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A novel approach for the valorization of wine lees as a source of compounds able to modify wine properties

Alberto De Iseppi [a], Matteo Marangon [a,*], Simone Vincenzi [a,b], Giovanna Lomolino [a], Andrea Curioni [a,b], Benoit Divol [c]

[a] Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Viale Dell'Università, 16, 35020, Legnaro, Padova, Italy
[b] Centre for Research in Viticulture and Enology (CIRVE), Viale XXVIII Aprile 14, 31015, Conegliano, Italy
[c] South African Grape and Wine Research Institute, Department of Viticulture and Oenology, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa

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ABSTRACT

Wine lees, a sludge-like material mainly consisting of yeast cells, are rich in mannoproteins. However, no reports on the extraction of mannoproteins from wine lees to be used as winemaking additives are available. This study aimed at developing efficient methods for yeast glycocompounds extraction from wine lees, and to assess their impacts when added back to wine. White wine lees were extracted using physical (autoclave and ultrasonication) and enzymatic (a β-glucanases) approaches. The autoclave extract was the richest in glycocompounds and showed the highest impact on wine tartrate stability and foaming properties. Conversely, the extracts containing less glycocompounds (ultrasonication and enzymatic) both improved wine protein stability upon heat test and had a low effect on wine foaming. In general, wine lees can be considered as a valuable source of substances to be used as winemaking additives thus opening the way to a new type of exploitation of these by-products.

1. Introduction

Within the sector of alcoholic beverages, winemaking is one of the most important industries in terms of volume, value and cultural significance. In 2018, the world wine production was estimated at 279 million hL, with Italy, France and Spain accounting for about 50% of it (OIV - International Organisation of Vine and Wine, 2017). Clearly, wine production requires a large amount of natural resources (viz. water, energy, chemicals, microorganisms), and the efficiency of the wine supply chain is diminished by its by-products, which include grape pomace, grape stalks and wine lees. It is estimated that processing 100 tons of grapes generates 20–22 tons of different by-products (Oliveira & Duarte, 2016; Pérez-Bibbins, Torrado-Agrasar, Salgado, Oliveira, & Domínguez, 2015). In this context, several strategies have been proposed for the recovery and valorization of grape stalks and pomace (containing both grape skins and seeds) (García-Lomillo & González-SanJosé, 2017; Nerantzis & Tataridis, 2006), while to date, wine lees have not received the same level of attention (De Iseppi, Lomolino, Marangon, & Curioni, 2020; Pérez-Bibbins et al., 2015; Pérez-Serradilla & de Castro, 2008; Ye, Harrison, Cheng, & Bekhit, 2016). Wine lees are a sludge-looking material mostly made of dead and living yeast cells, yeast debris and other particles that progressively precipitate at the bottom of wine tanks as soon as alcoholic fermentation ceases (Hwang, Shyu, & Hsu, 2009). The high organic matter content (900–35,000 mg/L) and chemical oxygen demand (around 30,000 mg/L) render wine lees environmentally harmful if not adequately disposed of (de Bustamante & Temiño, 1994; Pérez-Bibbins et al., 2015). Despite the proposal of several recovery and valorization strategies for the extraction of ethanol, tartaric acid and polyphenols from wine lees (Rivas, Torrado, Moldes, & Domínguez, 2006; Romero-Díez et al., 2019), only a few studies focused on the wine lees' solid fraction, that basically consists of yeast biomass. Some authors used this biomass for the production of culture media with mixed results (Kopsahelis et al., 2018; Salgado, Carballo, Max, & Domínguez, 2010). Moreover, yeast biomass resulting from brewing have been tested as a source of mannoproteins and β-glucans, both cell wall polysaccharides proposed as food emulsifiers and thickeners (Kalinga & Mishra, 2009; Silva Araújo et al., 2014). Moreover, yeast mannoproteins, which are made of high proportion of mannose and a smaller protein part (Goncalves, Heyraud, Norberta

Abbreviations: DAD, Diode Array Detector; HRSEC, High-resolution size-exclusion chromatography; HS, foam stability height; MW, Molecular Weight; OIV, International Organization of Vine and Wine; RID, Refractive Index Detector; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; HM, maximum foam height; TS, time taken for the foam to collapse after gas flow interruption.

* Corresponding author.
E-mail address: matteo.marangon@unipd.it (M. Marangon)

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de Pinto, & Rinaudo, 2002; Vidal, Williams, Doco, Moutounet, & Pellerin, 2003) have been studied as additives to prevent protein and tartrate precipitation in wines (Guise et al., 2014; Lomolino & Curioni, 2007; V.; Moine-Ledoux & Dubourdieu, 2002; Ribeiro, Fernandes, Nunes, Filipe-Ribeiro, & Cosme, 2014). Since the use of mannoproteins has been approved by the International Organization of Vine and Wine (OIV) (OIV - International Organisation of Vine and Wine, 2005), yeast mannoprotein preparations are now commercially available for use in winemaking. In fact, it has been demonstrated that mannoproteins can act as protective agents against wine protein instability, tartrate crystal formation and growth, thereby improving wine stability as a whole (Gerbaud, Gabas, Blouin, & Crachereau, 2010; Guise et al., 2014; V.; Moine-Ledoux & Dubourdieu, 2002). However, the way in which mannoproteins stabilize the wine has not been fully unraveled yet (Dufrechou, Doco, Poncet-Legrand, Sauvage, & Vernhet, 2015; Dupin et al., 2000; Lankhorst et al., 2017). Additionally, yeast mannoproteins have been shown to improve the foaming properties of sparkling wines probably because they are able to enter the liquid/air interface of wine bubbles thus reducing their surface tension (Blasco, Viñas, & Villa, 2011; Martínez-Lapuente, Guadalupe, Ayestarán, & Pérez-Magariño, 2015; Vincenzi, Crapisi, & Curioni, 2014). Finally, mannoproteins could increase the viscosity of the liquid of the wall of the bubbles, thus preventing their coalescence and contributing to foam stability and persistence (Blasco et al., 2011). Moreover, by affecting viscosity, mannoproteins may also positively affect wine mouthfeel (Gawel, Smith, & Waters, 2016; Li, Bindon, Bastian, Jiranek, & Wilkinson, 2017; Li, Bindon, Bastian, & Wilkinson, 2018).

All the mannoproteins-based oenological additives are typically manufactured from yeast cultures grown in bioreactors, whereas no attempts have been made to extract these components from wine lees taken during winemaking. As a matter of fact, while mannoproteins extraction from beer lees has been suggested (Cameron, Cooper, & Neufeld, 1988; Dikit, Maneerat, & H-kittikun, 2012; Dikit, Maneerat, Musikasang, & H-kittikun, 2010), no studies have attempted to extract cell wall components from wine lees' yeast biomass.

In this study, three different extraction methods, exploiting both physical and enzymatic approaches, were applied on lees recovered after the alcoholic fermentation of a white wine. In designing the extraction protocols and the choice of reagents, potential costs and applicability on large-scale were also taken into account. The composition of the extracts obtained was determined. Thereafter, their impact on wine protein and tartrate stability and their foaming properties were assessed.

2. Materials and methods

2.1. Reagents used

All the reagents used were sourced from Sigma-Aldrich (Saint Louis, MO, USA) unless otherwise specified. In particular, Phenol, PageRuler Plus Prestained Protein Ladder, Schiff reagent and SYPRO® Ruby Protein were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Wine lees production

Wine lees were recovered from a wine produced in the experimental cellar of the Department of Viticulture and Oenology at Stellenbosch University (South Africa). Sauvignon blanc grapes originating from the Overberg wine region (Western Cape, South Africa) were destemmed and pressed, and fermentation was conducted by adding to the must 25 g/hL of *Saccharomyces cerevisiae* Uvaferm HPS™ (Lallemand Inc, Montreal, Canada). The fermentation was carried out at 18 °C. When fermentation ceased, the wine was racked, and the lees collected from the bottom of the tank, washed twice with distilled water and centrifuged for 15 min at 4225 × *g*, 4 °C. The pellet obtained, mainly constituted of yeast biomass, was stored at −18 °C until further use.

2.3. Extraction of glycocompounds

For each extraction, 5 g of thawed lees (wet weight) were used. Each protocol was conducted at the pH value that allowed obtaining extracts having the best impact on wine properties in terms of solubility and protein stability as assessed by preliminary studies (data not shown). A schematic overview of the three protocols is represented in Fig. 1.

Physical extraction (Autoclave): the lees were suspended in 40 mL of McIlvaine buffer, pH 3.4 with 20 mM potassium metabisulphite and then autoclaved (121 °C, 20 min).

Ultrasonication extraction: the lees were suspended in 40 mL of McIlvaine buffer, pH 5 with 20 mM potassium metabisulphite and incubated at 35 °C for 30 min before being sonicated (Sonic Ruptor 4000, Omni International, Kennesaw, GA, USA) for 5 min at 50% of the maximum power.

Enzyme extraction: the lees were suspended in 40 mL of McIlvaine buffer, pH 5 with 20 mM potassium metabisulphite and incubated at 37 °C for 30 min. Thereafter, 35 mg of Glucanex® (Novozymes Co., Bagsvaerd, Denmark) were added and the suspension incubated at 37 °C for 3 h.

At the end of each extraction, the suspension was centrifuged (10509 × *g*, 10 min 4 °C) and the supernatant frozen at −18 °C overnight. After thawing, an insoluble fraction was separated by centrifugation (4105 × *g*, 10 min, 4 °C) and freeze dried. The obtained supernatant was then added with pure ethanol until it reached a concentration of 70% (v/v) and incubated overnight at −18 °C. Then, the sample was centrifuged (10509 × *g*, 30 min, 4 °C) and the pellet obtained freeze-dried.

Wine lees, glycocompounds extracts and insoluble fractions for each of the three extraction methods were oven dried (80 °C for 24 h) and the resulting weights were used to calculate the extraction yields.

2.4. Electron microscopy

Whole yeast cell and debris were first coated with a 15-20-nm gold layer in a sputter coater Quorum Q150R E (Quorum Technologies Ltd, Laughton, UK) and then observed under a FEI Quanta 200 variable pressure-environmental/ESEM microscope (Field Electron and Ion Company, Hillsboro, OR) equipped with a backscattered electron detector and an energy dispersive X-ray detector EDAX Element-C2B (EDAX Inc, Mahwah, NJ).

2.5. High-resolution size-exclusion chromatography by (HRSEC)

Both protein and polysaccharide concentrations in the three extracts and in the insoluble fractions resulting from the freezing/thawing of the extracts (see Fig. 1) were determined using a modified version of the high-resolution size-exclusion chromatography (HRSEC) procedure described by González-Royo et al. (González-Royo et al., 2017).

Determination of polysaccharides by HRSEC-RID. The polysaccharides' quantity and molecular distribution were measured by analyzing the freeze dried extracts by HRSEC (Ayestarán, Guadalupe, & León, 2004). Briefly, 1 mg of samples was dissolved in 1 mL of the running buffer (50 mmol/L aqueous solution ammonium formate), and sterile filtered (0.22-μm acetate cellulose filters, Millipore) directly into HPLC glass vials. Then, 10 μL were injected into the chromatographic system. The analyses were carried out in an Agilent 1260 series II quaternary pump LC (Agilent Technologies) equipped with a RID detector. Samples were held at 4 °C prior to injection in a temperature controlled auto-sampler. The separation was carried out at 20 °C using two gel perme-

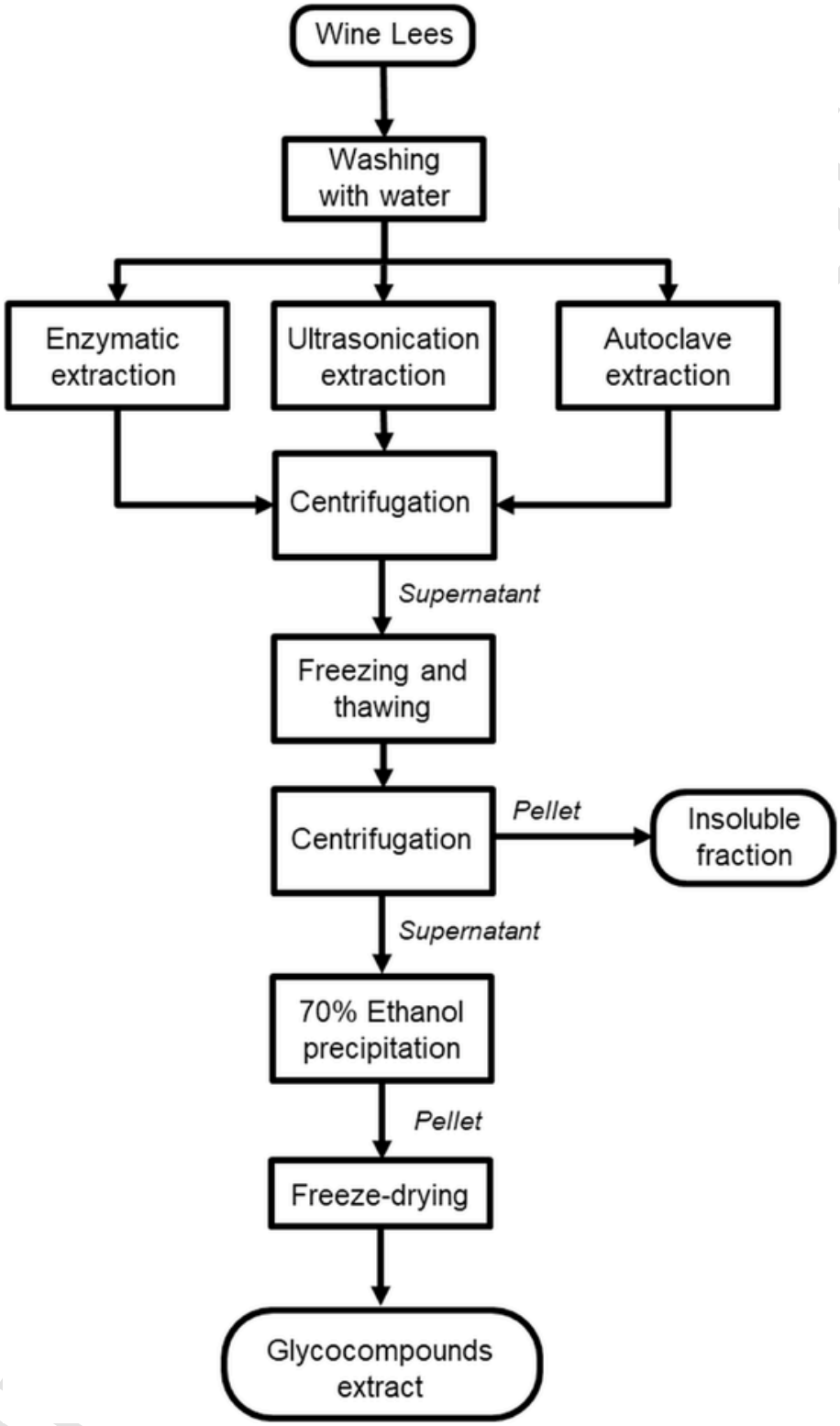

Fig. 1. Schematic representation of the different steps involved in the glycocompounds extraction from wine lees.

ation HPLC columns placed in series (PL-Aquagel-OH 50 and 40, Agilent). The mobile phase was applied at a constant flow of 0.6 mL/min for 70 min, and the temperature of the RID cell was kept at 35 °C. The molecular weight distribution of the extracts' polysaccharides was identified using a qualitative calibration curve made with 10 pullulan standards at MW ranging between 342 and 805,000 Da, while pectin and dextran were used in the range between 0 and 2 g/L to create the calibration curve for polysaccharide quantification.

Quantification of proteins by HRSEC-DAD. The protein concentration of the freeze-dried glycocompounds extracts and insoluble fractions was measured adapting a method previously described (González-Royo et al., 2017). Briefly, 1 mg of each extracts was dissolved in

1 mL of running buffer (300 mmol/L ammonium acetate) and sterile filtered (0.22-μm acetate cellulose filters, Millipore) directly into HPLC glass vials. Then, 100 μL of sample was injected into the chromatographic system that was equipped with a DAD detector monitoring at 230, 280 and 320 nm. The elution was performed in isocratic mode at a flow rate of 0.6 mL/min for 70 min. The separation was carried out at 20 °C using a PL-Aquagel-OH 40 gel permeation HPLC column (Agilent). The proteins were quantified according to the peak area for each fraction using the external standard method with bovine serum albumin in a range between 0 and 10 mg/mL.

2.6. Total polysaccharides quantification within the insoluble fraction

Total polysaccharide content of the insoluble fractions was measured using a modified version of the phenol–sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Segarra, Lao, López-Tamames, & De La Torre-Boronat, 1995). Each tartrate extract was added at 50 μg/mL in a water/phenol solution prepared by dissolving phenol (Honeywell International Inc., Wabash, USA) at 2% (v/v) in distilled water. The suspension was then heated (50 °C, 30 min) until complete solubilization of the tartrate crystals. Then, 400 μL of the samples were transferred into a new vial and added with 1 mL of pure sulphuric acid. After 30 min, the absorbance was measured at 490 nm. A calibration curve was prepared using a serial dilution of glucose (0–100 mg/L) prepared in the water/phenol solution.

2.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins within each extract were fractionated according to their molecular weight (MW) by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% total acrylamide gel. Each sample was prepared by solubilizing 2.5 mg of freeze died extract in 43.5 μL of distilled water and 6.5 μL of loading buffer (45% (v/v) 100% glycerol; 20% (v/v) 20% Sodium dodecyl sulphate; 17.5% (v/v) 0.5 M Tris/HCl pH 6.8; 12.5% (v/v) 2-mercaptoethanol; 5% (v/v) milliQ water) containing 0.1 mg/mL bromophenol blue. After heating at 95 °C for 10 min, 25 μL of this solution were loaded into the gel. The Glucanex enzyme was also analyzed for comparison purposes and loaded at the same concentration as the glycocompounds extracts. Standard MW was PageRuler Plus Prestained Protein Ladder (range 10–250 kDa). The analysis was conducted at constant amperage (48 mA). Protein fractions were stained using SYPRO® Ruby Protein Stain according to the manufacturer's instructions while glycosylated proteins were revealed using the Periodic Acid–Schiff (PAS) technique (Zacharius, Zell, Morrison, & Woodlock, 1969). Images of the gels were acquired at 300 dpi resolution with a ChemiDoc™ XRS molecular imager (Bio-Rad Laboratories).

2.8. Assessment of extracts' impact on wine properties

The effects of different extracts on several wine properties were tested. In particular, the extracts' effect on protein stability was assessed on the same Sauvignon blanc wine (pH 3.4) from which the wine lees were initially separated. The tartrate stability test was carried on a Pinot grigio wine (11.5% (v/v) Ethanol, 2.3 g/L tartaric acid, pH 3.8), while the foaming properties test was conducted on a model wine (12% (v/v) Ethanol, 2.5 g/L tartaric acid, pH 3.4). For the three tests, the same concentration of extract (0.5 g/L) was used.

2.9. Protein stability test (Wine lees extract)

The three extracts were solubilized in wine and incubated overnight at an ageing cellar-like temperature of 15 °C. Glucanex was also tested (at 0.05 g/L) to assess if the sole enzyme possessed a heat stabilizing effect. The heat stability was tested by heating the wines at 80 °C for 2 h, followed by a cooling step at 15 °C for 20 h before the haze produced was measured by calculating the difference between the heated and unheated samples in the absorbance values at 540 nm (Waters, Wallace, & Williams, 1991) by means of a Lambda 25 UV/Vis spectrophotometer (PerkinElmer, Waltham, MA). Samples were considered to be protein unstable when the difference in absorbance between heated and unheated controls was >0.02 absorbance unit (AU) (Waters, Wallace, & Williams, 1992).

2.10. Protein stability test (yeast culture extract)

With the aim of obtaining a model extract, a culture of the strain used for wine lees production (*S. cerevisiae* "Uvaferm HPS") underwent the ultrasonication extraction protocol. The extract was then solubilized in pH 5 McIlvaine buffer and incubated at 35 °C with 0.1 g/L Protease from *Aspergillus saitoi* (Sigma Aldrich). The latter commercial protease was used to simulate the proteolytic activity within Glucanex. At different time points (from 0 to 4 h), an aliquot of the solution was sampled, heated at 85 °C for 15 min to inactivate the protease and then centrifuged at $10{,}509 \times g$ for 5 min to remove possible insoluble material. The so-obtained supernatant was added to the Sauvignon blanc wine reaching an initial extract concentration of 0.5 g/L. Wine with no protease and no extract was used as control after the addition of a correspondent volume of buffer to check for dilution effect. After overnight incubation at 15 °C, the test was carried out as described in the previous section.

2.11. Tartrate stability

Wines added with the three extracts were analyzed with the Tartarcheck apparatus (Ing. C. Bullio, Modena, Italy), previously calibrated with a standard conductivity solution. During the test, 20 mL of sample were equilibrated at 0 °C, followed by the addition of 300 mg of micronized potassium hydrogen tartrate. The decrease in conductivity (ΔμS/cm), keeping the temperature at 0 °C and under continuous stirring, was measured during 10 min. White wines with values higher than 50 ΔμS/cm are considered unstable (Malacarne, Bergamo, Bertoldi, Nicolini, & Larcher, 2013). A yeast mannoproteins-containing product commercialized to improve tartrate stability (Mannostab, Laffort, Bordeaux, France) was also tested for comparison purposes at the same dosage (0.5 g/L).

2.12. Foaming properties

For this test, a modified Mosalux method was used (Brissonnet & Maujean, 1991; Crumpton, Rice, Atkinson, Taylor, & Marangon, 2018). The three extracts were solubilized in 40 mL model wine and then poured in a chromatography column (25 mm diameter, 500 mm in length, with a 16–40 μm glass sintered disc at the base) equipped with a 2-mm bore glass valve. A plastic pipe connected the column with a flowmeter regulator set at 100 mL/min and this to a CO_2 cylinder set at 1 bar pressure. Starting from the upper base of the glass filter, a measuring tape was attached to the outside of the column. Thanks to this, after pouring, the wine height was recorded as starting point. After the CO_2 flow started, the height of the produced foam was also recorded every 15 s for 7 min. This protocol allows measuring the maximum foam height (HM), foam stability height (HS) and the time taken for the foam to collapse after gas flow interruption (TS). Model wine with no extract addition was taken as control.

3. Results and discussion

3.1. Extraction yields and extracts composition

The extraction yields of the different methods along with data from protein and polysaccharides quantification are shown in Table 1.

Extraction yields did not differ significantly amongst the extraction methods tested, being generally higher than those reported in previous studies on the extraction of mannoproteins (Costa, Magnani, & Castro-Gomez, 2012; Silva Araújo et al., 2014) and cell wall soluble polysaccharides from yeast (De Iseppi et al., 2019). This discrepancy could be due to the fact that the yeast strain used in this work was specifically selected for its high content of cell wall's polysaccharides, while this was not the case for the strain used in the above-cited manuscripts. Polysaccharides were the main components in all the extracts. However, the measured quantities were higher than the extract weighed for the analysis (1000 mg/L), an occurrence probably due to the HPLC calibration method that used pectin and dextran as standards. The extraction methods adopted here were designed to extract mannoproteins which have been shown to possess molecular weights in the range from 5 to 800 kDa (Doco, Vuchot, Cheynier, & Moutounet, 2003; Rodrigues, Ricardo-Da-Silva, Lucas, & Laureano, 2012; Saulnier, Mercereau, & Vezinhet, 1991). According to this wide molecular weights' range, mannoproteins could be supposed to be present in all the polysaccharide fractions detected by HPLC. Nevertheless, the large proportion of oligosaccharides found in all extracts strongly suggests that native mannoproteins were partially hydrolyzed. However, a significantly higher content of high MW polysaccharides could be found in the autoclave extract compared to the ultrasonication and enzyme ones. This is consistent with previous studies in which this method was suggested for the extraction from yeast of high and medium molecular weight mannoproteins for potential food applications (Cameron et al., 1988; Costa et al., 2012; Silva Araújo et al., 2014). In all the extracts, proteins were present in concentrations ranging from 2.9 to 6.5%. These percentages are consistent with the yeast mannoproteins composition previously reported by others (Gonçalves, Heyraud, De Pinho, & Rinaudo, 2002). Nevertheless, compared to a previous study (De Iseppi et al., 2019) describing similar extractions from a yeast culture, the protein contents reported here were between 8 and 2 times lower (Table 1). This outcome could be due to the differences in both the extraction procedure and yeast cell's growth conditions that, in this case were recovered after wine fermentation while in the previous study were from a laboratory yeast culture.

The insoluble crystals-containing precipitate formed after freezing and thawing the extracts before the ethanol precipitation step (Fig. 1) became soluble upon heating, thereby allowing its composition to be investigated (Table 2).

Results showed that this fraction was predominantly made of tartaric acid and contained also smaller quantity of citric acid (Table 2). Some proteins and polysaccharides could also be visualized on SDS-PAGE gels, likely deriving from small amounts of extract remaining in the precipitate (Fig. S2). These data suggest that this insoluble fraction could be exploited for the recovery of tartaric acid, an acidifier used in many sectors (Salgado, Rodríguez, Cortés, & Domínguez, 2010). In this context, the autoclave extraction was the most efficient, likely because the high temperature applied resulted in a higher degree of solubilization of the tartrate salts present in the wine lees.

3.2. Characterization of the extracts through SDS-PAGE analysis

The three extracts (and the Glucanex enzyme) were analyzed by SDS-PAGE and gels were stained to detect both proteins (Sypro stain) and glycocompounds (PAS staining) (Fig. 2).

When looking at the gel stained for glycocompounds, the autoclave extract presented two bands with mobility corresponding to ≈250 kDa and ≈70 kDa. This result indicates that components containing sugars are able to enter the electrophoretic gel probably thanks to the presence of a protein part in the molecules.

Moreover, when comparing the PAS lane with the gel stained for proteins, faint protein bands were detected at the same level of the PAS-stained bands, an occurrence compatible with the possible presence of proteins associated with the sugars. In this context, the natural candidates are yeast mannoproteins, which have been shown to enter SDS-PAGE gels at least in part (Vincenzi, Marangon, Tolin, & Curioni, 2011). These findings, alongside the information about the composition of the extracts (Table 1) indicate that the autoclave extract is the richest in mannoproteins of high and medium MW. On the other hand, a diffuse Sypro-stained area between 10 and 15 kDa was detectable only for the autoclave extract, indicating the presence of protein fragments, which may be related to the thermal treatment, as previously observed with extraction conditions involving a heating step (De Iseppi et al., 2019).

After staining for proteins, the ultrasonication extract presented two bands, one with MW higher than 250 kDa and one between 70 and 55 kDa. In addition, a few faint bands were observed at lower molecular weights. Faint bands with corresponding electrophoretic mobility were found at the top of the gel after staining for both proteins and sugars as also seen for the autoclave extract. Therefore, there seem to be mannoproteins also in the ultrasonication extract.

The enzyme extract, despite containing a quantity of high MW polysaccharides similar to that of the ultrasonication one (Table 1), showed a more intense PAS-stained band at the top of the gel. This result is consistent with findings from a previous study in which the enzyme extraction protocol proved to be suitable for the isolation of high molecular weight glycocompounds from yeast culture (De Iseppi et al., 2019). However, it must be noted that many of the protein bands of the enzyme extract are also present in the Glucanex enzyme (Fig. 2, compare Sypro stains for enzyme extract with that of Glucanex enzyme preparation), thus indicating that residual Glucanex components remain in the enzyme extract. Despite this, the PAS staining revealed a band with the same mobility of those detected for the autoclave and ultrasonication extracts indicating that, also in this case, this band likely comprised high MW mannoproteins.

Table 1
Extraction yields, concentrations of proteins and polysaccharides (g/100 g dry lees) obtained from the different extraction methods tested. Polysaccharides: High MW: 1100–180 kDa; Medium MW: 180–40 kDa; Low MW: 40–7.5 kDa; Oligosaccharides: 7.5–1 kDa. Analysis was performed in triplicate. Mean values followed by the same letter are not significantly different at $p \leq 0.05$ by analysis of variance (ANOVA) and Tukey's test.

Method	Extraction yield	Protein	Polysaccharides (% of the total)				
			Total	High MW	Medium MW	Low MW	Oligosaccharides
Autoclave	18.8 [a]	6.5 [a]	124.4 [ab]	2.6 [a]	3.7 [a]	3.0 [a]	90.9 [b]
Ultrasonication	20.3 [a]	2.9 [b]	102.0 [b]	1.2 [b]	1.3 [b]	2.1 [a]	95.4 [a]
Enzyme	22.7 [a]	2.9 [b]	137.9 [a]	1.2 [b]	1.7 [c]	2.4 [a]	94.7 [a]

Table 2
Yields and composition of the insoluble fraction formed after freezing and thawing the extracts. Analyses were performed in triplicate. Mean values followed by the same letter are not significantly different at $P \leq 0.05$ by analysis of variance (ANOVA) and Tukey's test.

Method	Yield	Protein	Polysaccharides	Tartaric acid	Citric Acid
	(g/100 g dry lees)	(g/100 g dry insoluble fraction)			
Autoclave	23.6 [a]	3.3 [a]	12.1 [a]	62.7 [a]	6.3 [a]
Ultrasonication	7.2 [b]	2.1 [b]	8.9 [a]	62.7 [a]	3.3 [b]
Enzyme	11.5 [b]	2.2 [b]	8.1 [a]	73.0 [a]	5.0 [ab]

Considering the recovery of both high MW mannoprotein in the soluble fraction and of tartaric acid in the insoluble one, the autoclave extraction may be considered as the best protocol for a potential industrial exploitation of white wine lees.

3.3. Electron microscopy

The wine lees used in this experiment were submitted to scanning electron microscopy analysis in order to visualize the impact that the different treatments had on the structure of yeast cells (Fig. 3).

When compared to the control sample (before the extraction treatment), the yeast cells showed signs of degradation that differed among the three treatments adopted. In particular, compared to the control, the autoclave-treated yeast cells clearly showed the highest level of damage, displayed by a high quantity of distorted cells and cell debris. In contrast, yeast cells subjected to ultrasonication and Enzyme treatments showed to be less damaged even though part of the external porous layer of the cells appeared to be smoother than that of the control. These findings are in agreement with previous reports in which the degree of cell degradation was related to the level of degradation of the external layer of the cell walls which contains the mannoproteins (Bzducha-Wróbel et al., 2014; De Iseppi et al., 2019; Liu, Wang, Cui, & Liu, 2008; Magnani et al., 2009). Therefore, the level of damage visible in Fig. 3 seems to correspond to quantitative and qualitative data discussed above (Table 1 and Fig. 2), which showed that the autoclave extract contained the highest amount of mannoproteins.

However, the degree of cell damage was less severe than that reported in a previous study using yeast cells grown in optimal laboratory conditions (De Iseppi et al., 2019). Indeed, the harsh fermentation conditions to which yeast cells have been exposed to in this study (e.g. low nutrients, high ethanol content, presence of sulphur dioxide) could have resulted in a cell wall reinforcement and thus to a limitation of the wall's permeability, a hypothesis in agreement with previous reports (Klis, Boorsma, & De Groot, 2006).

In conclusion, treating the wine lees with the autoclave method showed the greater degree of damage, a fact that justifies the highest recovery of total glycocompounds found following this procedure.

3.4. Protein stability test

Although their precise mechanism is not fully understood, the positive role of mannoproteins in protein stabilization of white wines is well known (Dupin et al., 2000; Lomolino & Curioni, 2007; Moine-Ledoux & Dubourdieu, 1999), and to this aim winemakers

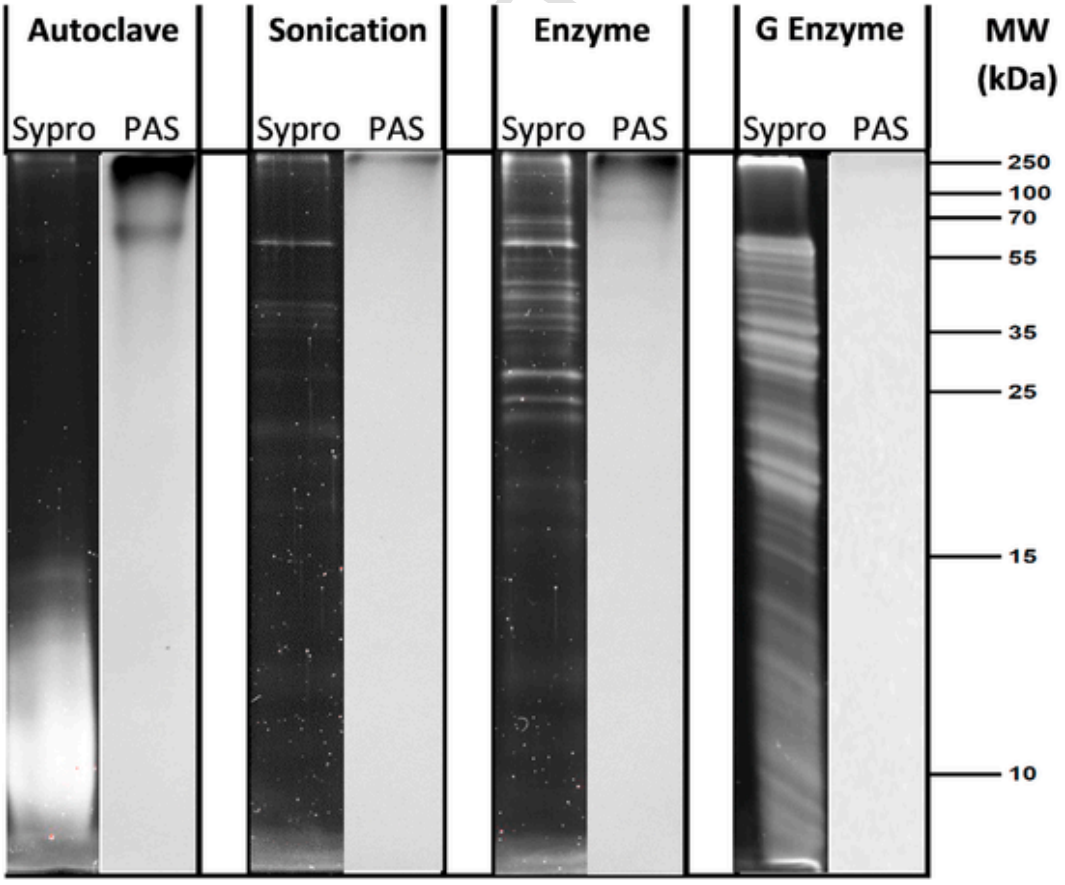

Fig. 2. SDS-PAGE analysis of the autoclave, ultrasonication and Enzyme extracts. Gels were stained for proteins and glycocompounds with the Sypro and PAS methods, respectively. G enzyme: Glucanex preparation used for the enzyme extract, shown for comparison.

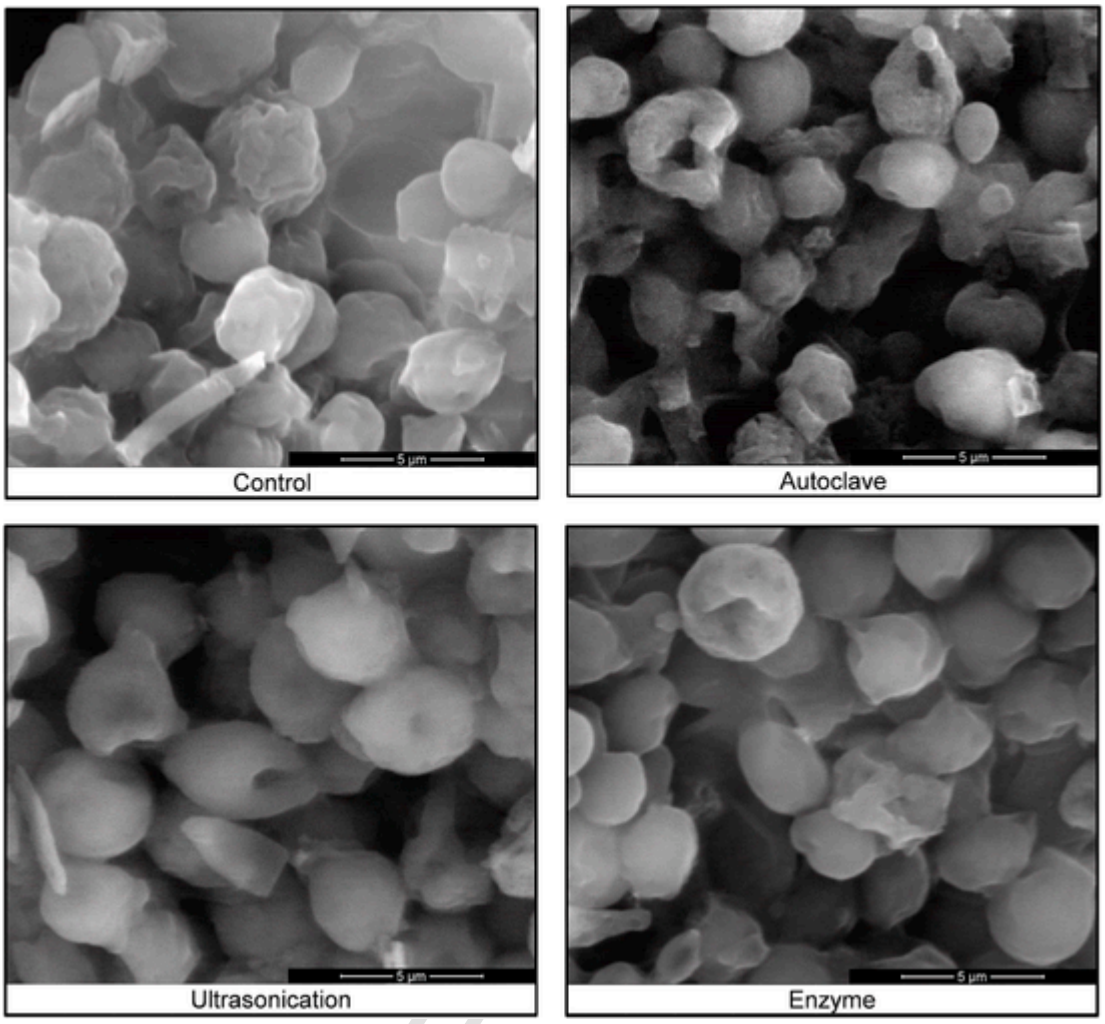

Fig. 3. Micrographs of wine lees before (control) and after the three different treatments.

use to maintain wine in contact with yeast lees for extended periods of time to favor its self-stabilization.

Therefore, considering that the three extracts described above are rich in polysaccharides, including mannoproteins, their effects on protein stability was assessed on a heat unstable white wine (Fig. 4). Before conducting the heat test, all the extracts resulted soluble in wine as assessed by measuring the wine turbidity after they were added (Fig. S1).

Upon heating, the turbidity of the wines supplemented with 0.5 g/L of ultrasonication and enzyme extracts were significantly lower than that of the control. This reduction, that was close to 7% for both extracts, confirms the stabilizing effect that yeast extracts have on wine protein stability (Dupin et al., 2000; Ribeiro et al., 2014). Unexpectedly, despite being the richest in high and medium molecular weight polysaccharides, the autoclave extract did not significantly alter wine turbidity upon heating.

The limited haze reduction obtained using the extracts may be attributed to the fact that they contain a certain amount of non-glycosylated proteins (Fig. 2), that would contribute to the haze formed, thus lowering/masking the stabilizing effect of the extracts. This hypothesis can partially explain the higher haze-reducing activity of both the ultrasonication and enzyme extracts (Fig. 4). Indeed, ultrasonication is a gentler treatment than autoclaving as confirmed by the electron microscopy results (Fig. 3). This fact should limit the extraction of proteins, as indicated by the protein concentration (Table 1) and SDS-PAGE (Fig. 2) results.

The enzyme extract, which was similar to the ultrasonication extract in terms of degree of cell damage, protein and polysaccharide profiles, also led to a significant decrease in turbidity (Fig. 4). This effect was not attributable to the degradation of the wine proteins by the proteolytic activity of the Glucanex preparation, since this had no effect when added to the wine (Fig. 4).

In conclusion, despite some significant reduction in turbidity compared to the untreated wine, the level of stabilization achieved using these extracts is too low to allow for a substantial reduction of the bentonite doses used in commercial winemaking.

In a previous study, it was hypothesized that the wine haze reducing effect observed for a yeast extract obtained by using Glucanex was due to its proteolytic activity which generated an invertase fragment responsible for the stabilizing activity towards protein haze formation (Moine-Ledoux & Dubourdieu, 1999). To verify this, a stability test was performed using a yeast extract obtained from a culture of the same strain (Uvaferm HPS) used for the production of the wine lees. To prepare the extract, ultrasonication was selected since it showed to provide an extract containing mannoproteins but with a low content of proteins that would interfere with the results of the heat test. To test the impact of the proteolytic activity, a pure aspartic protease was then added to the extract previously solubilized in a pH 5.0 buffer. During the incubation (at 35 °C), aliquots of the solution were sampled at dif-

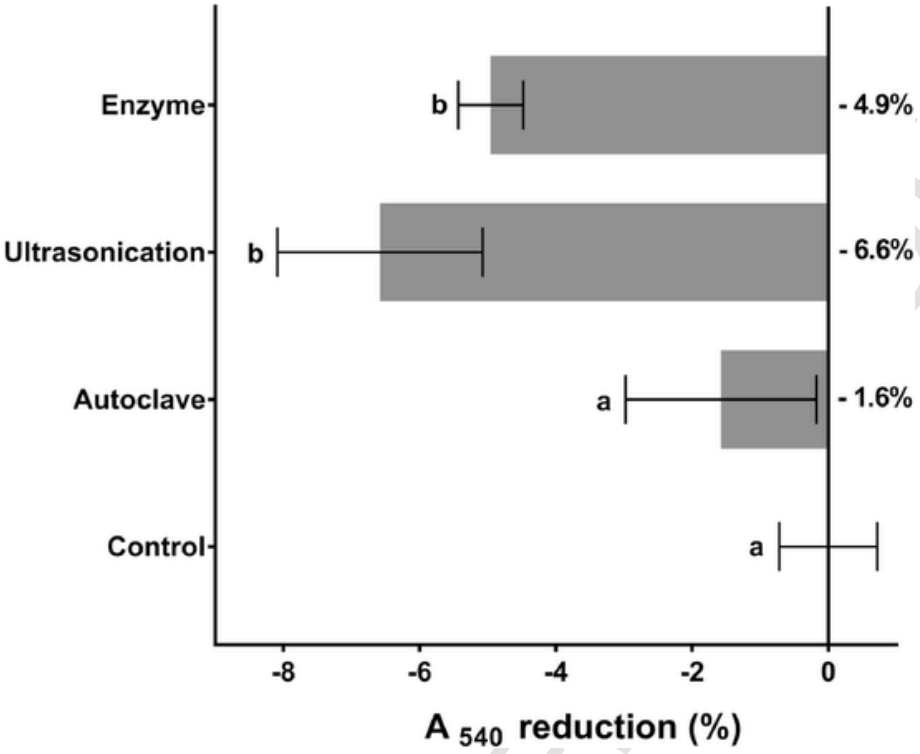

Fig. 4. Protein stability test of unstable Sauvignon blanc wine added with 0.5 g/L of the yeast lees extracts. The reduction in absorbance of the Glucanex alone was not significant and it was subtracted from that of the Enzyme extract. Data are expressed as percent difference in A_{540} from the control (wine with no added extracts, A_{540}: 0.175). Different letters indicate significant differences at $p \leq 0.05$ by analysis of variance (ANOVA) and Tukey's test.

ferent time intervals, heated (85 °C for 15 min) to inactivate the protease, and added to the Sauvignon blanc wine, that was then heat tested for protein stability (Fig. 5).

A constant decrease in haze formation was detected for samples incubated until 1 h of contact with the protease. This behavior seems to confirm that the proteolytic activity contributes to protein stabilization by yeast extracts (Moine-Ledoux & Dubourdieu, 1999). However, incubations of the extracts with the protease for more than 1 h resulted in a progressive reduction of the stabilizing effect. This could be due to the release of yeast heat-unstable proteins deriving from a prolonged protease activity. These data are in line with those of Fig. 4 where the ultrasonication extract showed the same reduction of haze formed of the sample at time zero shown in Fig. 5 (around 7%).

After 3 h of incubation with the protease, the reduction in turbidity was the same shown by the enzyme extract that was in fact prepared by incubating it for 3 h with the wine lees (Fig. 4), during which the Glucanex's protease should have had the time to act together with the main β-glucanase activity present in the preparation.

Even if the results presented here are not very promising for winemaking applications, the data reported from the extracts obtained from the laboratory-grown yeast strain and those produced starting from the wine lees are comparable. This may suggest that white wine lees could be considered as an alternative to the industrially produced yeast biomass used to prepare mannoprotein-based wine additives.

3.5. Tartrate stability test

The three yeast extracts produced in this study were added to a Pinot grigio wine not previously stabilized for tartrate salts precipitation, and the resulting tartrate stability was evaluated (Fig. 6).

Amongst the samples tested, the autoclave extract was the only one able to induce a significant decrease in tartrate precipitation (−11%), a reduction that, however, was not sufficient to fully stabilize the wine. Conversely, all the other extracts, including the commercial one, showed no stabilizing effects, a finding in line with previous reports, in which some commercial mannoproteins showed weak or no effect (Greeff, Robillard, & du Toit, 2012; Guise et al., 2014). According to the latter authors, the impact of yeast extracts on tartrate precipitation varies from none to decisive depending on both its mannoprotein composition and the wine tested. In fact, the autoclave extract displayed the highest mannoprotein content, thus suggesting that this factor is indeed responsible for the improved cold stability observed, although a possible contribution of specific proteins contained in the extract cannot be excluded. In any case, even if not able to fully stabilize a wine, mannoprotein-based additives offer the possibility to reduce the energy cost of other practices as cold treatments and electrodialysis (Lasanta & Gomez, 2012). In this context, the use of wine lees as a source of mannoproteins should further contribute to the sustainability of the winemaking process.

3.6. Foaming properties

Also the foaming proteins of sparkling wines have been related to the presence of yeast mannoproteins (Martínez-Lapuente et al., 2015). Therefore, the effects of the three yeast lees extracts on wine foaming properties were tested in model system. This approach was adopted in order to eliminate the effect of wine proteins as these are known to strongly contribute on wine foaming (Vincenzi et al., 2014). These properties have been evaluated in terms of foam volume and stability (Table 3).

The data revealed that the autoclave extract was the best performing for all the parameters investigated. This result is consistent with those reported in a previous study (Núñez, Carrascosa, González, Polo, & Martínez-Rodríguez, 2006) in which heat extracted yeast mannoproteins induced better foaming abilities than those extracted enzymatically. This is attributable to the fact that the autoclave extract

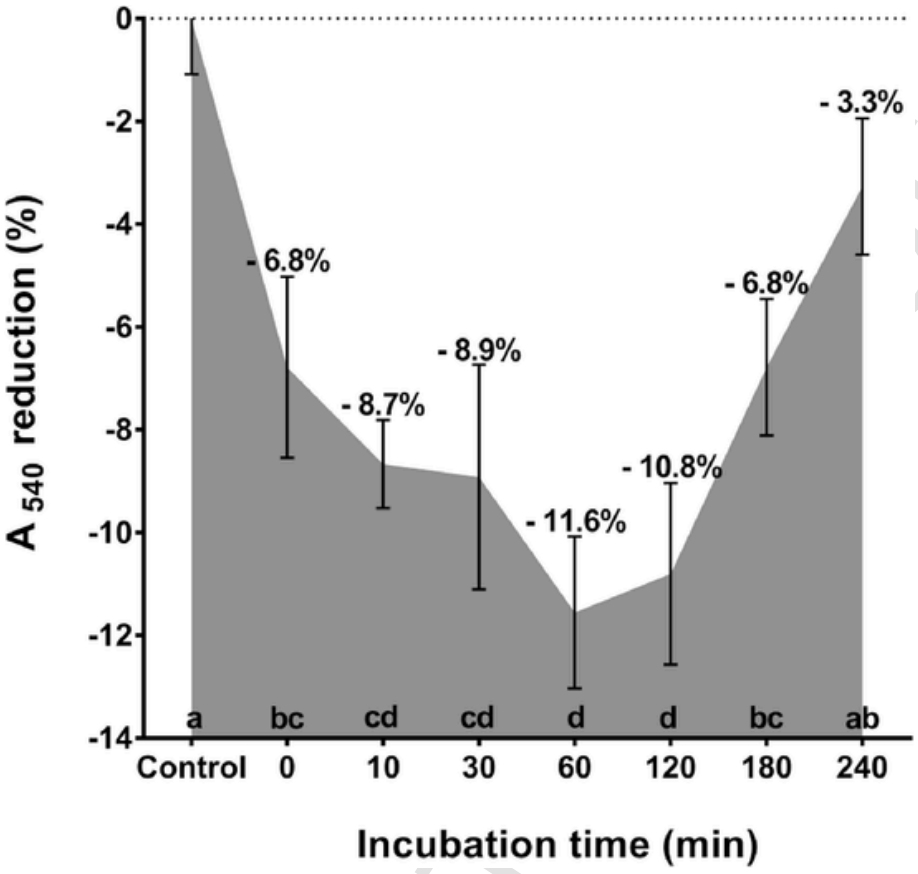

Fig. 5. Heat test results for a Sauvignon blanc wine added with 0.5 g/L of an extract obtained by ultrasonication of *S. cerevisiae* Uvaferm HPS cells and incubated with a protease from Aspergillus saitoi for 0, 10, 30, 60, 120, 180 and 240 min. The same untreated wine (no protease, no extract) was used as control after the addition of 0.5 g/L of buffer alone to avoid dilution effects. The horizontal dotted line indicates the turbidity level of the control wine.

contains the largest amount of both mannoproteins and proteins (Table 1), two of the main foam-promoting components in wine (Martínez-Lapuente et al., 2015; Vincenzi et al., 2014). This result is relevant as it has been reported that high molecular weight mannoproteins released from yeast contribute to wine foaming properties in sparkling wines (Vincenzi et al., 2014). Indeed, it can be noticed that the ultrasonication and enzyme extracts (which contained comparable high molecular weight polysaccharide amounts) both presented a similar behavior, showing lower values for all the foam parameters measured. Furthermore, in both cases, no differences were detected from the model wine alone for the time needed to the foam to disappear after stopping the gas flow (TS). This parameter, which would be indicative of the persistence of the foam collar in the glass (Gallart, Tomás, Suberbiola, López-Tamames, & Buxaderas, 2004), was instead more than ten times higher for the autoclave extract, confirming its positive effects on foam characteristics.

4. Conclusions

In this study, three extraction protocols were designed and compared to extract glycocompounds from wine lees collected at the end of the alcoholic fermentation of a real white wine. This was carried out to assess whether this winemaking by-product could be a source of valuable compounds to be used for the improvement of wine stability and sensorial properties.

Despite the fact that all the extracts impacted at least some of the wine properties tested, none of them was equally effective for all the applications tested. In particular, lees extracted by autoclave seem to be more suitable for wine tartrate stabilization, for the improvement of wine foaming and for the recovery of tartrates from its insoluble fraction. Conversely, lees extracted by ultrasonication and enzymatic methods seem to be more effective to aid in the protein stabilization of heat-unstable wines.

Interestingly, the commercial product used as control in the assessment of wine tartrate stability performed worse than the autoclave extract obtained from wine lees, thus suggesting the possibility of exploiting these by-products as a source of valuable substances to be used as winemaking additives.

Furthering the knowledge on wine lees as a source of compounds to be used in winemaking could lead to a better exploitation these by-products, thus contributing to an improvement of the circular economy approach within the wine industry.

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Declaration of competing interest

None.

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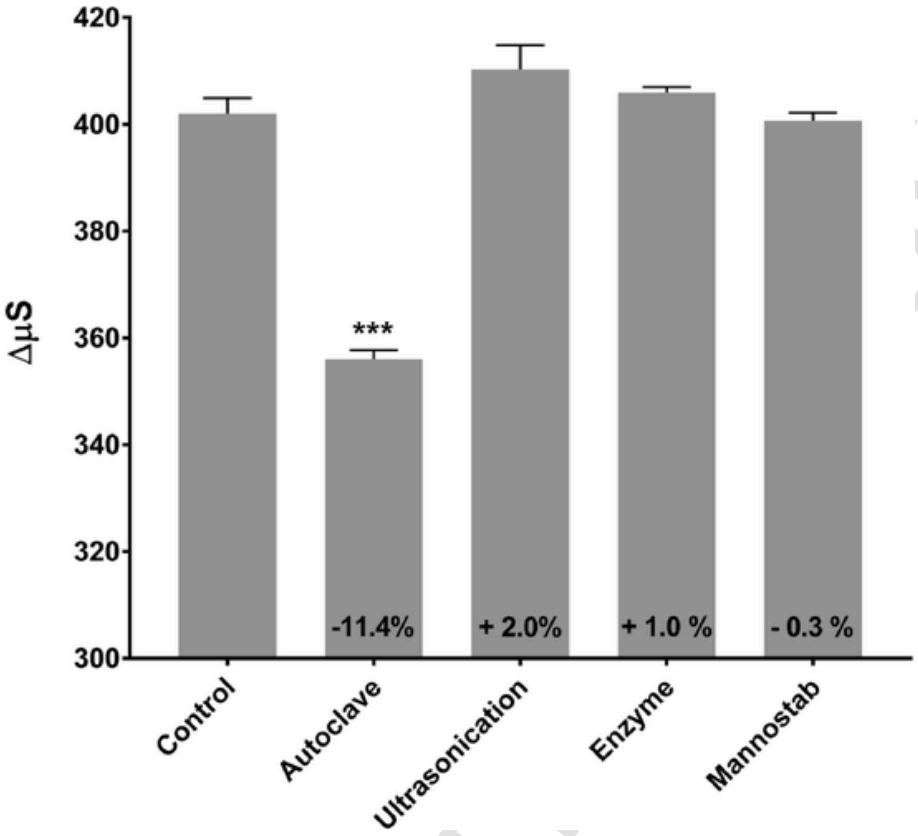

Fig. 6. Tartrate stability test (Tartarcheck apparatus) of a Pinot grigio wine containing 0.5 g/L of the yeast extracts. Mannostab®, a commercial mannoprotein-based wine additive used to improve wine tartrate stability, was tested for comparison at the same concentration. Data are expressed as the difference in the conductance values measured at the beginning and at the end of the analysis. Mean values with three stars (***) are significantly different from the control sample at $p \leq 0.01$ by analysis of variance (ANOVA) and Tukey's test.

Table 3
Maximum foam height (HM), foam stability height (HS) and time for foam disappearance (stability time, TS) of model wine (MW) supplemented with 0.5 g/L of the three wine lees extracts. Mean values followed by the same letter are not significantly different at $p \leq 0.05$ by analysis of variance (ANOVA) and Tukey's test.

Samples	HM (cm)	HS (cm)	TS (sec)
Model Wine alone	3.3 ^{c}	0.9 ^{d}	2 ^{c}
MW + Autoclave extract	8.8 ^{a}	3.2 ^{a}	58 ^{a}
MW + Ultrasonication extract	5.7 ^{b}	1.4 ^{bc}	5 ^{c}
MW + Enzyme extract	5.4 ^{bc}	1.7 ^{b}	5 ^{c}

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110274.

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