Innovative Food Science and Emerging Technologies 86 (2023) 103381

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Efficacy of fungoid chitosans from *Aspergillus niger* and *Agaricus bisporus* in controlling the oxidative browning of model white wines

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ARTICLE INFO

Keywords:
Browning
Catechin
Fenton reaction
White wine
Chitosan
Aspergillus Niger
Agaricus bisporus
Oligomeric polysaccharide

ABSTRACT

The efficacy of two water-insoluble chitosans from *Aspergillus niger* and *Agaricus bisporus*, in controlling the browning of model white wine solutions was assessed and compared with respect to sulfite addition (70 mg/L). A water-soluble oligomeric preparation from *Agaricus bisporus* was also included to test the effect of solubility and reduced molecular weight on the antibrowning capability of the polysaccharide. Chitosans were added at 0.5 g/L and 1 g/L. Color development, iron oxidoreductive equilibrium and generation of phenolic intermediates were monitored. Results demonstrated a significant and comparable anti-browning efficacy of both insoluble formulations (up to 85% reduction in browning development with respect to control samples), which mainly acted by chelating iron (up to around 4.4 mg/g of chitosan) and shifting its oxidoreductive equilibrium toward the reduced form. Oligomeric chitosan was ineffective for this purpose as it completely lacked chelating activity, which it is proposed, depended on its negligible interaction with tartaric acid. Data on browning and oxidation-related phenolic intermediates also revealed that sulfite promotes browning once it is completely oxidized.

Industrial relevance: Following its very recent European authorization as novel food, chitosan from *Agaricus bisporus* has been evaluated for the first time and compared in wine-like conditions with the already known water-insoluble chitosan from *Aspergillus niger*. A further novelty are the data on water-soluble chitosan preparations, not yet permitted in wine but potentially interesting due to the potentially higher specific surface once in solution. The results, apart from providing information on a recently introduced source for enological chitosan, can be useful to producers and winemakers in deciding among fungoid preparations aimed to control the browning of products.

1. Introduction

Chitosan (KT) is a polymer obtained from the partial deacetylation of chitin (poly b(1→4)-2-acetamido-2-deoxy-D-glucose) (Fig. 1), a widely diffused homopolysaccharide extracted from natural sources such as the exoskeleton of arthropods or the cell wall of yeasts and fungi (Dutta, Dutta, & Tripathi, 2004; Rinaudo, 2006; Struszczyk, 2002a). Due to their physical-chemical and biological properties, such as biocompatibility, metal chelation, film-forming capabilities, antioxidant and fungistatic behavior, chitin and chitosan have been the focus of interest in a wide range of industrial and biomedical applications in recent decades (Dutta et al., 2004; Gamage & Shahidi, 2007; Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Milhome, Ribeiro, Nascimento, Carvalho, & Queiroz, 2009; Struszczyk, 2002b).

Indeed, the partial deacetylation of chitin imply the generation of free amino groups, randomly distributed along the chitosan backbone (Fig. 1), whose reactivity markedly differentiate the two polymers. In fact, due to the protonation of the -NH2 group, at a pH of <6, KT exhibits a unique polycationic character and increased chelating and antimicrobial activities (Aranaz et al., 2009), making it particularly attractive for the food industry.

The degree of acetylation (DA) and molecular weight (MW) may also affect chitosan behavior since higher bactericidal and antioxidant properties were reported for low MW and low DA chitosan formulations (Sahariah & Másson, 2017; Yang, Shu, Shao, Xu and Gu, 2006).

In addition, solubility itself may depend on the molecular weight of chitosan, with the transition from acid solubility (typical for higher molecular weight) to water solubility (lower molecular weight) reportedly occurring at molecular weights between 4.67 and 3.82 KDa (Tian, Tan, Li, & You, 2015).

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https://doi.org/10.1016/j.ifset.2023.103381
Received 22 January 2023; Received in revised form 2 May 2023; Accepted 9 May 2023
Available online 10 May 2023

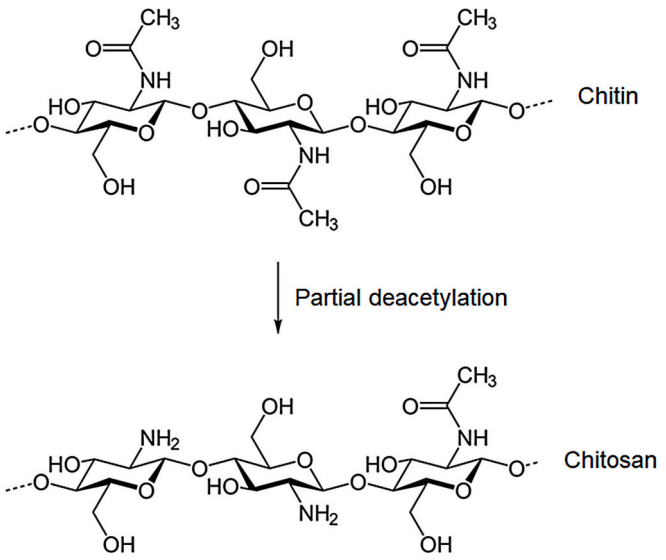

Fig. 1. Chemical structure of chitin and chitosan.

In 2011, water insoluble KT has been admitted in oenology as processing aid with clarifying and metal chelating properties, for the adsorption of contaminants or antimicrobial purposes (EU Commission, 2011).

Due to allergenicity concerns, only chitosan from *Aspergillus niger* had been authorized in wine (European Commission, 2019), excluding shrimps or crustaceans as eligible source for KT extraction. Very recently, fungoid KT from *Agaricus bisporus* has also been admitted in winemaking (European Commission, 2022) following its introduction in the European list of novel foods even if, to our knowledge, its oenological behavior has not been investigated yet.

Oxidation of stored wines is quite an articulate phenomenon, largely driven by the redox cycle of iron (Danilewicz, 2021; Li, Guo, & Wang, 2008), which involves the initial activation of oxygen by Fe^{2+} to generate hydroperoxyl radical and then H_2O_2 (Danilewicz, 2007). If not eliminated by antioxidants in solution (SO_2, ascorbic acid or glutathione, for instance), hydrogen peroxide ignites the Fenton pathway where it is reduced by Fe^{2+} to hydroxyl radical which eventually oxidizes ethanol and tartaric acid (two main constituents of wine) to acetaldehyde and glyoxylic acid respectively (Elias & Waterhouse, 2010). Phenolics with catechol or pyrogallol moieties (e.g. (+)-catechin, (−)-epicatechin, gallic or caffeic acids) fuel this oxidative cascade by reducing Fe^{3+}, generating supplementary hydrogen peroxide and *O*-quinones. These latter are highly reactive toward nucleophiles such as other phenolics, thiols or amines whose interaction ultimately drive to polymerization, browning and flavor changes of wines (Li et al., 2008). In model wine solutions containing (+)-catechin, the described pathway lead to the formation of yellow/brown pigments identified as xanthylium cations, coming from the oxidative condensation of two (+)-catechin molecules bridged by glyoxylic acid (Barril, Clark, & Scollary, 2008; Es-Safi, Le Guernevé, Fulcrand, Cheynier, & Moutounet, 2000).

Tartaric acid itself plays a further role in oxidation as it may lower the reduction potential of Fe^{2+}/Fe^{3+} couple by strongly co-ordinating Fe^{3+}, hence promoting Fe^{2+} oxidation at the expenses of the O_2/H_2O_2 couple, giving rise to the already mentioned O_2 activation (Coleman, Boulton, & Stuchebrukhov, 2020; Danilewicz, 2014).

In this overall context, SO_2 acts as an effective antioxidant because it i) reduces quinones back to the original o-diphenols, ii) quickly scavenges hydrogen peroxide before it oxidizes other wine constituents and iii) binds with carbonylic compounds (e.g. acetaldehyde or glyoxylic acid) responsible for side-reactions which negatively impact the color and the sensory features of oxidized wine (Elias & Waterhouse, 2010; Li et al., 2008).

In the last years, some investigations shed light on the intriguing capability of KT to acts as an antioxidant in wine-relevant conditions thanks to its radical scavenging properties, its metal chelation activity and the absorption of both native and oxidized phenolic species (Castro Marín et al., 2021; Castro Marín, Colangelo, Lambri, Riponi, & Chinnici, 2021; Chinnici, Natali, & Riponi, 2014). This would enlarge the oenological potential of such a polymer, in particular for the production of sulfite-free wines (Castro Marín et al., 2019), a subject of growing interest among producers, researchers and consumers concerned by the possible adverse effects of sulfites on human health (that include dermatitis, asthma or bronchoconstriction, among others) (Vally, Misso, & Madan, 2009).

The aim of this work was hence to compare for the first time the performance of oenological fungoid KT from *Aspergillus niger* and *Agaricus bisporus* in controlling the oxidative decay of model wine solutions in a typical Fenton-like environment where iron, dissolved O_2, and tartaric acid are contextually present. Model wines with and without sulfite acted as positive and negative control respectively. An oligomeric hydrosoluble KT derived from *Agaricus bisporus* was also included to test the effect of this alternative formulation on antibrowning efficacy. Color development, decline of (+)-catechin, generation of phenolic intermediates and iron speciation were used to monitor the progression of oxidative phenomena.

2. Materials and methods

2.1. Reagents

Water insoluble KT from *Aspergillus Niger* (AN), (viscosimetric molar mass = 31.6 KDa) was obtained from Tecnofood srl (Santa Maria della Versa – PV, Italy) while KT from *Agaricus bisporus* was supplied by ChiBio Biotech, (Quingdao, China) as water insoluble (AB) (viscosimetric molar mass = 37.0 KDa) and water soluble (ABsol) oligomeric formulations (viscosimetric molar mass = 1.2 KDa). The deacetylation degree of all the KT formulations was 85–90% according to the suppliers. HPLC grade acetonitrile and acetic acid were obtained from Merck (Darmastdt, Germany). Water was of MilliQ quality. (+)-catechin, (+)-tartaric acid, Fe(II) sulphate heptahydrate, ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*'-disulfonic acid monosodium salt hydrate), ascorbic acid and EDTA were obtained from Sigma-Aldrich (Milan, Italy).

2.2. Model wine solutions and Fenton environment

4 L of a solution containing 4 g/L (+)-tartaric acid and 12% (*V*/V) ethanol was prepared and adjusted to pH 3.10 with 2 M NaOH. The solution was stirred overnight in an open flask at room temperature to be saturated with O_2, before (+)-catechin addition (90 mg/L). Trials were arranged by transferring 40 mL of this solution in 125 mL inert glass bottles so that air remained in the headspace. An aqueous solution of Fe (II) sulphate heptahydrate was then added to each bottle to give a final concentration of 5 mg/L Fe. When appropriate, 70 mg/L sulphur dioxide (as potassium salt), 0.5 and 1 g/L of each powdered KT formulation were separately added to generate the following solutions: CT (model solution), SO_2 (model solution + sulphur dioxide), AN 0.5 and AN 1 (model solution +0.5 or 1 g/L insoluble KT from *Aspergillus niger*, respectively), AB 0.5 and AB 1 (model solution +0.5 or 1 g/L insoluble KT from *Agaricus bisporus*, respectively), ABsol 0.5 and ABsol 1 (model solution +0.5 or 1 g/L water soluble KT from *Agaricus bisporus*, respectively). KT doses were selected taking into account its mean dosage in winemaking (0.5 g/L) and the maximum admitted dose for chelating and clarification purposes (1 g/L) (EU Commission, 2011). Before being closed with silicone septum and airtight aluminum caps, 100 mM of H_2O_2 was added to the bottles to start the Fenton reaction. Storage was carried out in the dark at room temperature. To facilitate the resuspension of insoluble KT formulations, they were manually shaken for 2 min at the starting of the

trial and every 2 days during the experiment. Triplicate bottles were set up and analyzed in duplicate ($n = 6$), for browning development, total and free SO_2, titratable acidity, the decline of (+)-catechin, the generation of phenolic intermediates, the content of Fe^{2+} and Fe^{3+}, as described below.

2.3. Browning development, SO_2 and acidity parameters

The experiments lasted 30 days during which 3 bottles each of the cited eight thesis were taken at the time points 24, 48, 120 h and 30 days (24 bottles each sampling time). This time series was adopted to focus on the initial phases of the oxidation after hydrogen peroxide addition which represent the key steps for the generation of the yellow pigments with time (Guo, Kontoudakis, Scollary, & Clark, 2017).

Browning development was monitored following the increase in absorbance at 440 nm by using a Jasco 810 spectrophotometer (Tokyo, Japan). Free and total SO_2, pH and titratable acidity (TA) were determined following the official OIV methods (OIV, 2021). All the analysis were done after centrifugation (5 min @ 1200 *g*) and removal of the suspended KT, so as to emulate the winemaking practise and taking into account the removal of compounds by KT itself.

2.4. (+)-catechin and phenolic intermediates generated during oxidation

(+)-catechin, together with compounds generated from its oxidation were quantified according to Chinnici et al. (Chinnici, Sonni, Natali, & Riponi, 2013, 2014) on a Jasco apparatus (Tokyo, Japan) equipped with a quaternary pump Jasco PU-2089, an autosampler Jasco AS-2057, a Jasco UV/VIS MD 910 PDA and a Jasco FD 2020 fluorescence detectors and a Poroshell 120 SB C18, 2.7 mm, 150 × 4.6 mm I.D. C18 column (Agilent, Palo Alto, CA), operating at 30 °C with a flow of 0.8 mL/min. Eluents were 0.2% acetic acid in HPLC grade water (eluent A) and 0.2% acetic acid in HPLC grade acetonitrile (eluent B), with a elution program as follows: from 98% to 95% A in 9 min., from 95% to 90% A in 6 min., from 90% to 82% A in 4 min., from 82% to 80% A in 3 min., from 80% to 70% A in 3 min., from 70% to 50% A in 3 min., from 50% to 0% A in 2 min. A post run of 5 min was applied. Identification was accomplished by comparing UV spectra and retentions times as defined in the previously cited papers (Chinnici et al., 2013, 2014).

Quantification of (+)-catechin was carried out at 280 nm based on a calibration curve made with (+)-catechin solutions of known concentrations. Phenolic intermediates generated by oxidation were monitored according to their maximum absorption wavelengths (440 nm for xanthylim ions and their ethyl esters) or their fluorescence response at λex 280 nm and λem 345 nm (for carboxymethine-linked dimers) and quantified as peak area.

2.5. Fe^{2+}/Fe^{3+} content of model wines

Iron speciation study was based on a previously devised spectrophotometric method (Nguyen & Waterhouse, 2019). For Fe^{2+}determination, to 1.0 mL of sample, 10 μL of ferrozine solution (3.5% *w/v* in water) and, after mixing, a 1.5 mL of 0.005% w/v EDTA (in 10% hydroalcoholic solutio) were added. Reading of the absorbance at 562 nm against a blank (2.5 mL 10% hydroalcoholic solution +10 mL ferrozine) was taken within 1 min from the addition. Total iron analysis followed the same procedure except 1.5 mL ascorbic acid 0.1 mM in 10% hydroalcoholic solution (*w/v*) was pipetted in place of EDTA and readings were done once the absorbances stabilized following the complete reduction of Fe^{3+} to Fe^{2+} and its complexation with ferrozine (about 1 h). Standard solutions of Fe^{2+} in 10% hydroalcoholic solution (0.1–6 mg/L) subjected to total iron procedure were used to quantify total and reduced iron in samples by means of an external calibration curve. Fe^{3+} amounts were calculated by subtracting Fe^{2+} from total Fe. All the readings were duplicated.

2.6. Determination of viscosimetric molar mass of chitosans

An Ubbelhode Capillary Viscosimeter type 531/10 I was used for the determination of average molecular mass. A 1 g/L chitosan was prepared in 0.3 M acetic acid/0.2 M sodium acetate solution (pH 4.6), stirred overnight to facilitate KT dissolution and filtered with 0.45 nm cellulose acetate syringe filters. The flow time of both the solvent (t_s) and the KT solutions (t) was taken at 25 °C each with 5 replicates.

The viscometric molecular weight [M] was calculates according to the Mark-Houwink-Sakurada equation $[\eta] = KM^a$, where [η] = intrinsic vicosity, K = 0.074 mL/g and a = 0.79 (Rinaudo, Milas, & Dung, 1993). Intrinsic viscosity was obtained after a series of single point measurements to determine the near-zero concentration (equal to 1 g/L at our conditions), e.g. the lowest concentration permitting consistent readings of the time needed for solutions to flow through the capillary (110–125 s) (Pamies, Hernández Cifre, Del Carmen López Martínez, & García De La Torre, 2008). Once measured the mean flow time, [η] can be calculated using the Solomon-Ciuta equation (Solomon & Ciută, 1962): $[\eta] = \frac{2^*[\eta_{sp} - ln(\eta_r)]}{c}$ with $\eta_{sp} = (t\text{-}t_s)/t_s$ (specific viscosity), $\eta_r = t/t_s$ (relative viscosity) and c = concentration of chitosan solutions (g/mL). Viscosity-average molecular weight can be expressed as g/mol or KDa (1 KDa = 1000 g/mol).

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and presented as the mean ± standard deviation (SD). Post-hoc comparison (Tukey's test) was used to highlight significant differences with $p < 0.05$ using XLStat ver.2016 statistical package (Addinsoft, Paris, France).

3. Results and discussion

3.1. Effect of chitosan on titratable acidity and pH of model wines

To monitor the extent of KT interaction with tartaric acid, both the titratable acidity and the pH of the solutions were determined during the experiment. As shown by Fig. 2A, the addition of chitosan affected the acidity parameters of model wines depending on the formulation. Regardless of the source, insoluble KT formulations (AB and AN) provoked a prompt, comparable and dose dependent diminution of titratable acidity, up to about 0.5 g/L (for 1 g/L KT addition) and 0.25 g/L (for 0.5 g/L KT addition) reduction, since the first 24 h.

This behavior was not surprising as the removal of carboxylic acids from aqueous solutions after their sorption onto KT has already been demonstrated and proposed to be due to i) the ionic interaction between positively charged amines and carboxylic anions and/or ii) the hydrophobic interaction of KT internal domain with the hydrocarbon chain of acids (Shamov, Bratskaya, & Avramenko, 2002). Gyliene et al., for instance, found that at pH 4, one g of crustacean KT can adsorb up to 1 mM tartaric acid (Gyliene, Nivinskiene, & Vengris, 2008). The same authors did also find lower but appreciable sorption at pH > 6.3 where KT is not protonated, hence accounting for supplementary mechanisms such as hydrophobic or chemical interaction between the -NH_2 groups of KT and the -COOH groups of the acids, with the generation of new amide bonds. In our case, the per gram sorption was somewhat higher than the one reported by Gyliene and colleagues, the differences being probably due to experimental conditions (differences in pH, presence of ethanol, KT source and molecular weight). It is worth to mention that in another study on red wines we found changes in TA of about 0.28 g/L after the addition of 0.5 g/L KT from *Aspergillus niger* (Castro Marín, Colangelo, et al., 2021).

Surprisingly, soluble KT (ABsol) gave almost opposite results as it slightly increased TA of solutions when added at 1 g/L (Fig. 2A). To explain this finding, one should consider that, in this case, KT totally solubilized in the model wines, and after centrifugation, no precipitate

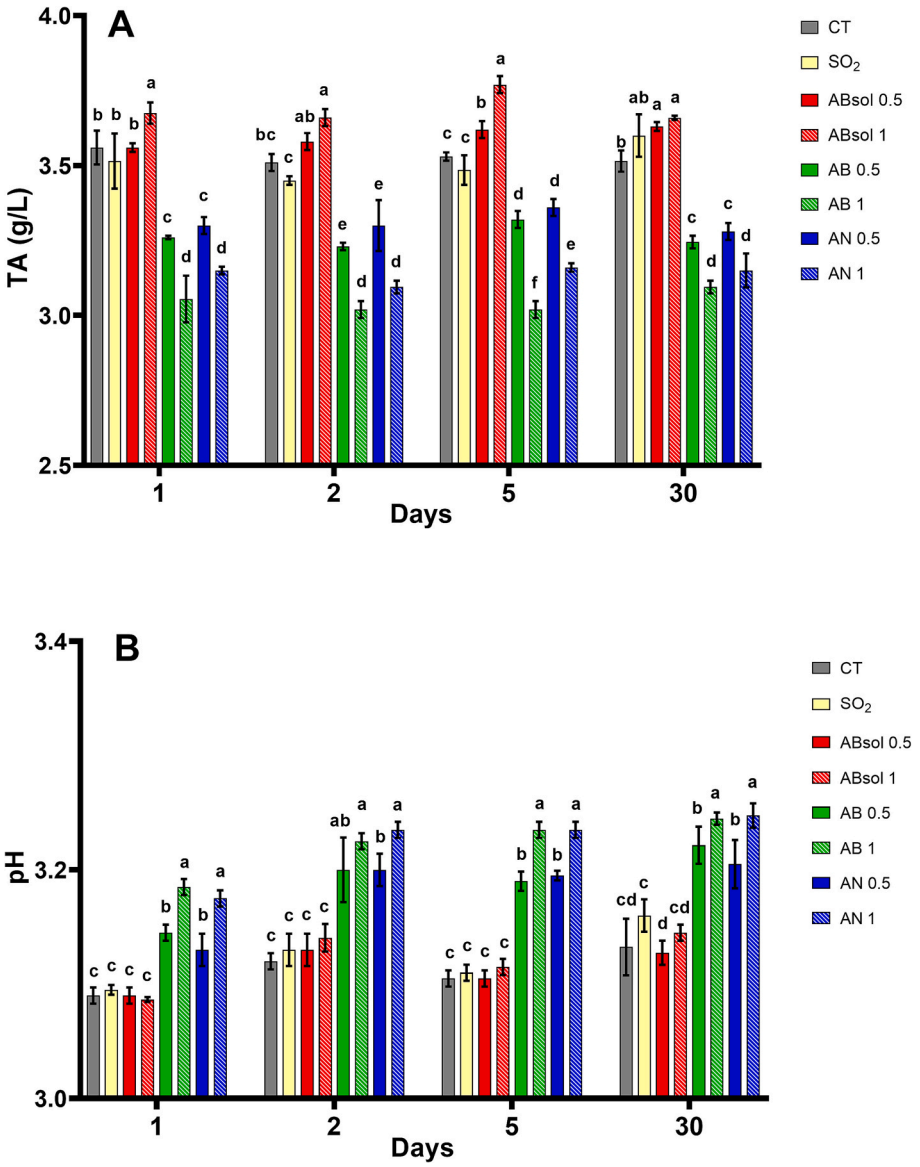

Fig. 2. Evolution of titratable acidity (TA) (panel A) and pH (panel B) of solutions during the experimentation. Data represent mean values ±SD (n = 6). For each sampling time, different letters indicate significant differences at $p < 0.05$.

was formed. Therefore, any potentially weakly bonded carboxylic acid and each -NH_3^+ group of KT would have been titrated during analyses. The greater number of amine groups in ABsol 1 samples with respect to ABsol 0.5 would further justify the tendentially higher acidity of the former. In addition, contrarily to higher MW KT, internal domain of oligomers may not have an adequate hydrophobicity to interact with tartaric acid backbone (Shamov et al., 2002).

The pH of model wines changed according with TA variations (Fig. 2B) as a consequence of the removal of organic acids from the medium. Insoluble formulations raised the pH by 0.11 and 0.15 units after 30 days and KT addition of 0.5 g/L and 1 g/L respectively, while ABsol did not significantly affect this parameter. Apart from the sorption of carboxylic acids, the increase of pH in KT aqueous suspensions could theoretically also be due to the protonation of the amine groups which decreases free [H+] in the medium (Gyliene et al., 2014).

3.2. Iron content of model wines and its speciation

As mentioned, Fe is a key factor in the Fenton chemistry which, in turn, dominates the chemical oxidation of wines. The extent of wine oxidation, in fact, is proportional to the amount of dissolved iron (Danilewicz, 2021; Elias & Waterhouse, 2010) and the claimed capability of KT to chelate this metal (Dutta et al., 2004; Gamage & Shahidi, 2007; Struszczyk, 2002a) might influence the overall kinetics. Accordingly, it has been proposed that the knowledge of Fe^{2+}/Fe^{3+} ratio may permit to estimate the overall redox state of a wine, representing a reliable index of the evolving equilibrium between oxidative and reductive reactions (Danilewicz, 2016).

As shown in Fig. 3, after 5 days of storage all the KT insoluble formulations had chelated Fe to a considerable extent, without a clear dose-dependency and independently from the fungoid source. For those formulations, residual Fe slightly changed at day 30, reaching chelation percentages comprised from 83% to 88% (for AB 0.5 and AB 1, respectively). Similar results were obtained in other studies in model solution and wines at comparable KT concentrations (Bornet & Teissedre, 2007; Chinnici et al., 2014). Once again, hydrosoluble oligomeric KT (ABsol) exerted unexpected results because its presence did not affect the amount of free Fe available to bind with ferrozine (Fig. 3). This result apparently contrasts with previous studies where KT oligomers (5–12 KDa) demonstrated higher chelating capacities with respect to medium

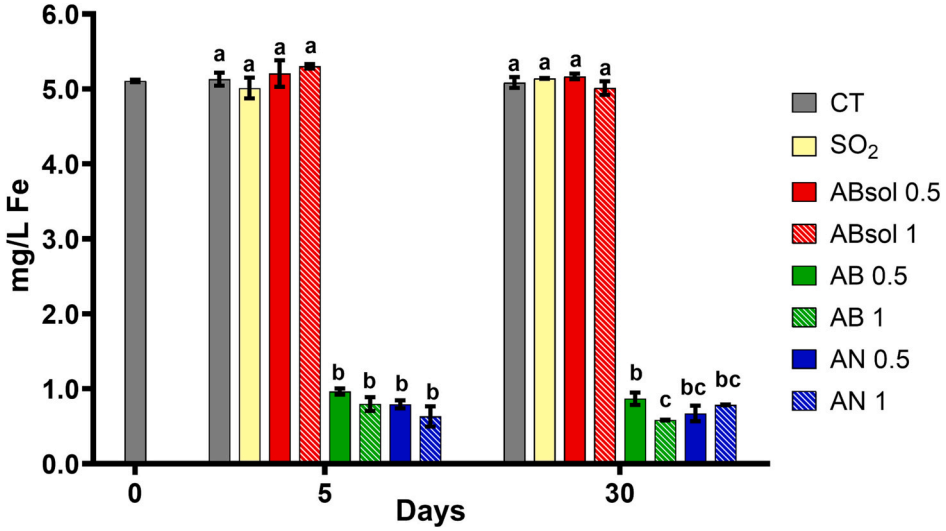

Fig. 3. Total Fe (mg/L) in the samples at days 0, 5 and. 30. Data represent mean values ±SD (n = 6). For each sampling time, different letters indicate significant differences at $p < 0.05$.

or high MW preparations (Chien, Sheu, Huang, & Su, 2007; Jung & Zhao, 2012). However, Rhazi et al. (Rhazi et al., 2002) established for KT a minimum polymerization degree equal to 6 (e.g. about 1.0–1.2 KDa) for noticeable complexation of copper, supposedly due to the need of a favorable spatial organization around the metal ion and to a high amount of molecular chain necessary to initiate the complexation. This would hence give a first plausible justification to our results with the 1.2 KDa hydrosoluble formulation. In addition, it has been speculated that in acidic solutions, chitosan alone may not behave as metal chelator because of the massive protonation of amine groups which impede the coordination with metal cations due to repulsive forces (Rocha, Ferreira, Coimbra, & Nunes, 2020). In these situations, the presence of polycarboxylic acids is thought to be crucial as they form negatively charged complexes with iron (Coleman et al., 2020) which eventually interact with -NH_3^+ groups of KT in a ternary KT-carboxylate-Fe complex, hence promoting metal adsorption (Castro Marín, Stocker, et al., 2021; Rocha et al., 2020). Accordingly, the negligible adsorption of tartaric acid by ABsol formulations (Fig. 2A), could be a further reason for the observed lack of iron chelation by KT oligomers.

Not only did KT formulations affect the total amount of Fe in solution, but they also impacted the oxidation state of Fe during the experiment (Table 1).

As expected, in CT samples the Fe^{2+}/Fe^{3+} ratio was found to be clearly shifted toward the oxidized form of the metal (up to 80–87% of total Fe), indicating strong oxidative environment all along the experiment.

On the contrary, at day 5 the samples added of sulfite only marginally diminished the percentage of Fe^{2+} with respect to time 0 (from 89% to about 85%), as a consequence of the scavenging of H_2O_2 by SO_2 and subsequent blocking of the Fenton cascade. This would decrease the amount of total sulfite (oxidized to sulphate) in the solutions as, in fact, we found for those samples (Table S1). Nevertheless, oxidation probably occurred to some extent, since bound sulfite contextually increased (Table S1) due to the binding with oxidation-derived aldehydes (acetaldehyde and glyoxylic acid) (Danilewicz, 2007; Elias & Waterhouse, 2010). Notably, AB 1 solutions showed 78% of Fe^{2+} after 5 days (Table 1), a value slightly lower than SO_2 but that, however, suggests the decreased rate of iron oxidation in those samples. Also, all the other model wines added of insoluble KT (AB 0.5, AN 1 and AN 0.5) had the prevalence of reduced Fe^{2+} over Fe^{3+}, even if to a lesser extent and commensurate to the dose of added KT. Taking into account that the rate of oxidation is proportional to the amount of total iron in solution (Danilewicz, 2021; Elias & Waterhouse, 2010), these results may well depend on the low amount of free Fe (Fig. 3) available to carry on the oxygen activation and the Fenton reactions e.g. it may depend on the chelating capability of KT. In addition, the scavenging activity against hydroxyl radical and H_2O_2 that insoluble KT may exert in wine relevant environment (low pH, presence of alcohol, phenolics and hydroxycarboxylic acids) (Castro Marín et al., 2019; Castro Marín, Stocker, et al., 2021) could have played a further role.

On the other hand, as it is obvious from Table 1, ABsol did not affect the oxidoreductive equilibrium of model wines, and the Fe^{2+}/Fe^{3+} ratio of those solutions was the same as CT, certainly due to the lack of iron chelation (Fig. 3) and, arguably, to a reduced radical scavenging activity of oligomeric formulation at those conditions. Indeed, when dealing with the scavenging activity of KT oligomers (< 10 KDa) as a function of molecular weight, the limited available literature provides inconsistent results when applied to acidic beverages (Chien et al., 2007; F. Yang

Table 1
Iron speciation in the samples at days 0, 5 and 30 (t = time). For each column, mean values (± SD) are shown ($n = 6$). In the same column, different letters indicate significant differences at $p < 0.05$.

	t0			t5			t30		
	Fe (II) mg/L	Fe (III) mg/L	Fe(II)%	Fe (II) mg/L	Fe (III) mg/L	Fe(II)%	Fe (II) mg/L	Fe (III) mg/L	Fe(II)%
CT	4.59 ± 0.03	0.53 ± 0.01	89.6 ± 1.02	0.69 ± 0.13^{bc}	4.65 ± 0.24^{a}	12.9 ± 1.90^{e}	1.03 ± 0.04^{ab}	4.06 ± 0.13^{a}	20.2 ± 2.36^{d}
SO_2				4.19 ± 0.22^{a}	0.77 ± 0.22^{b}	84.5 ± 2.29^{a}	1.25 ± 0.05^{a}	3.89 ± 0.06^{a}	24.3 ± 1.89^{c}
ABsol 0.5				0.78 ± 0.02^{b}	4.43 ± 0.16^{a}	14.9 ± 0.95^{e}	0.98 ± 0.00^{b}	4.19 ± 0.03^{a}	18.9 ± 1.85^{d}
ABsol 1				0.87 ± 0.09^{b}	4.44 ± 0.11^{a}	16.3 ± 2.02^{e}	1.02 ± 0.02^{b}	3.99 ± 0.10^{a}	20.4 ± 2.54^{d}
AB 0.5				0.56 ± 0.04^{c}	0.40 ± 0.05^{bc}	58.6 ± 2.24^{d}	0.23 ± 0.02^{d}	0.71 ± 0.13^{b}	24.6 ± 3.01^{c}
AB 1				0.62 ± 0.15^{cd}	0.17 ± 0.08^{d}	78.4 ± 2.72^{b}	0.19 ± 0.01^{d}	0.39 ± 0.01^{c}	33.1 ± 2.56^{b}
AN 0.5				0.46 ± 0.03^{d}	0.33 ± 0.01^{c}	58.0 ± 1.10^{d}	0.24 ± 0.10^{d}	0.43 ± 0.08^{c}	35.8 ± 2.31^{b}
AN 1				0.40 ± 0.06^{d}	0.20 ± 0.07^{cd}	66.6 ± 1.85^{c}	0.35 ± 0.05^{c}	0.44 ± 0.01^{c}	44.4 ± 1.98^{a}

et al., 2017). It is worth noting that at day 30, the Fe^{2+} percentages of all the samples dropped considerably, indicating the prevalence of oxidation on the long-term, independently from the addition performed (Table 1). Sulfite itself was unable to prevent Fe from being oxidized because of its eventual complete disappearance as free SO_2 (Table S1).

Further, additional amounts of bound sulfite were consumed during oxidation. Considering that in our model wines SO_2 binders were almost exclusively represented by acetaldehyde and glyoxylic acid, this evidence confirms that even the strongly bound sulfite fractions may dissociate and participate to the overall antioxidant potential of a wine, as recently reported (Sacks, Howe, Standing, & Danilewicz, 2020). Overall, at day 30, in the presence of insoluble KT, the percentage of Fe^{2+} was the highest, especially for KT from *Aspergillus niger* at 1 g/L, indicating the slower rate of oxidation of those media.

3.3. Generation of (+)-catechin intermediates during oxidation and browning of samples

While attempting to study the chemical pathways involved in the browning of white wine, model solutions have often been employed. Indeed, since the late 1990s, the generation of yellow xanthylium pigments from the oxidative decay of (+)-catechin in the presence of glyoxylic acid (coming from tartaric acid oxidation) and iron has been elucidated in those matrixes (Es-Safi et al., 2000; Es-Safi, Le Guernevé, Fulcrand, Cheynier, & Moutounet, 1999). The cascade includes the transient formation of 4 isomeric carboxymethine-linked (+)-catechin dimers, which further undergo to dehydration to give xanthenes isomers whose eventual oxidation generates 6 isomeric yellow pigments and their respective ethyl esters (Clark, Prenzler, & Scollary, 2003; Es-Safi et al., 2000).

In hydroalcoholic model solutions, the ancillary formation of (+)-catechin dimers bridged by acetaldehyde (stemming from ethanol oxidation) was also established, but no evidence emerged so far that those dimers are able to generate yellow pigments equivalent to xanthylium cations (Clark et al., 2003).

The chromatographic analysis carried out on our solutions permitted the identification and semi-quantification of most of those intermediates as illustrated in Fig. S1. In Fig. 4, therefore, the amount of both the (+)-catechin dimers and the xanthylium pigments generated in the samples during the experimentation are shown as sum of peaks area of their respective isomers. As mentioned before, provided the presence of sufficient dissolved O_2, (+)-catechin dimers should evolve to yellow compounds, giving browning extents proportional to their quantity.

Apart from the solutions added with SO_2, (+)-catechin dimers were promptly generated in all the samples since the first 24 h (Fig. 4, panel A) due to the initial addition of hydrogen peroxide to ignite the Fenton

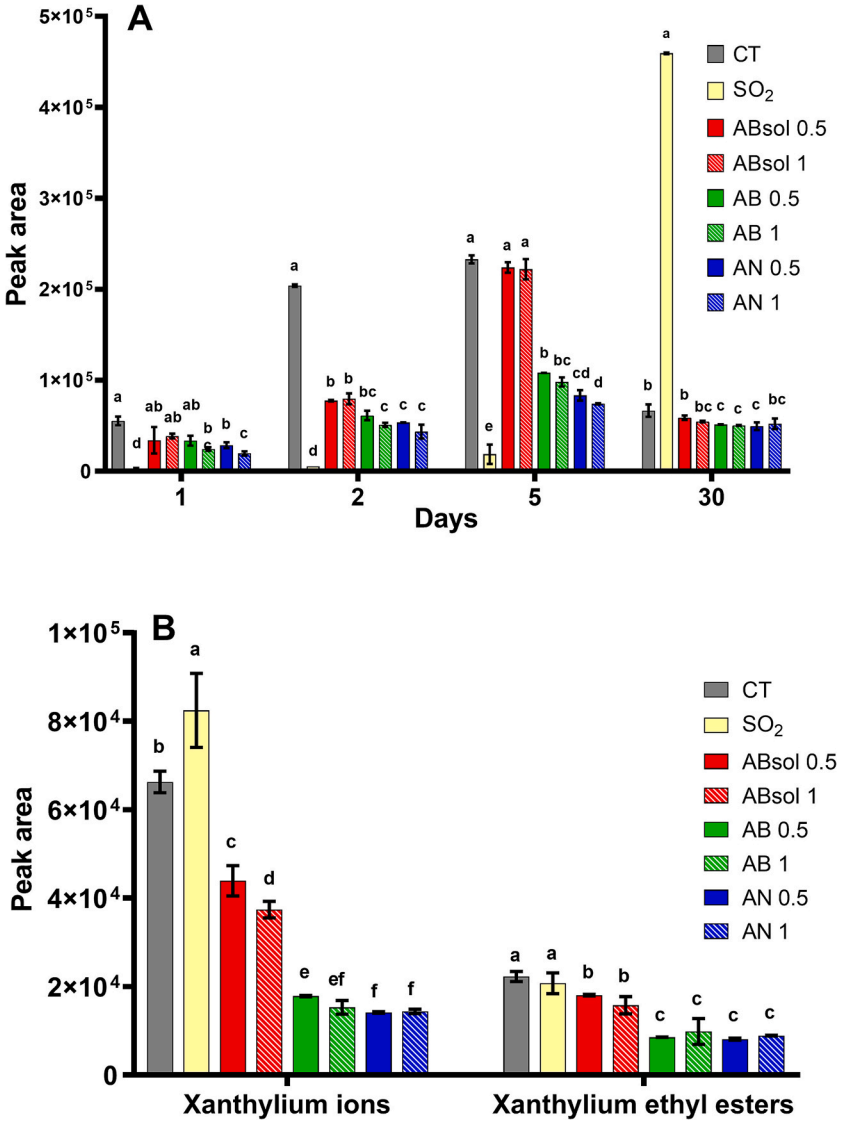

Fig. 4. Phenolic intermediates generated by (+)-catechin oxidation during the storage of model wines. Panel A: evolution of carboxymethine-linked (+)-catechin dimers (as sum of HPLC peak areas of the 4 isomers) during the experimentation. Panel B: Xanthylium pigments and their ethyl esters amounts (as sum of HPLC peak areas of the respective isomers) identified in the solution at day 30. Data represent mean values ±SD (n = 6). For each sampling time (panel A) and for each class of pigments (panel B), different letters indicate significant differences at $p < 0.05$.

reaction. CT and ABsol progressively produced the highest amount of those intermediates up to day 5, while insoluble KT formulations (AB and AN) generated significantly lower quantities of dimers with some differences only between AB 0.5 and AN 1 after 5 days.

On the other hand, it appears that the presence of sulfite strongly interfered with the production of dimers, most likely due to its scavenging of H_2O_2, thereby delaying iron oxidation (Table 1). A further reason would be that any glyoxylic acid possibly generated in those samples would have reacted with free SO_2, in this way becoming unavailable to participate to the formation of dimers.

Xanthylium pigments were found to be produced in little amounts only after 5 days, and solely in CT and ABsol 1 samples (data not shown). This lag period is comparable with the one found by Guo et al. (Guo et al., 2017) in wine-like solutions where the oxidative cascade had been boosted by glyoxylic acid addition and corresponded to the time needed for the generation and subsequent oxidation of (+)-catechin dimers at those conditions. Indeed, after 30 days of storage, xanthylium ions were largely produced in all model wines (Fig. 4, panel B), to which corresponded the generalized and expected depletion of (+)-catechin dimers (Fig. 4, panel A), with the notable exception of SO_2 samples.

In that case, in fact, the formation of dimers appeared to be delayed but, once begun, to proceed toward high accumulation of intermediates up to day 30. It is noteworthy that in both model and real wines, sulfite demonstrated to considerably increase O_2 consumption and redox cycling of (+)-catechin by reducing quinones back to their *O*-diphenol form while oxidizing to sulphate (Danilewicz, 2011). As suggested by table S1, once free sulfite is depleted bound sulfite may dissociate from acetaldehyde and glyoxylic acid which could therefore bridge (+)-catechin molecules to generate dimers. This may probably explain the generation of dimers in SO_2- containing solution only after day 5. Further, the mentioned high rate of oxygen consumption connected to (+)-catechin redox cycles could have played a further role, increase the amount of aldehydes and, hence, of dimers produced. Indeed, supplementary sampling points after day 5 could have provided further insights on the kinetic of dimers and pigments formation but, apart from being this subject beyond the scope of the present work, as discussed below the collected data are adequate enough to explain the color development of the solutions.

The extent of pigments generated at day 30 was the highest for SO_2, CT and ABsol samples (Fig. 4, panel B). Insoluble KT from *Agaricus bisporus* (AB) tended to form marginally higher amounts of pigments with respect to *Aspergillus niger* (AN) especially at 0.5 g/L.

At the end of the experimentation, percentages spanning from 31% to 49% of (+)-catechin were lost, depending on the sample (Table 2) but without an apparent correlation between the amount of intermediates and pigments formed during oxidation (Fig. 4) and the decrease of their initial precursor. However, while for CT and SO_2 the aforementioned loss may be deemed to be only due to oxidative phenomena (e.g. generation of dimers and xanthylium pigments), in the presence of KT (+)-catechin could be additionally removed via adsorption onto the polymer (Castro Marín & Chinnici, 2020; Chinnici et al., 2014). An indirect confirmation of the existence of this mechanism is the lower amount of residual (+)-catechin in all the solutions added with high KT doses (1 g/L) with respect to their respective low dosage (0.5 g/L) (Table 2) despite similar quantities of oxidation intermediates (Fig. 4). It is nonetheless unclear why soluble KT (ABsol 0.5 and ABsol 1) gave the highest loss of (+)-catechin and remains to be verified whether this may depend on its increased interaction with phenolics due to the augmented specific surface once solubilized.

Table 2 also shows the extent of browning of solutions after 5 and 30 days of storage. In that table, the optical densities at T0 are also reported to take into account the impact of KT and SO_2 on the initial color. KT formulations, in fact, appeared to turn the samples slightly yellower since the very beginning, probably due to the presence of residual soluble glucans (Dutta et al., 2004; Struszczyk, 2002a) while sulfite, as expected, bleached the solutions to some extent. The color of all the solutions started to increase at day 5, namely only once xanthylium ions had been generated, and continued up to the end of the storage to various extent depending on the sample. After 30 days, CT, SO_2 and ABsol 1 had the highest color increase, somewhat corresponding to the amount of xanthylium ions present in those samples (Fig. 4, panel B). It seems obvious, hence, that neither the presence of KT oligomer was effective in protecting the solutions from browning nor was sulfite once depleted its free fraction. Both the insoluble formulations, on the contrary, significantly contributed to reduce the oxidative yellowing of model wines, particularly in the case of AN 1. The prolonged presence of the polymer as a suspension permitted, on the long term, to obtain samples with the lowest degree of browning.

Table 2
Residual (+)-catechin content, corresponding percentage loss (in brackets), optical densities of samples (OD 440 nm) at t0, t5 and t30 (days) of storage and browning (Δ D.O. between t0 and t30) of samples. For each column, mean values (± SD) are shown (n = 6). In the same column, different letters indicate significant differences at $p < 0.05$.

	(+)-Catechin at t30		OD 440 nm at t0	OD 440 nm at t5	OD 440 nm at t30	Browning
	(mg/L)		(AU)	(AU)	(AU)	(Δ AU)
CT	63.07 ± 0.85^{a}	(−32.1%)	0.027 ± 0.004^{b}	0.044 ± 0.005^{a}	0.203 ± 0.003^{a}	0.176 ± 0.005^{a}
SO_2	63.26 ± 1.39^{a}	(−31.9%)	0.015 ± 0.005^{c}	0.017 ± 0.003^{c}	0.187 ± 0.007^{c}	0.172 ± 0.005^{a}
ABsol 0.5	54.15 ± 0.84^{c}	(−41.7%)	0.032 ± 0.004^{ab}	0.036 ± 0.003^{b}	0.189 ± 0.003^{b}	0.157 ± 0.004^{b}
ABsol 1	47.34 ± 1.62^{d}	(−49.0%)	0.034 ± 0.004^{a}	0.039 ± 0.007^{ab}	0.205 ± 0.005^{a}	0.171 ± 0.004^{a}
AB 0.5	63.27 ± 1.05^{a}	(−31.9%)	0.037 ± 0.002^{a}	0.039 ± 0.003^{ab}	0.071 ± 0.001^{d}	0.034 ± 0.002^{c}
AB 1	61.25 ± 0.94^{b}	(−34.1%)	0.035 ± 0.004^{a}	0.038 ± 0.003^{ab}	0.068 ± 0.003^{de}	0.033 ± 0.003^{c}
AN 0.5	64.09 ± 0.85^{a}	(−31.0%)	0.032 ± 0.001^{ab}	0.036 ± 0.002^{b}	0.064 ± 0.001^{de}	0.032 ± 0.001^{c}
AN 1	61.50 ± 0.97^{b}	(−33.8%)	0.036 ± 0.003^{a}	0.037 ± 0.002^{b}	0.062 ± 0.002^{e}	0.026 ± 0.003^{d}

4. Conclusions

Overall, the data discussed in this paper confirmed that insoluble, > 30 KDa MW fungoid KT can reduce the browning of model wines subjected to oxidation, with little differences between doses (0.5 or 1 g/L). The source of KT, whether from Aspergillus niger (AN) or Agaricus bisporus (AB), had only a marginal effect on the efficacy of the polymer, with AN formulations being slightly more effective, particularly at the highest dose. The role of oxidoreductive equilibrium of iron and its chelation by KT on the extent of Fenton reaction was highlighted. Water soluble oligomeric KT from *Agaricus bisporus*, on the other hand, was ineffective for that purpose, and the browning of those solutions was almost the same as the CTRL samples where no antioxidant was added. It is proposed that this behavior may depend on the negligible iron chelation demonstrated by soluble KT which, in turn, could be due to its inability to adsorb tartaric acid. To our knowledge, this is the first report on the use of soluble oligomeric KT in a wine-like medium. Sulfite, on the other hand, may increase browning once fully oxidized. Further research is needed to ascertain whether soluble KT may have increased antimicrobial capabilities with respect to insoluble formulations, as reported in other food matrixes, in this way potentially representing an additional tool for winemakers wanting to reduce the use of sulfite in

wines.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2023.103381.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Federico Baris: Investigation, Writing – original draft. **Antonio Castro Marín:** Conceptualization, Formal analysis. **Ana Cristina De Aguiar Saldanha Pinheiro:** Investigation, Resources. **Silvia Tappi:** Resources, Visualization. **Fabio Chinnici:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

No.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge Tecnofood srl (Santa Maria della Versa – PV, Italy) and ChiBio Biotech, (Quingdao, China) for providing the chitosan formulations.

Our acknowledgement also goes to Serena Belfanti for her help at the laboratory.

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