HOW CHITOSAN IMPACTS THE PHENOLIC AND VOLATILE COMPOSITION OF A RED WINE

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1. Introduction

During red winemaking, secondary fermentations may impact the overall quality of wines. These are conducted by microorganisms such as, lactic or acetic acid bacteria leading to malolactic or acetic fermentation. However, while a controlled development of some microorganisms such as lactic acid bacteria could be beneficial since it lowers the acidity and rounds the wine, the presence of acetic acid and certain yeasts may negatively impact the wine. A further example is the development of spoilage yeast *Brettanomyces bruxellensis*, responsible for the generation of volatile phenols (4-ethyl phenol, and 4-ethyl guaiacol), directly correlated to the consequent undesirable "horse sweat", "rubber" and "leather" character of red wines aged in oak barrels. This phenomenon may be particularly significant in low-sulfites wines, more susceptible to microbiological spoilage.

Recently, the attempts to find strategies to combat spoilage secondary fermentation have revealed that chitosan, a biopolymer made of 2-Amino-2-deoxy-poly-D-glucosamine and N-acetyl glucosamine, is an efficient tool to prevent unwanted microbial development [1]. Recent publications have demonstrated that treatments with 4-8 g/hl chitosan for 6-10 days could prevent from microbiological spoilage, thus avoiding the development of the undesirable sensory attributes mentioned above [2,3].

Chitosan, obtained from the deacetylation of chitin extracted from fungi, crustaceans or insects, has been recognized as GRAS (generally recognized as safe) by the Food and Drug Administration (FDA) for its biocompatibility, biodegradability and low toxicity and could therefore represent an interesting safer alternative to manage secondary fermentations in red wines.

However, little is known about the effect of chitosan treatments on the chemical composition of red wines, anthocyanins, phenolics and volatile compounds. Hence, this work aims to deepen into the physico-chemical and sensory features of a red wine treated with fungoid chitosan and how the color and aroma compounds could be affected by two distinct addiction modes (e.g. powder and pre-dissolved chitosan).

2. Material and Methods

2.1. Wine samples

Red wine samples from Sangiovese grapes were obtained from local winemakers.
Sets of different trials of 100 mL wines were arranged in triplicate into 100 mL glass bottles by adding 0.5 g/L of powder (KT) or pre-dissolved chitosan (fungal origin, 80-90% deacetylated, 10-30 KDa) in tartaric acid (KTS). A third set of samples without any addition was used as negative control. Wines were agitated twice a day for a week and kept at dark at laboratory temperature (about 20 °C). After treatment, samples were centrifuged at 4500g for 10 min before analysis. Subsequently, a parallel set of samples was prepared where wines were spiked with 1500 µg/L ethyl phenol and 500 µg/l ethyl guaiacol.

2.2. Phenolic compounds

Phenolic acid, flavanols and flavonols analysis [5] was carried out in a HPLC apparatus equipped with a quaternary gradient pump Jasco PU-2089, an autosampler Jasco AS-2057 Plus Intelligent Sampler and two detectors: A Jasco UV/Vis MD-910 PDA detector and a Jasco FP-2020 Plus Fluorescence detector. A C18 Poroshell 120 column was employed (Agilent technologies), 2.7µm, (4.6 x 150 mm), temperature oven of 35° C with a flow of 0.8 mL/min. Elution solvents were 2% acetic acid in HPLC grade water (Eluent A) and 2% acetic acid in HPLC grade acetonitrile (Eluent B). Gradient elution was as follow: from 98% to 95% A in 10 min, 95% to 90% A in 7 min, 90 to 82% A in 6 min, 82% to 80% A in 3 min, 80% to70% A in 3 min, 70% to 50% A in 3 min, 50% to 0% A in 4 min and finally to 98% A in 1 min. Quantification was performed by means of calibration curves previously obtained by duplicate injections of pure standards solutions at known concentrations. All the analyses were carried out by triplicate. For compounds lacking of pure standard, the calibration curves of structurally similar compounds were used.

2.3. Chromatic profile

Optical densities were determined at 420, 520 and 620 nm (1cm of optical path) using a Jasco 810 spectrophotometer (Tokyo, Japan) in order to calculate color density and hue. CIELab parameters L* (Lightness), a* (redness), b* (yellowness), Cab* (chroma) and Hab (hue) were analysed according to OIV methods [4]. Co-pigmentation was determined according to Boulton [6].

2.4. Volatile composition

Extraction of volatile compounds was carried out by following a validated method of by Lopez et al [7]. A 100 µL of internal standard (2-octanol) solution at 500 mg/L was added to 20 mL of wine and deposed on a Lichrolut EN cartridge previously activated. Analytes were eluted with 5 mL of dichloromethane and concentrated to two hundred µL under a stream of nitrogen before GC-MS analysis. The Trace GC ultra-apparatus coupled with a Trace DSQ mass selective detector (Thermo Fisher Scientific, Milan, Italy) was equipped with a fused silica capillary column Stabilwax DA (Restek, Bellefonte, PA, USA; 30 m, 0.25mm i.d., and 0.25 µm film thickness). The carrier gas was He at a constant flow of 1.0 mL/min. The GC programmed temperature was: 45 °C (held for 3 min) to 100 °C (held for 1 min) at 3 °C/min, then to 240 °C (held for 10 min) at 5 °C/min. Splitless mode injection (1 µL) was performed at 250 °C. Detection was carried out by electron ionization (EI) mass spectrometry in full scan mode,with ionization energy of 70 eV. Transfer line interface was set at 220 °C and ion source at 260 °C. Mass acquisition range was m/z 30–400. Compounds were identified by following a triple criterion: i) comparing their mass spectra and retention time with those of pure standards, ii) compounds lacking standards were identified after matching their respective mass spectra with those present in the commercial libraries NIST 08 and Wiley 7, iii) matching the linear retention index (LRI) obtained under our conditions, with already published LRI on comparable polar columns. Quantification of compounds was carried out via the respective total ion current peak areas after normalization with the area of the internal standard. Calibration curves were obtained by injections of standard solutions, subjected to the cited extraction procedure, containing a mixture of commercial standard compounds at concentrations between 0.01 and 200 mg/L, and internal standard at the same concentration as in the samples. The calibration equations for each compound were obtained by plotting the peak area response ratio (target compound/internal standard) versus the corresponding concentration. For compounds lacking reference standards, the calibration curves of standards with similar chemical structure were used. Analyses were done in triplicate.

2.5. Sensory analysis

Sensory analysis was performed by 22 panelists recruited at the Department of Agricultural and Food Sciences. Wine sensory attributes were based on testing cards already established by our research group for red wines spiked with volatile phenols. Selected sensory descriptors were: fruity, floral, herbaceous, smokey, animal, rubber and aromatic intensity for the aroma, and color intensity and limpidity of appearance. A preliminary triangular test was carried out followed by a quantitative descriptive analysis (QDA) on a structured scale from 1 (absent) to 5 (maximum).

2.6. Statistical analysis

Statistical analysis of dataset was performed by means of the XLSTAT Software package (Version 2013.2, France). One-way analysis of variance (ANOVA) followed by a post hoc comparison (Tukey's HSD test) was carried out.

3. Results and discussion

3.1. Phenolic acids

Phenolic acids composition after 7 days of treatment is outlined in table 1. When compared to control, chemical families of hydroxycinnamic acids, benzoic acids and flavanols were slightly reduced after treatments with both chitosan formulations (KT and KTS). This evidence could be correlated to the presence of amine (-NH_2) and hydroxy (-OH) groups on chitosan formula, that allows the formation of weak bonding (hydrogen bonding or van der Waals forces) between these groups and phenolic acids [8,9]. Moreover, at wine acidic pH, electrostatic interactions between phenolate form of phenolic acids and chitosan surface may also be involved [10].

	C	KT	KTs
Flavanols			
Procyanidin B1	8.46 ± 0,25 a	8.31 ± 0,16 a	8.37 ± 0,23 a
Catechin	25.52 ± 1,34 a	25.14 ± 1,15 a	25.33 ± 0,97 a
Procyanidin B3	3.50 ± 0,10 a	3.44 ± 0,06 a	3.50 ± 0,10 a
Procyanidin B2	7.10 ± 0,43 a	7.02 ± 0,37 a	7.05 ± 0,27 a
Epicatechin	17.57 ± 1,03 a	17.35 ± 0,89 a	17.40 ± 0,69 a
Sum flavanols	62.15 ± 3,15 a	61.27 ± 2,61 a	61.64 ± 2,24 a
Benzoate derivatives			
Gallic acid	38.21 ± 0,85 a	37.71 ± 0,21 a	38.05 ± 0,47 a
Ethyl gallate	8.28 ± 0,33 a	8.29 ± 0,19 a	8.32 ± 0,07 a
Protocatechuic acid	7.97 ± 0,10 a	8.01 ± 0,26 a	7.96 ± 0,17 a
Ellagic acid	5.27 ± 0.06 a	3.02 ± 0.02 b	3.17 ± 0.03 b
Sum benzoates	59.73 ± 0.74 a	57.03 ± 0.56 a	57.5 ± 0.24 a
Hydroxycinnamate derivatives			
cis-coutaric	2.91 ± 0,07 a	2.91 ± 0,02 a	2.97 ± 0,01 a
trans-coutaric	7.57 ± 0,14 a	7.49 ± 0,03 a	7.55 ± 0,01 a
p-coumaric acid	1.97 ± 0,14 a	1.71 ± 0,02 b	1.71 ± 0,02 b
cis-Caftaric acid	2.36 ± 0.02 a	2.21 ± 0.04 a	2.21 ± 0.02 a
trans-Caftaric acid	24.54 ± 0,44 a	23.32 ± 0,11 b	23.72 ± 0,17 b
GRP	5.44 ± 0,05 a	4.55 ± 0,04 c	4.63 ± 0,02 b

Fertaric acid	2.08 ± 0,02 a	2.04 ± 0,03 a	2.05 ± 0,01 a
Caffeic acid	5.07 ± 0,32 a	4.30 ± 0,01 b	4.31 ± 0,02 b
Sum hydroxycinnamates	49.56 ± 0,96 a	46.27 ± 0,22 b	46.94 ± 0,24 b
Flavonols			
Flavonol derivative	1.23 ± 0,01 a	1.23 ± 0,01 a	1.24 ± 0,01 a
Dihydromyricetin 3-O-rhamnoside	1.41 ± 0,01 a	1.39 ± 0,01 a	1.40 ± 0,02 a
Myricetin glucuronide	1.87 ± 0,06 a	1.76 ± 0,04 a	1.78 ± 0,05 a
Myricetin-3-glucoside	4.24 ± 0,15 a	4.09 ± 0,08 a	4.12 ± 0,11 a
Myricetin 3-rhamnoside	1.97 ± 0,07 a	1.91 ± 0,02 a	1.92 ± 0,02 a
Quercetin glucuronide	5.95 ± 0,10 a	5.68 ± 0,03 b	5.75 ± 0,02 b
Quercetin glucoside	4.55 ± 0,09 a	4.46 ± 0,04 a	4.52 ± 0,02 a
Syringetin-3-glucoside	1.57 ± 0,03 a	1.57 ± 0,02 a	1.57 ± 0,00 a
Myricetin	2.27 ± 0,09 a	2.13 ± 0,07 b	2.21 ± 0,04 ab
Quercetin	6.90 ± 0,28 a	6.23 ± 0,14 b	6.40 ± 0,36 ab
Sum flavonols	31.97 ± 0,25 a	30.46 ± 0,08 c	30.91 ± 0,16 b
Stilbenes			
Resveratrol glucoside	0.89 ± 0,01 a	0.89 ± 0,00 a	0.89 ± 0,00 a
t-resveratrol	1.03 ± 0,01 a	1.02 ± 0,00 a	1.03 ± 0,01 a
Others			
Tyrosol	27.59 ± 0,71 a	27.36 ± 0,32 a	27.58 ± 0,34 a

Table 1. Concentration (mg/L) of phenolic acids, flavonols and flavanols identified and quantified in red wine samples after 7 days of chitosan treatments. In the same row, different letters indicate significant difference according to Tukey's test ($p<0.05$). C: Control; KT: 0.5 g/L of powder chitosan; KTs: 0.5 g/L of chitosan as a solution.

Analysis revealed that hydroxycinnamic acids were affected to a greater extent by chitosan treatments, especially compounds derived from caffeic acid such as caftaric acid and GRP, reaching removal levels up to 1.22 mg/L, and 0.89 respectively (table 1). Previous works suggested that this phenomenon could be attributed to a cooperative adsorption between hydroxycinnamates and molecules already adsorbed onto chitosan surface [10]. In addition, electrostatic interaction between negatively charged carboxylic groups of hydroxycinnamates and protonate amine groups of chitosan could be involved. This trend could be in some cases beneficial for red wines since hydroxycinnamic acids are the precursor of volatile phenols, the main compounds responsible for the "horse sweat" character of wines affected by Brettanomyces spp [11].

Regarding benzoates, ellagic acid showed a decreased content after fining treatments with both formulations. This evidence, as already studied by other authors, is probably linked to the planarity of the molecule that favored a setting up of hydrogen bonds with polar groups of chitosan [12]. A similar mechanism could also be evoked for the slight decrease observed for flavonols, another class of molecules with an extended planar structure. These compounds, together with anthocyanins, could be involved in the color expression of red wines by means of the co-pigmentation phenomenon, which will be evaluated in the following chapters.
Stilbenes, whose beneficial role as nutrition supplement due to their radical scavenging activity in human cells, were not reduced by the addition of chitosan to the wine.

3.2. Color profile and anthocyanins content of red wines after fining treatments

	C		KT		KTS	
% Co-pigmentation	9.7 ± 2.7	a	0.0 ± 0.1	c	6.1 ± 1.8	b
% Anthocyanins	36.5 ± 2.1	b	52.5 ± 4.0	a	46.9 ± 2.1	a
% Polymers	53.8 ± 0.7	a	47.5 ± 2.4	b	47.0 ± 0.8	b
CIELab parameters						
L*	19.43 ± 2.62	b	25.72 ± 0.11	a	24.1 ± 0.51	a
a*	48.17 ± 3.28	b	54.82 ± 0.14	a	52.8 ± 0.43	a
b*	34.98 ± 4.03	b	40.10 ± 0.08	a	37.9 ± 0.55	a
C*	57.83 ± 4.97	b	67.93 ± 0.16	a	65.0 ± 0.67	a
h_{ab}	33.50 ± 1.49	b	36.19 ± 0.05	a	36.7 ± 0.18	a
TPI (GAE) (mg/L)	1286 ± 17.43	a	1179 ± 19.23	b	1170 ± 4.14	b
Abs 420 nm (AU)	1.587 ± 0.02	a	1.423 ± 0.01	b	1.421 ± 0.01	b
Abs 520 nm (AU)	1.690 ± 0.02	a	1.528 ± 0.01	b	1.528 ± 0.01	b
Abs 620 nm (AU)	0.455 ± 0.02	a	0.376 ± 0.03	b	0.337 ± 0.04	b
Hue	0.939 ± 0.01	a	0.931 ± 0.01	b	0.930 ± 0.01	b
Color density (AU)	3.730 ± 0.04	a	3.327 ± 0.01	b	3.386 ± 0.02	b

Table 2. CIElab and colour parameters of red wines after 7 days of chitosan treatments. In the same row, different letters indicate significant difference according to Tukey's test ($p<0.05$). C: Control; KT: 0.5 g/L of powder chitosan; KTs: 0.5 g/L of chitosan as a solution.

CIElab color descriptors and co-pigmentation index are outlined in table 2. As shown, both chitosan formulations reduced significantly co-pigmentation in sample wine. Anthocyanins amount (data not shown) seemed not to be influenced by the presence of chitosan. Hence, since anthocyanins, directly correlated to color contribution have not been adsorbed, this evidence could be linked to the lesser content of cofactors responsible of co-pigmentation such as hydroxycinnamic acids, flavanols and ellagic acid (table 1) as outlined above[13]. These results are in line with reduced values of total color on KT and KTs samples, due to the reduction of the absorbance related to yellow (420nm), red (520nm) and blue (620nm) nuances (table 2).

A significant change was observed on CIElab parameters after fining treatments (table 2). Results reported that the presence of chitosan led to an increased luminance (L*), and yellowness (b*). These evidences are partially in line with previous experiments [14], where a decrease in co-pigmentation should be linked to lighter color and lower saturation (C*). However, in our experiments, samples treated with chitosan exhibited higher C* and b* values. Furthermore, color perception was determined by calculating ΔE^*ab where $\Delta E^*ab>3$ indicates differences visible to human eye [15]. Analysis showed a value of ΔE^*ab equal to 8.5 between C and KTS and 3.49 for KT and KS, demonstrating that all the samples may be distinguishable each other.

3.3. Volatile and sensory profile of samples treated with chitosan

About 80 compounds were identified and quantified after solid phase extraction of wines, belonging to three chemical families, namely acids, alcohols and esters.
Apart from slight differences on certain single compounds, gas chromatographic analysis revealed no overall impact of the treatments on wine aroma profile (figure 1). In order to emulate a contamination by *Brettanomyces bruxellensis*, additional trials were arranged where wines were spiked with ethyl phenols before fining treatments. Results demonstrated reductions of 4-ethyl phenol and 4-ethyl guaiacol of 10 and 40 µg/L respectively for KT and 55 and 45 µg/L on treatments with KTS (data not shown). Judging by the results, higher performance of KTs could be correlated to an increased specific surfaced induced by solubilization, leading to an increase in the adsorption capacity of the polysaccharide. Greater adsorptive effect for 4-ethyl guaiacol on both treatments

further suggested a stabilizing effect between the polar methoxy group of this compound and chitosan surface. However, it is necessary to state that those reductions were absolutely not enough to remediate to the “brett” character of wines and that remaining amounts of ethyl phenols were still largely prevalent during olfaction. Because of these limited absorption behavior, chitosan could only be proposed as a remediating agent for red wines containing low levels of that phenols, in an attempt to bring them below their odor threshold.

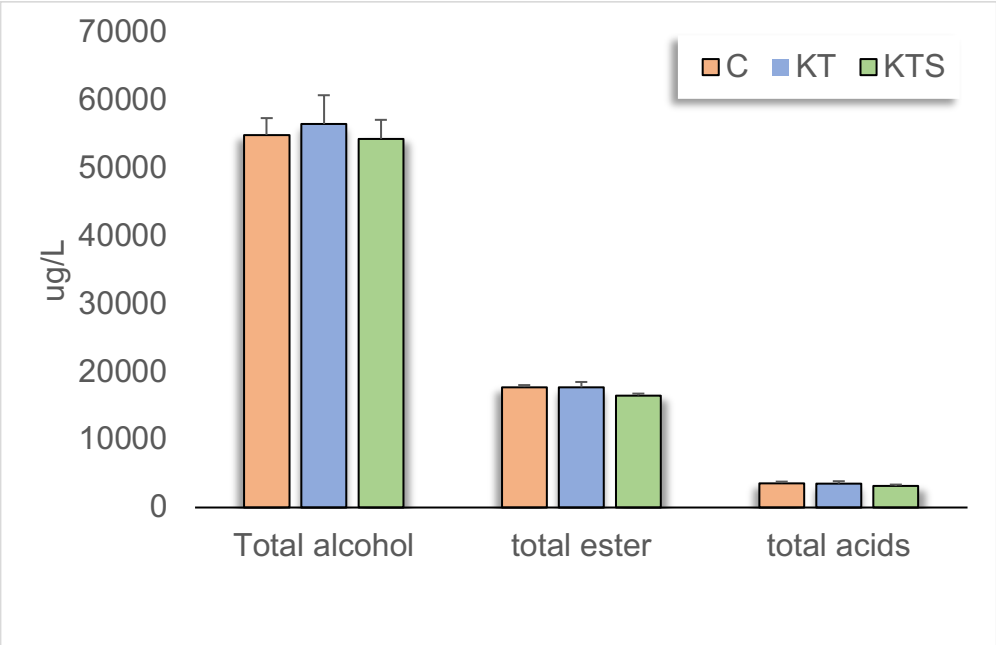

Figure 1. Concentration of volatile compounds (µg/L) in red wines after 7 days of treatment. C: Untreated, KT: Solid chitosan, KTS: Dissolved chitosan.

Sensory analysis on wines added of ethyl phenols (figure 2) confirmed the above results. After a triangular test, panelists were able to distinguish both chitosan treatments from the control, but not between chitosan formulations, because of little differences in color brilliance (data not shown). However, the high presence of ethyl phenols used in the experiment decreased the impact of other positive sensory attributes, causing no significant differences between our samples, apart from limpidity and herbaceous notes which were higher for KTS treated wines.

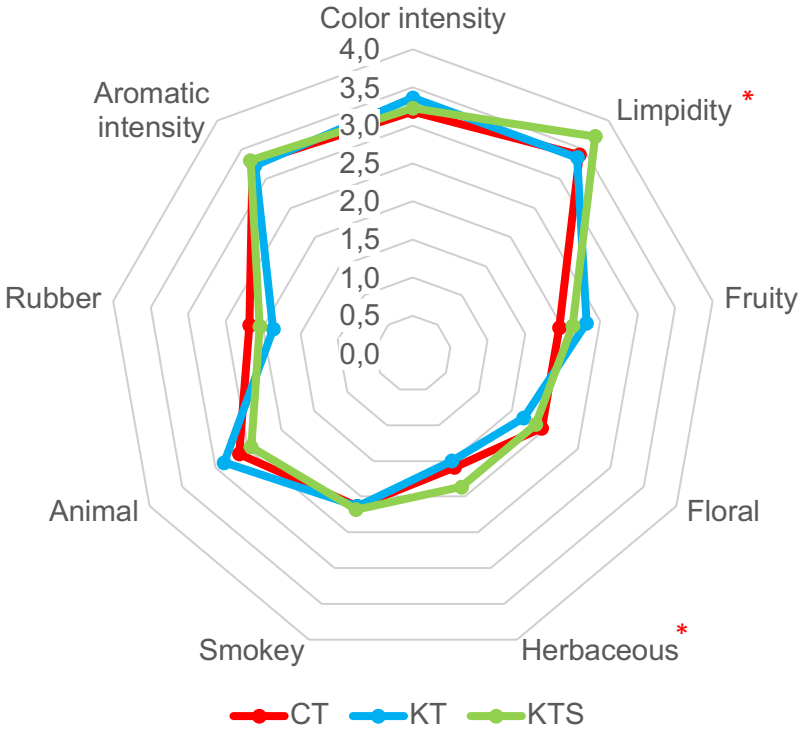

Figure 2. Sensory analysis and discriminant evaluation of red wines after 7 days of treatment. CT: Untreated, KT: Solid chitosan, KTS: Dissolved chitosan.

4. Conclusion

Based on the above results, it could be concluded that in our conditions, 0.5 g/L of chitosan could marginally influence the overall color of red wines, mainly by affecting the cofactors responsible of co-pigmentation, namely hydroxycinnamic acids, flavanols and ellagic acids. Regarding volatile compounds, addition of chitosan did not negatively impact volatile profile, and also contributed to little reduce the content of volatile phenols. Only little differences were observed to be possibly linked to the way of addition and based on these results it can be affirmed that, when used in red wines to reduce either contaminants or microbial spoilage, chitosan does not substantially affect the overall physical-chemical features and quality of wines.

Summary

Chitosan is gaining interest in red winemaking thanks to its ability to inhibit the development of Brettanomyces spp. yeast, or other undesired wine microbial threats. However, little is known about potential side-effects of its addition on the physico-chemical parameters of red wines. To fill the gap on this subject, this work focused on changes in color, phenolic and volatile composition of red wines treated for 7 days with 0.5 g/L of fungoid chitosan, added in both undissolved and dissolved form. When compared to untreated samples, minor changes in phenolic compounds were observed in chitosan added wines, mainly involving hydroxycinnamic acids and flavonols, with reductions of 3 mg/L and 1.5 mg/L respectively. Ellagic acid, however, was absorbed up to 2 mg/L, which reduced his content by 40%. Since some of these compounds actively participate to co-pigmentation with anthocyanins, the color of wines was influenced accordingly. Chitosan marginally absorbed some aroma compounds, including ethyl esters and volatile phenols whose amounts were slightly but significantly decreased after treatment. Visual and olfactive comparison of samples confirmed that, at the dose adopted, chitosan is suitable to be used in red winemaking for microbial or physical stability purposes, not severely impairing the quality parameters of the final wines.

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