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The use of chitosan as alternative to bentonite for wine fining: Effects on heat-stability, proteins, organic acids, colour, and volatile compounds in an aromatic white wine

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ABSTRACT

This investigation focuses on the use of chitosan treatment simulating a bentonite fining in order to detect any modification of the wine haze potential by simultaneously evaluating the secondary effects on untargeted fixed and volatile compounds. A significant removal of chitinases was observed after fining an aromatic white wine with 1 g/L of a fungoid chitosan. Even if the more stable thaumatin like protein fractions were not significantly affected, the heat stability of the fined chitosan wine samples were highly improved in the 55–62 °C range. Among the secondary effects of a fining treatment, a reduction of tartaric acid, malic acid, potassium and iron was showed. All the free terpenols with the exception of α-terpineol were reduced in significant amounts, whilst the glycosylated forms and the fermentative aroma compounds were not affected by a 1 g/L chitosan addition.

1. Introduction

In recent years, several reports have attempted to characterize the factors affecting protein instability and haze formation in white wines, which can reduce or invalidate their commercial value. The most abundant haze-forming proteins in wine include chitinases (Waters, Hayasaka, Tattersall, Adams & Williams, 1998), along with thaumatin-like (TL) proteins and β-glucanases (Esteruelas et al., 2009). Wine instability may also be influenced by non-protein factors including the wine pH, ionic strength (Dufrechou, Poncet-Legrand, Sauvage, & Vernhet, 2012; Lambri, Dordoni, Giribaldi, Riva Violetta, & Giuffrida, 2013), ethanol content, concentrations of polysaccharides (Jaeckels, et al., 2016; Lambri, Dordoni, Silva & De Faveri, 2010), polyphenols (Esteruelas et al., 2009), and sulfates (Marangon et al., 2011).

The risk of wine haze is traditionally lowered with the addition of sodium-activated bentonite. The adsorption of proteins by bentonite is unspecific and, in addition, bentonite fining results in the removal of other elements, such as aroma compounds (Lambri et al., 2010; Lambri, Dordoni, Silva, & De Faveri, 2013) and polyphenols (Dordoni, et al., 2015), thus negatively affecting the quality of wines. Alternative methods to bentonite fining have been proposed including the use of proteases, ultrafiltration, polysaccharides (Ferreira, Picarra-Pereira, Monteiro, Loureiro & Teixeira, 2002), and chitin (Vincenzi, Polesani & Curioni, 2005). Chitin is a promising fining agent due to capacity to retain its biological activity in wine and its selectiveness toward chitinases (Vincenzi et al., 2005). However, EU regulation prohibits the use of chitin in winemaking, while allowing the use of chitosan (Commision regulation (EU) 53/2011), a polymer obtained through the deacetylation process of chitin in alkaline conditions (Bornet & Teissedre, 2008).

Chitosan is structurally linear; it is basic and positively charged below pH 6.5. As a result of the deacetylation process, it is composed of β-1,4 linked D-glucosamine and *N*-acetyl-glucosamine. In contrast with chitin, that is highly hydrophobic, chitosan is soluble in acidic solutions according to its degree of deacetylation (Vårum, Ottøy & Smidsrød, 1994) and shows properties of high interest in the food industry, i.e film formation, metal ions chelation, antioxidant and radical scavenging activity, and antimicrobial activity (Bornet & Teissedre, 2008; Crini, Morin-Crini, Fatin-Rouge, Deon, & Fievet, 2017; Dutta, Tripathi, Mehrotra, & Dutta, 2009; Rocha, Coimbra, & Nunes, 2017). Fungoid chitosan from *Aspergillus niger* is the only type of chitosan accepted in winemaking (Commision regulation (EU) 53/2011) and the addition to wines is till now aimed at controlling *Brettanomyces* spp population (Chinnici, Natali & Riponi, 2014), and for the removal of ochratoxin A, iron, lead, cadmium, and copper (Bornet & Teissedre, 2008). The limit of chitosan addition ranges from 10 g/hl to 500 g/hl according to the goal (Commision regulation (EU) 53/2011); for fining purposes this limit is set at 100 g/hL (O.I.V., 2009a).

A recent investigation has assessed the viability of chitosan as

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support for enzyme treatments on wine proteins and promising reduction of haze potential in Sauvignon blanc has been reported by cross linking pineapple stem bromelain onto chitosan beads (Benucci, et al., 2016). However, few authors have studied the effect of chitosan as single adjuvant in white wines. Chinnici et al. (2014) underlined the capacity of chitosan to act as a substitute of SO_2, reducing browning and protecting thiols from oxidation, at least in model wine solutions. Reduced browning was already observed by Spagna, et al. (1996), who hypothesized the removal of procyanidins and cinnamic acids from wine up to 30%.

Within the literature on chitosan application in winemaking, data concerning its effect on the removal of heat unstable proteins from wines and on related consequences toward haze potential and stability are missing. The hypothesis at the base of this work is that chitosan, being a derivative of chitin, could be capable of interacting with grape chitinases, the most widespread haze-forming proteins in wine (Marangon et al., 2011; Waters et al., 1998). The trials were applied onto an aromatic white wine to assess if chitosan as single adjuvant was capable of removing haze forming proteins, if there were other modifications in the wine general composition and if molecules like phenolic compounds, minerals, and volatiles could be affected by a chitosan fining treatment.

2. Materials and methods

2.1. Chitosan sample and characterization

Chitosan sample was supplied by L'Enotecnica s.r.l. (Nizza Monferrato, Italy) and analysed in triplicate as acetylation degree (O.I.V., 2009b). Carbon and nitrogen analysis was carried out to characterize the purity of the chitosan by means of the Dumas combustion method (AOAC, 2000) with Vario Max CN Element Analyzer (Elementar Analysensysteme GmbH, Germany). The results, expressed as C%, were calculated taking as a reference the C% of a pure chitosan with the same degree of deacetylation. The morphology of the chitosan powder was analysed by observation at the scanning electron microscope FEI/Philips XL-30 Field Emission ESEM (Philips, Eindhoven, Netherlands), after drying at 40 °C for 48 h in an oven. The dried samples were analysed after sputter-coating with gold under argon atmosphere (25 mA, 120 s).

2.1.1. Chitosan solubility

Solubility tests were performed in triplicate in hydro-alcoholic solutions and in wine-model solutions. Hydro-alcoholic solutions (HS) were composed by 9% and 13% (v/v) ethanol in distilled water and pH was adjusted at 3.64 with hydrochloric acid to simulate the value of the wine used for the trial. Wine model solutions (WMS) were constituted of tartaric acid in 9% and 13% (v/v) hydroalcoholic solutions buffered at pH 3.64 with sodium hydroxide. Chitosan (1 g) was added to 1 L of both HS and WMS and maintained at 20 °C under stirring (150 rpm) for 12 h. To check the solubility of chitosan in time, aliquots of 100 mL were collected after 30 min, 1 h, 2 h, 6 h and 12 h for being vacuum-filtered on 0.45 μm membranes (Sartorius Stedim Biotech GmbH, Heidelberg, Germany). The membranes remained 24 h in oven at 105 °C and then they were put in vacuum-dryer at 21 ± 2 °C and weighted until constant weight.

2.1.2. Chitosan interaction with organic acids

To search for the interaction of chitosan with wine organic acids, model solutions were arranged in triplicate as follows: four single solutions each one containing acetic acid (A), tartaric acid (T), malic acid (M), lactic acid (L); two double solutions each one with tartaric acid + malic acid (TM), tartaric acid + lactic acid (TL); two triple solutions each one composed of tartaric acid + malic acid + acetic acid (TMA), and of tartaric acid + lactic acid + acetic acid (TLA). Chitosan (1 g) was added in triplicate to 1 L of each solution and maintained at 20 °C under stirring (150 rpm) for 12 h. Then solutions were vacuum-filtered on 0.45 μm membranes (Sartorius Stedim Biotech GmbH, Heidelberg, Germany) and analysed in triplicate for tartaric, malic, lactic, and acetic acid by HPLC Thermo 3000 Series (Thermo Electron Corporation, Waltham, MA, USA) equipped with a UV detector (UV100) set to 210 nm. The analyses were performed isocratically at 0.8 mL/min and 65 °C with a 300 × 7.8 mm i.d. cation exchange column Aminex HPX-87H and a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA), using 0.0026 N H_2SO_4 as mobile phase.

2.2. Wine sample

Wine samples were obtained by small-scale (20 L) fermentations of must from 2015 *Vitis vinifera* L. cv. Moscato grapes, harvested at commercial maturity and inoculated with 20 g/hL of EC118 *Saccharomyces cerevisiae* strain (Lallemand Inc., Montreal, Canada). In order to avoid interactions between SO_4^{2-} and proteins (Chagas, Ferreira, Laia, Monteiro & Ferreira, 2016), no sulphites were added and only 200 mg/L of dimethyl dicarbonate (Sigma, Milan, Italy) were put in wine 36 h after the end of alcoholic fermentation to avoid any malolactic fermentation.

2.2.1. Wine characterization and identification of proteins

In order to control the fermentation, both reducing sugar and ethanol concentration were monitored (O.I.V., 2017). Final wine was characterized in triplicate by effective alcohol degree, pH, titratable and volatile acidity, dry extract, reducing sugars, total SO_2, and ashes (O.I.V., 2017).

Protein profile of the final wine was determined using Nano LC/QTOF proteomic mass-spectrometry through the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA), following manufacturer's recommendations and using bovine γ-globulin as standard. Thereafter, a bottom-up proteomic analysis was carried out as previously reported (Lucini & Bernardo 2015). Briefly, an accurate amount (50 μg) of proteins was reduced with dithiotreitol, alkylated with iodoacetamide and overnight digested using porcine trypsin (Promega, Madison, WI, USA). Peptides were finally analysed using nanoscale liquid chromatography (Agilent 1260 Chip Cube source - Agilent Technologies, Santa Clara, CA, USA) coupled to a hybrid quadrupole-time-of-flight (Agilent 6550 IFunnel Q-TOF – Agilent Technologies, Santa Clara, CA, USA) mass spectrometer. In the LC/QTOF system, peptides were enriched onto a C18 trapping column and back flush eluted into a 150 mm separation column (ProtID chip, Zorbax 300SB-C18, 5 μm pore size). Separation was achieved using an acetonitrile gradient (from 3 to 70% v/v in 50 min, then hold for 15 min), with 0.3 μL min^{-1} elution flow. The QTOF was operated in Auto-MS/MS mode (tandem MS data-dependent acquisition and positive ionization mode, 20 precursors per cycle); SCAN was done in the range from 300 to 1700 *m/z*, with 4 spectra per second. MS/MS spectra of peptides were used for protein inference using Spectrum Mill MS Proteomics Workbench (Rev B.04; Agilent Technologies) against the proteome of *Vitis vinifera* (UniProt, downloaded July 2016). Carbamidomethylation of cysteine was set as a fixed modification, trypsin selected as digestion enzyme, and two missed cleavages per peptide were accepted. The database was concatenated with the reverse one and 1% false discovery rate validation was carried out. Lbel-free quantitation was carried out using summed peptide abundance.

2.3. Wine fining with chitosan

One g of chitosan was added in triplicate to 1-litre aliquots of wine (fined samples) so as to obtain the highest dose admitted by the regulation for wine fining (O.I.V., 2009a) which can be considered as the most common dose used for sodium-activated bentonite treatment (Lambri et al., 2010; Lambri, Dordoni, Giribaldi, Riva Violetta, &