was performed on an LC-2010 AHT liquid chromatographic system (Shimadzu, Kyoto, Japan), equipped with an integrated autosampler and UV–Vis detector. Compounds were separated on a 4.6 × 250 mm Vydac C8 column (Altech, Milan, Italy), coupled with a 4.6 × 5 mm precolumn (Altech, Milan, Italy) with the same stationary phase, and thermostated at 35 °C. The mobile phase was composed of 83% (v/v) solvent A (0.1% trifluoroacetic acid in 8% acetonitrile solution) and 17% (v/v) solvent B (0.1% trifluoroacetic acid in 80% acetonitrile solution). A linear gradient was set as follows: solvent B was increased from 17% to 49% in the first 7 min, from 49% to 57% from 7 to 15 min, from 57% to 65% from 15 to 16 min, from 65% to 81% from 16 to 30 min, and then held at 81% for 5 min before re-equilibrating the column in the starting conditions for an additional 6 min. The injection volume was 100 μL and the flow rate was set to 1 mL/min.

The peaks were detected at 210 nm and qualitative analysis was carried out as reported in literature (Marangon et al., 2009); peaks with a retention time between 9 and 12 min were assigned to the TL protein classes, whereas peaks eluted from 18.5 to 24.5 min were assumed to be chitinases. Protein quantification was done through a calibration curve of Bovine Serum Albumin (BSA) at different concentration (50-1000 ppm).

2.6.3. Total polyphenols content

The total polyphenols content (TPC) was determined using Folin–Ciocalteau reagent. Briefly, the reaction mixture contained 100 μL of sample, 500 μL of the Folin–Ciocalteau reagent, 4 mL of water, and 2 mL of a sodium carbonate–water solution (15% w/v). After 2 h of reaction at room temperature, the absorbance was read at 750 nm using a UV–Vis spectrophotometer (Shimadzu UV-1650, Italy). The TPC was calculated by a calibration curve, obtained with different standard solutions of gallic acid in the range of 50–500 mg/mL (R2 = 0.99). The results were expressed as the mg gallic acid equivalent (GAE) per liter (mg $_{GAE}$/L).

2.6.4. Spectrophotometric determinations

The optical densities at 420 nm, related to wine yellow color, were measured. All the determinations were carried out in a UV-Vis spectrophotometer (Shimadzu UV 1650, Milano, Italy), using distilled water as a control. All the measurements were performed in triplicate.

2.6.5. Polyphenol Oxidative Medium (POM) test

The predisposition of wines towards browning was determined by the so-called POM-test proposed by Müller-Späth (1992), with slight modification. Briefly, 5 mL of wine were heated at 60 °C for 1 h, after addition of 25 μL of a 3% (v/v) hydrogen peroxide solution. The browning was estimated based on the percent increase of the absorbance at 420 nm, using the following equation:

$$POM\ (\%) = \frac{(O.D.420_{H_2O_2} - O.D.420_{H_2O})}{O.D.420_{H_2O}} \times 100$$

where O.D.420$_{H2O2}$ is the absorbance with H_2O_2 addition and O.D.420$_{H2O}$ with H2O addition. All the analyses were carried out with a UV–vis spectrophotometer (Shimadzu UV 1650, Tokyo, Japan).

2.6.6. SPME analysis GC-MS aroma compounds

The volatile composition of control and treated samples was characterized by SPME–GC–MS. Analyses were carried out using a a GCMS-QP2020 NX GC-MS system (Shimadzu, Kyoto, Japan) with a 2800T autosampler (HTA S.r.l., Brescia, Italy). Wines samples (10 mL) and two internal standards (100 μL of n-dodecanol 0.106 g/L and 50 μL of ethyl heptanoate 0.094 g/L in ethanol) were introduced in 20 mL glass vials sealed with PTFE/silicone septa, with 3 g of NaCl. Vials were preconditioned for 15 min at 40 °C before microextraction, and SPME was run at the same temperature for 15 min, using a 2 cm 50/30 lm divinylbenzene/carboxen/polydimethyl-siloxane fiber (Supelco, Bellefonte, PA, USA). A J&W DB-Wax capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness (Agilent Technologies Inc., Santa Clara, CA, USA) was used for the GC separation, with the following operating conditions: 40 °C for 5 min, then 4 °C min^{-1}, up to 240 °C, with a final holding of time of 10 min. Injection was performed in splitless mode with 5 min of splitless time; injection port and transfer line were set at 250 and 240 °C respectively. Carrier gas was helium, at a linear flow rate of 0.9 mL/min. The mass spectrophotometer was set in SCAN mode at the range m/z ratio 30-350. The process of identifying volatile compounds involved recording electron impact mass spectra at 70 eV and comparing them with those found in the mass spectrum libraries (Wiley 6 and NIST 107, 20 and 21) of the software GCMS solution by Shimadzu. On the basis of n-alkane retention periods, linear retention indices were also computed and compared to those documented in the literature (http://webbook.nist.gov/chemistry/). Using the internal standard method, semi-quantitative analysis was carried out with a response factor of 1.00. Furthermore, the odour activity value (OAV) of each identified compound was calculated as the ratio of concentration over its perception threshold and used to indicate the contribution of different volatile compounds to the overall aroma in the wine (Capone et al., 2013).

2.7. Statistical analysis

The experimental trials were performed in triplicate, and the results were expressed as mean ± standard deviation. Minitab 17 software (Minitab Inc., State College, Pennsylvania, US) was used for statistical analysis using one-way analysis of variance (ANOVA) and at Tukey's honest significant difference multiple comparison test was performed for revealing significant differences between groups (*p-value* <0.05).

3. Results and discussion

3.1. Chitosan characterization

The chemical characterization of chitosan isolated from mushroom stems by subcritical water (SCW) processing is reported in Table 2. The obtained SCW chitosan exhibited a moisture content of 15 ± 1 %, which slightly exceeds the 7–12 % range typically observed for dried commercial or crustacean-derived chitosan, yet remains consistent with the stability expected of a dry product (Ssekatawa et al., 2021; Szymańska & Winnicka, 2015). The low ash content (2.48 ± 0.09 %) and residual protein level (1.43 ± 0.38 mg/g) reflect the efficacy of the SCW treatment in removing inorganic matter and proteins, comparable to values achieved following conventional acid–alkali demineralization and deproteinization procedures (Marzieh et al., 2019; Thamer et al., 2025). The limited protein residue is corroborated by the FT-IR spectrum (Fig. 1), which shows the absence of the characteristic amide I absorption band at 1700–1740 cm^{-1} attributable to C=O stretching in proteins (Hassainia et al., 2018). FT-IR analysis further enabled estimation of the degree of acetylation and the crystallinity index. The chitosan exhibited

Table 2
Chemical characterization of chitosan obtained by subcritical water (SCW) from mushroom *(Pleurotus ostreatus)* stems.

Parameters		SCW chitosan
Moisture	*(%)*	15 ± 1
Ash content	*(%)*	2.48 ± 0.09
Proteins content	*(mg/g)*	1.43 ± 0.38
Particle size distribution[a]	*(μm)*	1.33 ± 0.36
ζ potential[a]	*mV*	+69.84 ± 1.75
Degree of deacetylation	*(%)*	82 ± 3
Cristallinity index	*(–)*	0.29 ± 0.05
Solubility[a]	*(mg/L)*	2.18 ± 0.26

[a] estimated in a wine model solution constituted by a tartaric acid buffer solution (pH 3.2) with 12% of ethanol.

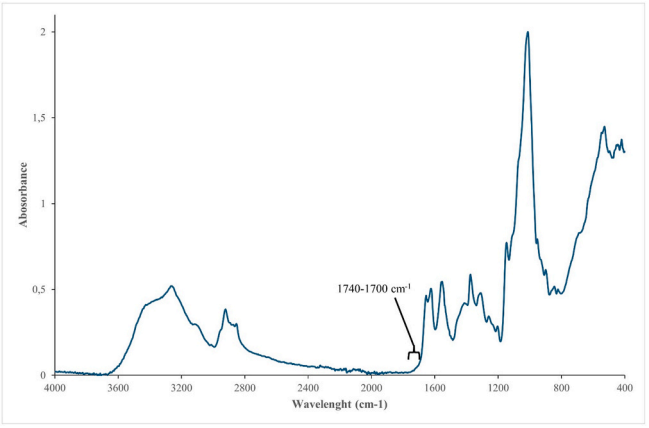


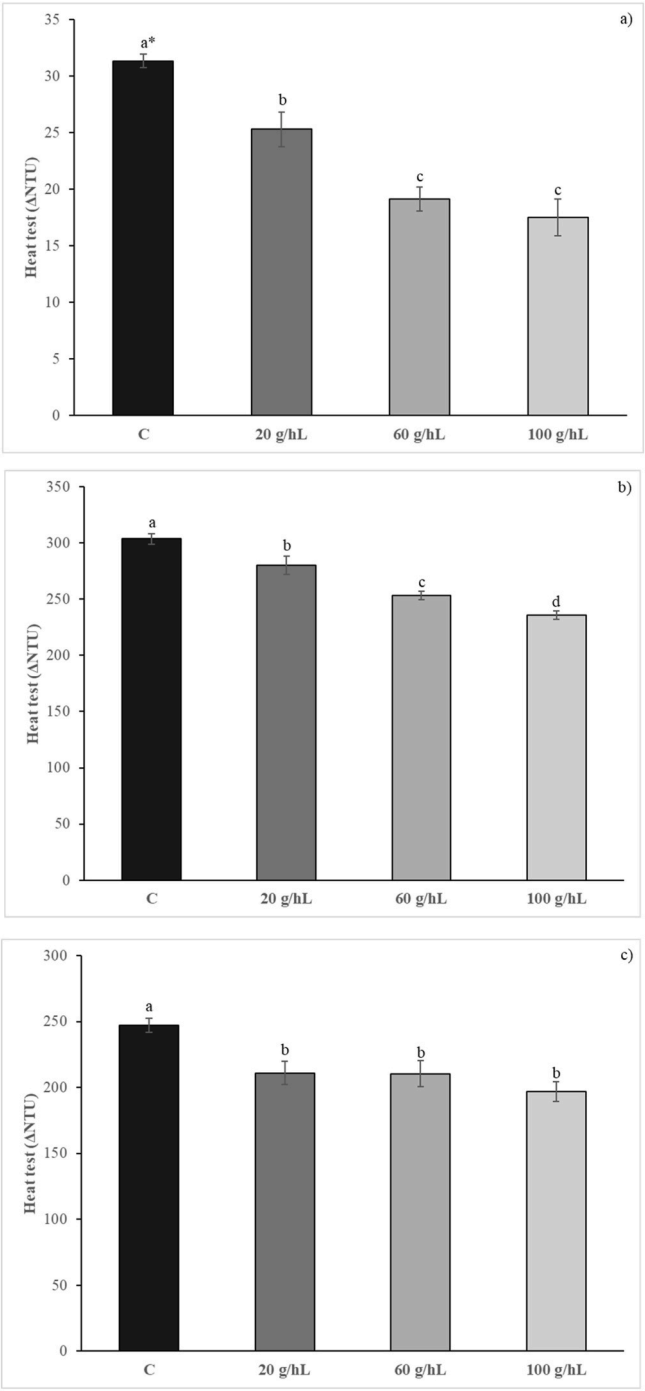
Fig. 1. FT-IR spectra of chitosan obtained from mushroom stems by subcritical water process.

a DA of 82 ± 3 %, comparable to the range (80–90 %) generally reported for *Agaricus bisporus* and other fungal chitosan, values regarded as optimal for achieving a high density of protonated amino groups and enhanced bioactivity (Westlake et al., 2025; Wu et al., 2019). In line with evidence that fungal chitosan is typically less crystalline than their crustacean analogues (Iqbal et al., 2024), the CI value of 0.29 ± 0.05 suggests a predominantly amorphous morphology, which likely enhances the accessibility and reactive potential of amine functionalities.

In a wine-like tartaric buffer (pH 3.2, 12 % v/v ethanol), the SCW chitosan dispersed as micron-sized particles (1.33 ± 0.36 μm) displaying a highly positive ζ-potential (+69.84 ± 1.75 mV). These values are comparable to, or higher than, those previously reported for protonated fungal chitosan, implying good colloidal stability and strong electrostatic affinity toward charged wine constituents. The solubility of SCW chitosan in the wine-like medium was low (2.18 ± 0.26 mg/L), comparable to bentonite and other established fining adjuvants. Such limited solubility minimizes residual impact and facilitates removal by conventional filtration, thus identifying SCW-derived fungal chitosan as a technically viable and environmentally sustainable biopolymer for clarification and stabilization applications in wine and other acidified beverages (Vendramin et al., 2021).

3.2. Protein instability

Protein stability tests assess white wine haze risks from PR-protein precipitation and colloids, guiding fining agent dosages (Cosme, Inês, et al., 2020). These quantify total proteins or solubility loss via heat/chemicals (Ribéreau-Gayon et al., 2021), with the heat test as industry's most reliable haze predictor (Esteruelas et al., 2009). Fig. 2 shows the results of heat tests conducted on three white wines, Graševina (a), Malvasia (b), and Traminer (c), before (C) and after the addition of mushroom stem-derived chitosan at increasing dosages (20, 60, and 100 g/hL). The heat test performed on untreated wine samples revealed different levels of protein instability across different grape varieties. Malvasia and Traminer wines exhibited significantly higher protein instability, with heat test values of 304 ± 5 and 247 ± 6 respectively, compared to Graševina, which showed a much lower value of 31 ± 1. The application of chitosan derived from mushroom stems resulted in a significant reduction of protein instability across all tested white wine samples. The most substantial effect was observed in Graševina variety, which exhibited a 44% decrease in instability at the highest treatment level of 100 g/hL. In contrast, Malvasia and Traminer responded less markedly, showing an average reduction of approximately 21% under the same chitosan dosage. Furthermore, the effect of chitosan concentration varied depending on the wine variety. In both Graševina and Malvasia, significant differences were observed across the



* Different letters indicate significant differences (p<0.05)

Fig. 2. Protein instability evaluated by heat test of different white wines (a – Graševina; b - Malvasia; c - Traminer), before (C) and after addition of chitosan at different concentrations (20, 60, and 100 g/hL).
* Different letters indicate significant differences ($p < 0.05$).

tested dosages, and increasing the dosage allowed achieving lower heat test values. In contrast, Traminer did not exhibit notable changes in protein instability when the dosage exceeded 20 g/hL, suggesting a saturation threshold or reduced responsiveness to higher chitosan levels. The heat test results confirm that chitosan derived from mushroom stems exhibits a partial effectiveness in stabilizing unstable proteins in white wines. While significant reductions in protein instability were observed—particularly in Graševina and, to a lesser extent, in Malvasia

and Traminer—the treatment does not allow the complete stabilization. This outcome aligns with findings reported in the literature, suggesting that although chitosan can improve protein stability and it may need to be complemented by additional fining agents or process adjustments (Chagas et al., 2012; Gruppi et al., 2024; Lárez Velásquez, 2023). However, the efficiency of mushroom stem–derived chitosan can be compared with that of commercial chitosan obtained from *Aspergillus niger*. Arenas et al. (2021), for example, reported a 40–45% decrease in protein instability, expressed as a reduction in heat test values, when applying commercial *A. niger* chitosan within the limits established by the International Organisation of Vine and Wine (100 g/hL). Their study was carried out on Albariño white wine, which exhibited an initial protein instability comparable to that observed in the Graševina wine analyzed in the present study.

Protein haze formation in wine is a multifactorial process. In addition to the influence of matrix parameters and interactions with other macromolecules, the concentration and type of pathogenesis-related proteins play a pivotal role. Some authors have suggested that haze is primarily associated with total protein content, with wines containing higher overall protein concentrations being more prone to instability. Conversely, other studies report that protein instability is not solely determined by the total protein content, since individual protein fractions display different behaviors and contribute unequally to haze formation (Cosme, Fernandes, et al., 2020). The total PR-proteins content and the concentrations of the two main classes of them, thaumatin-like proteins and chitinases, measured before and after the addition of mushroom stem–derived chitosan at different dosages (20, 60, and 100 g/hL), are reported in Table 3. The content of PR proteins in untreated wines differed among varieties, and a strong linear correlation (R^2 = 0.9954) was observed with protein instability as measured by the heat test. The addition of chitosan significantly reduced PR protein levels in all varieties, with maximum reductions of 37%, 51%, and 12% observed in Graševina, Malvasia, and Traminer wines, respectively, at the highest dosage (100 g/hL). A significant effect was already observed at a dosage of 20 g/hL; however, further increases produced different outcomes depending on the wine variety. In Graševina and Traminer, raising the dosage above 60 g/hL did not result in any additional significant effect, whereas in Malvasia an increase from 60 g/hL to 100 g/hL led to a marked decrease in PR protein content, from 169.95 ± 25.32 to 109.89 ± 6.86 mg/L.

Table 3
Total PR-proteins, Chitinases, Taumatin-like protein content of untreated (0 g/hL) and treated wines with different dosages of mushroom stem-derived chitosan (20, 60, and 100 g/hL).

Wine	Chitosan dosage	Chitinases	TLPs	PR proteins
	(g/hL)	(mg/L)	(mg/L)	(mg/L)
Graševina	**Control**	7.19 ± 0.33 a[a]	24.07 ± 1.39 a	31.26 ± 1.76 a
	20 g/hL	5.17 ± 0.68 b	21.06 ± 2.42 a	26.23 ± 3.00 ab
	60 g/hL	2.97 ± 0.43 c	17.64 ± 0.43 b	20.61 ± 0.06 b
	100 g/hL	2.48 ± 0.23 c	17.08 ± 1.04 b	19.56 ± 1.28 b
Malvasia	**Control**	54.98 ± 8.90 a	170.38 ± 12.18 a	225.35 ± 7.69 a
	20 g/hL	38.87 ± 9.38 b	143.40 ± 3.99 b	182.28 ± 12.92 b
	60 g/hL	34.47 ± 6.68 b	135.48 ± 18.85 b	169.95 ± 25.32 b
	100 g/hL	14.22 ± 2.33 c	95.67 ± 4.53 c	109.89 ± 6.86 c
Traminer	**Control**	7.37 ± 0.28 a	201.65 ± 5.80 a	209.02 ± 2.54 a
	20 g/hL	5.73 ± 0.37 b	185.35 ± 5.74 b	191.08 ± 6.00 b
	60 g/hL	5.86 ± 0.41 b	184.56 ± 4.00 b	190.42 ± 4.40 b
	100 g/hL	6.08 ± 0.32 b	178.09 ± 5.12 b	184.17 ± 5.44 b

Each data represent the mean of three replicates ± standard deviation.
[a] Different letter within column and same wine variety indicate significant differences ($p < 0.05$).

After the addition of chitosan, the strong linear correlation between heat test values and PR protein content disappeared, suggesting differences in the behaviour and interactions of chitosan with specific PR protein fractions. Notably, Malvasia exhibited a markedly higher initial chitinase content (54.98 ± 8.90 mg/L) compared with Graševina (7.19 ± 0.33 mg/L) and Traminer (7.37 ± 0.28 mg/L). The effectiveness of chitosan in protein stabilization of wine has been attributed to a dual mechanism of action. It has been elucidated that the effectiveness of chitosan in protein stabilization in wine can been attributed to a selective interaction with chitinases. The proposed mechanism relies on the presence of a cysteine-rich chitin-binding domain, which enables the interaction with chitosan and the subsequent removal of the complexed proteins together with insoluble polysaccharides. It is worth mentioning that based upon the former mechanism, chitosan has been proposed as specific ligand for affinity precipitation and recovery of plant chitinases (Castro Marín et al., 2021). The results in Table 3 indicated also a particular effectiveness of mushroom stems-derived chitosan against thaumatin-like proteins, achieving a significant reduction in all three wines. However, in Malvasia wine the effect was more pronounced, and a dosage of 100 g/hL led to a 44% reduction of the initial content (from 170.38 ± 12.18 mg/L to 95.67 ± 4.53 mg/L). This phenomenon may be attributed to the lower pH of Malvasia (3.17 ± 0.01) compared to Graševina (3.57 ± 0.01) and Traminer (3.56 ± 0.01), as acidic conditions promote protonation of chitosan amino groups, thereby destabilizing protein colloidal suspensions and enhancing flocculation. Specifically, chitosan has been proposed to act as a cross-linker of the pectin network, interacting electrostatically with this anionic polymer that normally stabilizes protein colloidal suspensions, ultimately accelerating the clarification process (Taştan & Baysal, 2017; Wang et al., 2017).

Beyond the characteristics of the wine matrix, the effectiveness of chitosan toward both protein fractions could also be attributed to specific properties of the polymer itself. Indeed, it is well established that parameters such as the degree of acetylation and the crystallinity index strongly influence chitosan bioactivity. Our previous recent study demonstrated that subcritical water (SCW) processing significantly modifies these structural features, yielding chitosan with a lower crystallinity index and a higher proportion of amorphous regions compared to the conventional method. This altered structure may expose a greater number of functional groups, thereby enhancing the polymer's ability to interact with proteins and improving wine stabilization. Moreover, FT-IR spectra revealed configuration changes induced by SCW, including decreased intensities of amide I, II, and III bands and weakening of the O–H and N–H stretching vibrations, indicating a disruption of the hydrogen-bonding network. Such rearrangements contribute to the reduction of crystalline order and the prevalence of amorphous domains, further supporting the enhanced reactivity and bioactivity of SCW-derived chitosan (Natolino et al., 2025).

3.3. Color and polyphenols

The color and phenolic profile of wine are key determinants of its overall quality. Color intensity defines its visual appeal, while polyphenols exert a pivotal influence on sensory attributes such as astringency, bitterness, mouthfeel, and color stability (Kumar & Suhag, 2024). The results of total polyphenol content (TPC), the optical densities measured at 420 nm (O.D. 420 nm), and the oxidation stability evaluated by a specific browning assay (POM test), of white wines before and after addition of different dosages of mushroom stems-derived chitosan

are reported in Table 4.

The addition of chitosan, even at increasing concentrations, did not alter significantly the total polyphenol content or the optical density at 420 nm in any of the white wines tested. Consistent results have been reported by other authors using commercial chitosan derived from *Aspergillus niger*, who found that it neither impaired the antioxidant activity of wine phenolics nor removed significant amounts of non-reducing polyphenols (Colangelo et al., 2018). In contrast, other fining agents commonly employed in enology for the stabilization of white wines have been shown to negatively affect both color and phenolic composition. Bentonite, in particular, has been consistently associated with a depletion of chromatic intensity and a reduction in total phenolic content, especially hydroxycinnamic acids and, to a lesser extent, flavonoids, with reported decreases of about 7% in total phenols and 3.1% in flavonoids in Malvazija Istarska wine (Cosme, Inês, et al., 2020; Lukić et al., 2023). Arenas et al. (2021) further investigated the impact of different fining agents on Albariño white wine and observed a decrease in absorbance at 420 nm. In the same study, flavonoid phenols were reduced by 22.7% with sodium bentonite when pre-fermentative maceration was applied, while total phenolics significantly decreased by 18.6% with sodium bentonite and by 11.8% with calcium bentonite in the absence of maceration. Similarly, Italian Riesling wine treated with bentonite showed a 20% decrease in color intensity and a 4.8% reduction in total phenolics compared to untreated wine (Ma et al., 2020). Collectively, these findings highlight that while bentonite is effective for protein stabilization, its application may modify the chromatic and phenolic quality of white wines. Other protein-based fining agents, such as potassium caseinate and soybean protein, have also been reported to lower total phenols and reduce color intensity, although generally to a lesser extent than bentonite (Cosme, Inês, et al., 2020; Ma et al., 2020). Collectively, these findings highlight that, unlike bentonite and other conventional fining agents, chitosan appears to exert a milder effect on the phenolic composition and color stability of white wines. However, it should be emphasized that there is no absolute or uniform response to chitosan addition, as its effects can vary considerably depending on both the characteristics of the wine matrix and the physicochemical properties of the polymer.

Although no relevant effects were detected on total polyphenols (TPC) or O.D. 420 nm, a slight and gradual increase of the POM test values the oxidizability index, evaluated by Polyphenols Oxidative Medium (POM) test, was observed at the maximum dosage of chitosan (100 g/hL) for all the wine varieties, as reported in Table 4. POM test measurement consists in adding to wines an excess of H_2O_2 that completely oxidizes all oxidizable substances, including sulfites. This index was developed as a practical indicator for optimizing must hyperoxygenation: a high POM test value indicates that grape must contains a relevant concentration of oxidizable substances (primarily polyphenols), and this value generally decreases during hyperoxygenation treatments (Müller-Späth, 1992). In wine, a high POM test highlights that the wine retained its phenolic components well during processing or storage; therefore, even if showing a greater potential for browning, it has been better protected and less oxidized. In the present experiment, with the exception of Traminer, POM test values are quite low for the other two varieties; however, as the dosage of chitosan increased (Table 4), POM test also gradually increased, in agreement with the claimed antioxidant capacity reported in the literature (Castro Marín et al., 2019). The best results were obtained with an addition of 100 g/hL, that is the maximum dosage recommended by the International Organization of Vine and Wine (OIV) for must and wine fining and for the removal of heavy metals. With this added amount, increments were relevant: 102 % of the original value for Graševina, 171 % for Malvasia and 41 % for Traminer.

Table 4
Total polyphenol content (TPC), Optical density at 420 nm (O.D. 420 nm), and Polyphenols Oxidative Medium (POM) test of untreated (0 g/hL) and treated wines with different dosages of mushroom stem-derived chitosan (20, 60, and 100 g/hL).

Wine	Chitosan dosage	TPC	O.D. 420 nm	POM test
	(g/hL)	(mg_{GAE}/L)	(—)	(%)
Graševina	**Control**	252.2 ± 4.3 a[a]	0.086 ± 0.005 a	5.1 ± 0.1 b
	20 g/hL	243.8 ± 5.0 a	0.086 ± 0.004 a	6.3 ± 1.7 b
	60 g/hL	257.8 ± 15.4 a	0.094 ± 0.010 a	7.2 ± 1.9 b
	100 g/hL	242.2 ± 6.2 a	0.096 ± 0.006 a	10.3 ± 2.2 a
Malvasia	**Control**	264.8 ± 0.5 a	0.133 ± 0.005 a	2.1 ± 0.9 b
	20 g/hL	262.0 ± 2.9 a	0.125 ± 0.002 a	2.7 ± 0.4 b
	60 g/hL	260.4 ± 7.6 a	0.126 ± 0.001 a	3.3 ± 0.9 b
	100 g/hL	262.3 ± 6.4 a	0.131 ± 0.005 a	5.7 ± 0.6 a
Traminer	**Control**	210.6 ± 1.4 b	0.132 ± 0.005 a	40.2 ± 1.3 b
	20 g/hL	213.5 ± 4.0 ab	0.141 ± 0.015 a	45.9 ± 1.8 b
	60 g/hL	217.2 ± 4.2 ab	0.131 ± 0.02 a	43.3 ± 3.7 b
	100 g/hL	222.6 ± 5.0 a	0.141 ± 0.013 a	56.8 ± 2.0 a

Each data represents the mean of three replicates ± standard deviation.
[a] Different letter within column and same wine variety indicates significant differences ($p < 0.05$).

3.4. Aroma profile

The volatile composition of wine, encompassing over 1000 distinct aroma and flavour compounds, is fundamental to its sensory profile. The chemical classes, concentrations, and sensory attributes of these volatiles contribute to the complexity of wine flavour, which is a key factor in consumer preference (Duan et al., 2018; Sánchez-Palomo et al., 2019). The impact of clarification treatments on aroma compounds is governed by multiple variables, including the chemical properties of the target volatiles, the composition and the physicochemical characteristics of the fining agents, and their interactions with macromolecules previously associated with the fining process (Rihak et al., 2022).

The content of the main volatile classes, esters, alcohols, fatty acids, terpenes, and norisoprenoids, of Graševina, Malvasia, and Traminer wines before and after addition of different chitosan dosages is reported in Table 5. A significant and comparable reduction in total esters concentration was observed across all analyzed wine samples at the highest chitosan dosage (100 g/hL): −23% in Graševina, −20% in Malvasia, and −26% in Traminer. In Graševina and Malvasia, a notable decrease was also evident at the lowest dosage (20 g/hL), with an average reduction of approximately −12%, and a clear dose-dependent trend was observed, with esters reduction increasing alongside chitosan concentration. In contrast, Traminer showed no significant differences in ester concentration between the tested chitosan dosages, indicating that the effect of chitosan on the decrease of ester content in this variety seems to be independent on dosage. Esters are among the most important volatile compounds contributing to wine aroma, primarily formed through chemical esterification between alcohols and acids or biosynthesized by yeast strains during alcoholic fermentation (Robinson et al., 2014). The observed decrease in ester content aligns with findings from previous studies investigating alternative fining agents, such as pea and soybean proteins applied at concentrations of 0.3 and 0.5 g/L, which resulted in reductions of 17% and 22%, respectively (Ma et al., 2020; Rihak et al., 2022). However, the extent of ester loss in the present study remains lower than that reported for bentonite treatments, which led to a reduction of up to 48%, and other stabilization techniques such as membrane filtration, which caused a decrease of approximately 60% (Ma et al., 2020). Esters are typically classified into three subclasses, ethyl esters, acetate esters, and other minor ester types, each playing a distinct role in shaping the fruity and floral sensory attributes of wine (Robinson et al., 2014). Among these, ethyl esters represent the quantitatively dominant group, accounting for over 90% of the total ester

Table 5
Content of the main volatile classes of untreated Graševina, Malvasia, and Traminer wines (0 g/hL) and after treatment with different dosages of mushroom stem-derived chitosan (20, 60, and 100 g/hL).

Wine variety	Compounds		Treatment			
			Control	20 g/hL	60 g/hL	100 g/hL
Graševina	*Total esters*	(μg/L)	1370 ± 95 a[a]	1231 ± 53 b	1154 ± 52 bc	1062 ± 19 c
	Ethyl esters	(μg/L)	1243 ± 88 a	1132 ± 46 ab	1059 ± 45 bc	971 ± 17 c
	Acetate esters	(μg/L)	92 ± 6 a	67 ± 5 b	63 ± 5 b	60 ± 1 b
	Other esters	(μg/L)	34 ± 1 a	32 ± 2 ab	32 ± 1 b	31 ± 1 b
	Alcohols	(μg/L)	849 ± 111 a	690 ± 84 a	704 ± 47 a	723 ± 19 a
	Fatty acids	(μg/L)	108 ± 8 a	110 ± 8 a	90 ± 7 b	75 ± 3 c
	Terpenes	(μg/L)	9.9 ± 0.9 a	5.5 ± 0.6 b	5.4 ± 0.3 b	4.0 ± 0.2 b
	Norisoprenoids	(μg/L)	4.5 ± 0.5 a	3.7 ± 0.5 ab	3.4 ± 0.3 ab	2.8 ± 0.1 b
Malvasia	*Total esters*	(μg/L)	1878 ± 17 a	1624 ± 63 b	1457 ± 82 c	1509 ± 39 bc
	Ethyl esters	(μg/L)	1838 ± 14 a	1593 ± 60 b	1423 ± 80 c	1474 ± 38 bc
	Acetate esters	(μg/L)	14.8 ± 0.4 a	14.0 ± 0.5 a	14.1 ± 1.3 a	14.4 ± 0.9 a
	Other esters	(μg/L)	24.8 ± 1.8 a	17.0 ± 1.5 c	19.3 ± 1.4 bc	20.7 ± 0.6 b
	Alcohols	(μg/L)	1316 ± 10 a	1333 ± 61 a	1461 ± 138 a	1410 ± 132 a
	Fatty acids	(μg/L)	120 ± 7 a	115 ± 6 a	113 ± 8 a	115 ± 8 a
	Terpenes	(μg/L)	30.7 ± 0.6 a	28.4 ± 1.0 a	31.4 ± 2.7 a	28.6 ± 2.1 a
	Norisoprenoids	(μg/L)	3.68 ± 0.02 a	3.97 ± 0.11 a	3.75 ± 0.45 a	3.98 ± 0.04 a
Traminer	*Total esters*	(μg/L)	1545 ± 91 a	1139 ± 128 b	1142 ± 99 b	1143 ± 46 b
	Ethyl esters	(μg/L)	1390 ± 86 a	928 ± 108 b	936 ± 90 b	942 ± 41 b
	Acetate esters	(μg/L)	132 ± 1 a	128 ± 12 a	118 ± 6 b	116 ± 2 b
	Other esters	(μg/L)	89 ± 5 a	84 ± 8 a	89 ± 4 a	85 ± 3 a
	Alcohols	(μg/L)	1574 ± 131 a	1539 ± 53 a	1315 ± 119 a	1587 ± 78 a
	Fatty acids	(μg/L)	188 ± 5 a	182 ± 17 a	177 ± 5 a	183 ± 3 a
	Terpenes	(μg/L)	178 ± 20 a	183 ± 7 a	174 ± 11 a	187 ± 6 a
	Norisoprenoids	(μg/L)	12.0 ± 0.9 a	12.5 ± 0.5 a	10.8 ± 0.1 a	12.6 ± 0.1 a

Each data represents the mean of three replicates ± standard deviation.
[a] Different letter within line and same wine variety indicates significant differences ($p < 0.05$).

content. A reduction in their concentration following chitosan addition was observed consistently across all wine samples. Similar findings have been reported by other authors, suggesting that this effect may be linked to the specific interaction of chitosan with certain fractions of pathogenesis-related (PR) proteins (Lambri et al., 2013). However, it remains uncertain whether chitosan directly removes fermentative aroma compounds to the same extent as bentonite, which is known for its stronger binding affinity and more aggressive stripping of volatiles. The selective interaction of chitosan with specific bands of PR-proteins, coupled with its moderate impact on other protein classes, indicates a potential advantage in preserving the aromatic integrity of the wine (Colangelo et al., 2018). Evaluation of individual compounds significantly affected by chitosan addition, as reported in Table 6, revealed that the major ethyl esters include ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, and ethyl palmitate. Ethyl butanoate and palmitate were detected in Graševina and Malvasia wines and they were always under the odour threshold, also before chitosan addition, as indicated by the extremely low OAV value (below 0.004). All other ethyl esters are present well above their odour thresholds, with OAVs significantly exceeding unity. Notably, ethyl hexanoate in Traminer exhibits a reduction of over 80% following chitosan treatment at a dosage of 20 g/hL, decreasing from 435 ± 4 to 84 ± 7 μg/L. Nevertheless, its concentration remains above the odour threshold, with OAV values still exceeding 5.

The impact of chitosan treatment on acetate esters varied notably across the different wine varieties. Malvasia exhibited the lowest initial concentration of acetate esters (14.8 ± 0.4 μg/L), and no significant changes were observed following chitosan application. In contrast, Graševina and Traminer wines showed a marked reduction in acetate ester levels, decreasing from 92 ± 6 to 60 ± 1 μg/L and from 132 ± 1 to 116 ± 2 μg/L, respectively. Among the individual compounds, isoamyl acetate was identified as the predominant acetate ester, contributing characteristic banana-like sensory notes to the wines. Despite the significant reduction in isoamyl acetate concentration after treatment, its final levels remained above the odour threshold, with odour activity values (OAVs) consistently exceeding unity. This suggests that the perceptibility of this key aromatic compound seems to be preserved, maintaining its contribution to the overall sensory profile.

The other esters were detected at low concentrations and consistently below their respective odour thresholds. Consequently, their odour activity values (OAVs) remained well below unity, suggesting a limited contribution to the overall aromatic profile of the wine.

Alcohols are other key volatile compounds in wine, primarily produced through yeast metabolism during alcoholic fermentation. At concentrations exceeding 300 mg/L, they may contribute pungent sensory notes; however, at lower levels, they enhance the aromatic complexity by imparting pleasant nuances to the wine bouquet (Swiegers et al., 2005). The initial total alcohol content was 849 ± 111 μg/L in Graševina, 1316 ± 10 μg/L in Malvasia, and 1574 ± 131 μg/L in Traminer. Chitosan addition at varying dosages did not result in statistically significant changes in alcohol concentrations across the tested wine varieties.

Fatty acids have an enzyme origin during fermentation through yeast metabolism and were reported to contribute fruity, cheese, fatty, and rancid notes to wine aroma complexity at low concentrations (Ma et al., 2020). Chitosan treatments did not affect the fatty acid content in Malvasia and Traminer wines. However, a significant decrease was observed in Graševina, where total fatty acids dropped from 108 ± 8 to 75 ± 3 μg/L. In this variety, the main fatty acids identified were octanoic acid and decanoic acid, which were already present at low concentrations in the untreated wine, resulting in odour activity values (OAVs) consistently below unity. A significant effect of chitosan addition was also detected for terpenes and norisoprenoids, again exclusively in Graševina. These compounds are recognized as varietal aroma markers, primarily derived from grape metabolism (Robinson et al., 2014). In Graševina, terpenes—mainly linalool, α-terpineol, and citronellol—were found at low concentrations, well below their odour thresholds. Norisoprenoids, represented by β-damascenone with its characteristic rose and floral notes, showed a decrease after treatment; however, its concentration remained above the odour threshold, with OAV values exceeding unity, suggesting that its sensory impact may be preserved. In contrast, Malvasia and Traminer are both classified as

Table 6
Content, Odour threshold, and Odour activity value (OAV) of the volatile compounds significantly affected by mushroom stems-derived chitosan addition at different dosages.

Wine variety	Compound	Odour description[b]	Odour Threshold[b]	Content (μg/L)				Odour activity value (OAV)			
			(μg/L)	Control	20 g/hL	60 g/hL	100 g/hL	Control	20 g/hL	60 g/hL	100 g/hL
Graševina	*Ethyl butanoate*	*Flora, fruity*	20	10.6 ± 0.6 a[a]	8.4 ± 0.2 b	7.6 ± 0.4 b	7.8 ± 0.1 b	0.531	0.421	0.380	0.389
	Ethyl hexanoate	*Fruity, green apple*	14	226 ± 3 a	187 ± 5 b	170 ± 8 c	163 ± 1 c	16.169	13.391	12.165	11.644
	Ethyl octanoate	*Ripefruit,.pear. sweety*	240	687 ± 50 a	629 ± 16 ab	602 ± 27 ab	544 ± 12 b	2.862	2.619	2.509	2.266
	Ethyl dodecanoate	*Oily.fatty.fruity*	1500	213 ± 25 a	204 ± 17 b	185 ± 8 c	169 ± 3 c	0.142	0.136	0.123	0.113
	Ethyl palmitate	*Mildwaxy*	1000	0.72 ± 0.02 a	0.65 ± 0.05 a	0.34 ± 0.04 b	0.38 ± 0.05 b	0.001	0.001	0.000	0.000
	Isoamyl acetate	*Banana*	30	88 ± 5 a	63 ± 5 b	59 ± 5 b	56 ± 1 b	2.932	2.093	1.955	1.876
	β-phenethyl acetate	*Floral*	73	3.7 ± 0.2 a	3.9 ± 0.4 a	3.4 ± 0.2 ab	2.8 ± 0.1 b	0.050	0.054	0.047	0.038
	Acetic acid. hexyl ester	*pleasant fruity. pear.cherry*	670	21.7 ± 0.1 a	18.3 ± 0.6 b	17.6 ± 0.2 b	17.3 ± 0.3 b	0.032	0.027	0.026	0.026
	Methyl octanoate	*waxy. appleskin. fruity*	100-400	2.32 ± 0.02 a	1.20 ± 0.14 b	1.41 ± 0.13 bc	0.94 ± 0.05 c	0.009	0.005	0.006	0.004
	Octanoic acid	*Rancid. cheese. fatty acid*	500	75 ± 6 a	78 ± 5 a	66 ± 4 ab	58 ± 2 b	0.149	0.155	0.132	0.115
	n-Decanoic acid	*Fatty acid. dry. woody*	1400	29 ± 1 a	27 ± 2 a	19 ± 3 b	14 ± 1 b	0.021	0.019	0.014	0.010
	nerol. methyl ether	*Floral. sweet. citrus notes*	10-50	2.96 ± 0.15 a	0.37 ± 0.06 b	0.64 ± 0.07 bc	0.62 ± 0.06 c	0.099	0.012	0.021	0.021
	Linalool	*Flowery. Muscat*	25	4.68 ± 0.56 a	3.06 ± 0.37 b	2.99 ± 0.11 b	2.27 ± 0.06 b	0.187	0.123	0.120	0.091
	α-terpineol	*Floral*	250	0.19 ± 0.02 a	0.14 ± 0.02 ab	0.12 ± 0.01 b	0.10 ± 0.01 b	0.001	0.001	0.000	0.000
	Citronellol	*Rose*	100	1.43 ± 0.14 a	1.05 ± 0.11 b	0.71 ± 0.02 c	0.44 ± 0.02 d	0.014	0.011	0.007	0.004
	β-damascenone	*Rose. sweet. flowers*	0.14	4.52 ± 0.48 a	3.71 ± 0.52 ab	3.39 ± 0.32 ab	2.82 ± 0.10 b	32.304	26.467	24.180	20.112
Malvasia	*Ethyl octanoate*	*Ripefruits.pear. sweety*	240	980 ± 3 a	912 ± 23 b	843 ± 23 c	871 ± 5 bc	4.084	3.801	3.514	3.630
	Ethyl decanoate	Pleasantfruity	200	464 ± 4 a	326 ± 10 b	249 ± 24 c	242 ± 9 c	2.319	1.629	1.247	1.211
	Ethyl dodecanoate	*Oily.fatty.fruity*	1500	47.0 ± 2.0 a	24.7 ± 1.7 b	22.4 ± 0.3 b	22.2 ± 0.5 b	0.031	0.016	0.015	0.015
	Ethyl palmitate	*Mildwaxy*	1000	3.70 ± 0.02 a	1.69 ± 0.08 b	1.03 ± 0.08 c	1.28 ± 0.17 c	0.004	0.002	0.001	0.001
	Methyl octanoate	Waxy. appleskin. fruity	100-400	7.16 ± 0.11 a	6.40 ± 0.45 a	3.07 ± 0.05 b	2.48 ± 0.12 b	0.024	0.019	0.040	0.045
	Isoamyl caproate	Sweet. fruity. pineapple	1400	4.08 ± 0.02 a	0.79 ± 0.02 b	0.73 ± 0.05 bc	0.62 ± 0.09 c	0.003	0.001	0.001	>0.001
	Isoamyl octanoate	Sweet. fruity. coconut-like	70	12.0 ± 1.6 a	9.3 ± 1.1 ab	8.5 ± 0.7 ab	9.2 ± 0.4 b	0.171	0.133	0.120	0.131
	Isoamyl decanoate	*Sweet. fruity. slightly floral*	500	2.65 ± 0.03 a	1.57 ± 0.10 b	0.87 ± 0.09 c	0.96 ± 0.01 c	0.005	0.003	0.002	0.002
Traminer	*Ethyl hexanoate*	*Fruity. green apple*	14	435 ± 4 a	84 ± 7 b	76 ± 8 b	79 ± 1 b	31.055	5.970	5.438	5.648
	Ethyl-9-decenoate	*Fruity. waxy. or apple-like*	10	8.42 ± 0.39 a	1.18 ± 0.14 b	1.19 ± 0.02 b	1.07 ± 0.06 b	0.842	0.118	0.119	0.107
	Ethyl-9-hexadecenoate	*Mild waxy*	1000	2.65 ± 0.14 a	0.34 ± 0.03 b	n.d.	n.d.	0.0027	0.0003	n.d.	n.d.
	Isoamyl acetate	*Banana*	30	132 ± 1 a	128 ± 12 a	118 ± 6 b	116 ± 2 b	4.400	4.267	3.933	3.867

Each data represent the mean of three replicates ± standard deviation.
[a] Different letter within line and same wine variety indicate significant differences ($p < 0.05$).
[b] Odour description and thresholds reported in the literature: Ma et al. 2020; Capone et al. 2013.

aromatic grape varieties, with Traminer showing a notably higher content of terpenes and norisoprenoids. In these wines, chitosan treatment did not produce significant changes, and the concentrations of these aroma compounds remained stable.

The more pronounced effect observed in Graševina wine could be associated with a greater efficacy of chitosan in removing unstable proteins in this matrix. This suggests that interactions between aroma compounds and wine proteins may play a significant role in modulating the impact of fining treatments on volatile composition. Previous studies have demonstrated that wine proteins—particularly pathogenesis-related proteins such as thaumatin-like proteins and chitinases—can bind to aroma compounds, influencing their volatility and sensory perception. The selective removal of these proteins by chitosan may therefore alter the equilibrium between free and bound aroma compounds, especially in varieties with lower intrinsic aromatic intensity (Waterhouse et al., 2016).

4. Conclusions

This study evaluated the enological potential of chitosan derived from mushroom stems, extracted via subcritical water treatment, as a sustainable fining agent for white wine stabilization. The treatment demonstrated efficacy in mitigating protein instability by selectively reducing pathogenesis-related (PR) proteins, including thaumatin-like proteins and chitinases. Although complete stabilization was not achieved, significant improvements observed in Graševina and Malvasia wines underscore the potential of mushroom-derived chitosan as part of a multi-step stabilization protocol.

Importantly, the application of chitosan preserved total polyphenol content and chromatic intensity, thereby overcoming the typical drawbacks associated with bentonite fining. Moreover, the increase of the POM test values at higher dosages confirms the ability of this fining agent to preserve wine phenolics against oxidative spoilage.

Volatile compound analysis via gas chromatography–mass spectrometry revealed a dosage-dependent decrease in ester concentrations, while levels of alcohols, fatty acids, terpenes, and norisoprenoids remained largely unaffected. The retention of varietal aromatic compounds and other key volatiles, at least above sensory thresholds indicate, a minimal impact on wine aromatic profile. Nonetheless, GC-MS data alone are insufficient to fully characterize sensory perception. Future investigations should incorporate structured sensory evaluations to assess changes in aroma, taste, and mouthfeel, ensuring alignment between chemical metrics and consumer experience.

In conclusion, chitosan obtained from mushroom stems through subcritical water extraction presents a promising eco-friendly alternative to conventional fining agents. It enables selective protein removal with minimal impact on phenolic composition, colour attributes, and aromatic integrity. Its integration into comprehensive stabilization strategies, supported by robust sensory validation, could advance sustainable and quality-preserving practices in modern enology.

CRediT authorship contribution statement

A. Natolino: Writing – original draft, Visualization, Validation, Supervision, Formal analysis, Data curation, Conceptualization. **T. Roman:** Visualization, Validation, Investigation, Data curation. **L. Tat:** Writing – original draft, Formal analysis, Data curation. **E. Celotti:** Visualization, Validation, Supervision. **P. Comuzzo:** Writing – original draft, Visualization, Validation, Supervision, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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