



# The macromolecular diversity of Italian monovarietal red wines

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## ABSTRACT

While red wine phenolics have been extensively studied, polysaccharides and proteins have not received the same level of attention, especially when considering Italian wines. In this study, for the first time, quantitative and qualitative data on the macromolecular (proteins and polysaccharides) and tannin composition of 110 monovarietal red wines from 11 of the most important Italian grape varieties are reported. The winemaking did not include any filtration, oak contact, fining treatments, or ageing on yeast lees. Results highlighted a great inter- and intra- varietal diversity. The protein content ranged between 0 and 159 mg/L, polysaccharides between 211 and 1081 mg/L and total tannins between 171 and 3746 mg/L, with averages of 41 mg/L, 497 mg/L and 1687 mg/L, respectively. Six varieties with protein content representative of the variability observed were selected and submitted to electrophoresis. Within each variety, the SDS-PAGE mobility of protein-tannin complexes was similar but showed two distinct patterns for wines of different varieties (higher mobility for Corvina, Teroldego and Raboso Piave, lower mobility for Nebbiolo, Aglianico, Cannonau), suggesting that the Italian monovarietal wines can be diverse also in their colloidal-forming structures. This can be explained by looking at the different percentages of protein-reactive tannins ( $T_{BSA}$ ) on the total tannin content ( $T_{MCP}$ ), which is a varietal characteristic.

**KEYWORDS:** red wine, macromolecules, proteins, polysaccharides, tannins, colloids, diversity

## INTRODUCTION

In the past decade, Italy has established itself as the country with the highest yearly wine production (International Organisation of Vine and Wine, 2019). Italian wine production is characterised by a multitude of monovarietal wines thanks to the over 600 autochthonous grape varieties currently registered (Bavaresco *et al.*, 2014). Several of these grape varieties are grown in significant amounts in different wine regions, resulting in the introduction on the market of very diverse wines able to satisfy the requests of different types of customers. Indeed, Italy currently produces 527 wines with geographical indication including 76 DOCG (Denominazione di Origine Controllata e Garantita) wines, 333 DOC (Denominazione di Origine Controllata) wines and 118 IGT (Indicazione Geografica Tipica) wines (FederDOC, 2020). Internationally the situation is quite different. While countries other than Italy possess a large ampelographic platform (e.g., Portugal), a few major French varieties (e.g., Chardonnay, Sauvignon blanc, Cabernet-Sauvignon, Pinot noir, Merlot and Syrah) dominate the world wine scene. However, in the past couple of decades, several Italian grape varieties have been increasingly planted in other countries (Wine Australia, 2018) by growers interested in their distinctive quality traits (e.g., aromas, colour, market appeal) or characteristics that would match well the local climatic conditions (e.g., acidity level, alcohol level, resistance to stress, phenological stages).

Despite the world relevance of Italian varieties, these have not been intensively researched as the French varieties previously mentioned. The recent establishment of “The Diversity of Italian Wines (D-Wines)” group seeks to rectify this issue by conducting collaborative projects aimed at the collection and analyses of large compositional datasets from Italian monovarietal wines sourced from their typical area of production (here indicated as “origin wines”). As a result of this effort, several articles focussing on the chemical and sensory profiles of Italian red wines have recently been published. Numerous classes of compounds were quantified in Italian red wines with different abundance in each of the monovarietal wines investigated, suggesting that these could be instrumental to understanding wine diversity, and several of them were putative markers for the identification of the cultivar (Arapitsas *et al.*, 2020; Giacosa *et al.*, 2021; Parpinello *et al.*, 2019a; Piombino *et al.*, 2020). However, data on the macromolecular composition of Italian red wines are scarcely available in the literature, while a better understanding of their characteristics, function and interactions would be welcome by winemakers due to the practical implications that these findings would have.

Proteins, polysaccharides and condensed tannins are wine macromolecules known to interact with each other to form colloidal particles (Bindon *et al.*, 2016; Gazzola *et al.*, 2012; Kassara *et al.*, 2019; Marassi *et al.*, 2021; Quijada-Morín *et al.*, 2014). These molecules, alone or

in their colloidal assemblies, are known to greatly affect many wine quality parameters, including stability, sensory attributes and colour (Del Barrio-Galán *et al.*, 2015; Jones-Moore *et al.*, 2022; Martínez-Lapuente *et al.*, 2020; Pascotto *et al.*, 2021; Vernhet, 2019). When looking at red wines, the classes of colloid-forming molecules mostly studied are polysaccharides and phenolics (Jones-Moore *et al.*, 2021; Mercurio *et al.*, 2007; Vidal *et al.*, 2003), even if only phenolics data are available for Italian varieties (Giacosa *et al.*, 2021; Parpinello *et al.*, 2019a; Parpinello *et al.*, 2019b).

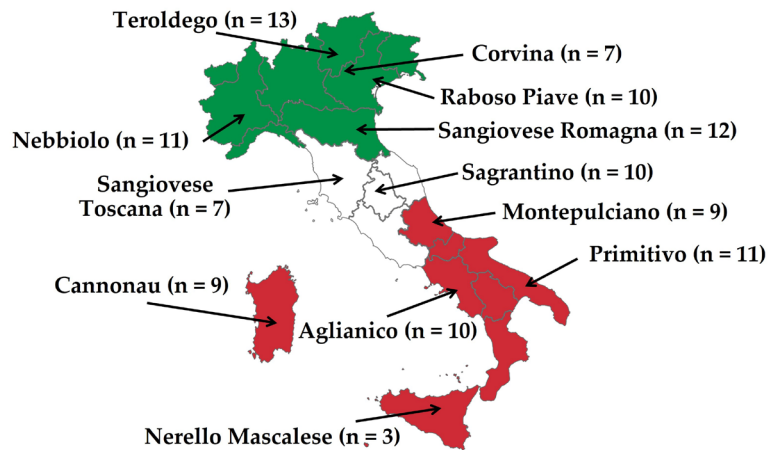
Red wine proteins have been scarcely investigated because of the analytical difficulties for their analysis in red wines, but also because of the common belief that, due to their intrinsic reactivity with tannins, wine proteins would bind to them to form insoluble complexes and precipitate during winemaking. However, recent studies have shown that red wines contain significant amounts of soluble proteins (Kassara *et al.*, 2022; Smith *et al.*, 2011; Wigand *et al.*, 2009), but data on Italian varieties are still scarcely available (Vincenzi *et al.*, 2005).

This study focused on the characterisation, in terms of proteins, polysaccharides and tannins, of the macromolecular profiles of red wines produced from 11 of the most representative Italian red grape varieties (origin wines). Additionally, the proteins’ state of the wines from 6 selected varieties was analysed in terms of electrophoretic mobility and interpreted in relation to the tannin composition of the wines.

## MATERIALS AND METHODS

### 1. Wine samples

A total of 110 red wines (vintage 2016) were sourced directly from several Italian commercial wineries located in typical and homogeneous areas of production, as shown in Figure 1. The wines were sampled from winery tanks in the early year 2017 and were all produced using a single variety sourced from single vineyards so that the varietal peculiarities could be maximised in the resulting wines. Fermentations were carried out with the yeast(s) of choice of each winery that provided the wines. The winemaking protocol did not include any filtration, oak contact, fining treatments, or ageing on yeast lees. Wines were clarified by static settling and racking only and were adjusted to 50 mg/L free SO<sub>2</sub> prior to bottling. Wines were stored at 13–15 °C in glass bottles sealed with Select Green 500 corks (Nomacorc, Rivesaltes, France) until analysis. A total of 11 grape varieties were selected according to their importance for each Italian region, which represent the typical autochthonous varieties used for red wine production. These were: Sangiovese (n = 19, of which 7 samples from Toscana and 12 from Romagna regions), Nebbiolo (n = 11), Primitivo (n = 11), Teroldego (n = 11), Aglianico (n = 10), Raboso Piave (n = 10), Sagrantino (n = 10), Cannonau (n = 9), Montepulciano (n = 9), Corvina (n = 7) and Nerello Mascalese (n = 3). These origin wines have been extensively characterised as part of the research activities of



**FIGURE 1.** Map of Italy indicating the region of origin of the monovarietal wines studied (“n” indicates the sample-set size for each wine group).

the D-Wines group (Arapitsas *et al.*, 2020; Giacosa *et al.*, 2021; Piombino *et al.*, 2020).

## 2. Protein and polysaccharide content determination

The determination of protein and polysaccharide content was performed colorimetrically. To remove wine’s phenolic compounds that would interfere with the colorimetric methods selected, red wine samples were pre-treated with polyvinylpyrrolidone (PVPP, Polyclar, Ashland) at 5 mg/mL. Wine samples were placed in an orbital shaker for one hour before separating the PVPP via centrifugation (3500g, 5 min, 4 °C, Mikro 200, Hettich). The supernatants were filtered (0.45 µm, PES syringe filters, Sartorius) and total proteins were quantified using a modified version of the protocol proposed by Smith *et al.*, 2011. Briefly, 500 µL of wine were added with 1 mL of cold acetone (Sigma-Aldrich) containing 10 % (w/v) of trichloroacetic acid (TCA; Scharlau, Barcelona, Spain). Samples were stored at –18 °C for 16 hours before the insoluble proteins were recovered by centrifugation (14,000g, 15 min, 4 °C). The so obtained pellet was washed once with 1 mL of acetone, vigorously mixed and centrifuged again (14,000g, 10 min, 4 °C). Then, pellets were dried by placing the open vials on a heating mantle (H2O3 Dry Bath, Coyote Bioscience Company) set at 65 °C for 30 minutes. Pellets were then solubilised with 500 µL of distilled water, and an aliquot of 100 µL was added with 1 mL of Bradford solution (AppliChem), mixed vigorously and the absorbance (595 nm) was measured spectrophotometrically (Jasco 7800) using yeast invertase (0–1000 mg/L, Sigma-Aldrich) as standard protein for the calibration curve.

The quantification of the total polysaccharides was performed by adapting a previously proposed method (Dubois *et al.*, 1956; Segarra *et al.*, 1995), as reported by Marassi and colleagues (Marassi *et al.*, 2021). Briefly, 20 µL of filtered and PVPP-treated wine were added with 500 µL of absolute ethanol (Sigma-Aldrich), stored at 4 °C for 16 h and

centrifuged at 14,000g for 30 minutes. The obtained pellets were dried by placing the open vials on a heating mantle set at 65 °C for 30 minutes. Pellets were then solubilised with 1 mL of a water/phenol solution prepared by dissolving phenol (Fluka) at 2 % (v/v) in distilled water. Then, 400 µL of the samples were transferred into a new vial and added with 1 mL of pure sulphuric acid (Sigma-Aldrich). After 30 minutes, the absorbance was measured at 490 nm. A calibration curve was prepared using a serial dilution of glucose (0–100 mg/L, Sigma-Aldrich) prepared in the water/phenol solution.

## 3. Tannin quantification assays

Total wine tannin contents were analysed by the methylcellulose precipitable ( $T_{MCP}$ ) tannin assay (Mercurio and Smith, 2008), while the iron/BSA reactive tannins ( $T_{BSA}$ ) were determined according to the method proposed by Harbertson and colleagues (Harbertson *et al.*, 2003). The tannin data here presented are reported, in aggregated form, in a previous article published by the D-Wines group (Giacosa *et al.*, 2021).

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

A total of 51 origin wines from 6 varieties with protein content representative of the variability observed were analysed by SDS-PAGE (Laemmli, 1970). Initially, red wine samples were pre-treated with PVPP at 5 mg/mL and placed in an orbital shaker for one hour before separating the PVPP via centrifugation (3500g, 5 min, 4 °C). The supernatants were filtered (0.45 µm, PES syringe filters, Sartorius) and 650 µL aliquots µL were added with 1.3 mL of the same TCA/acetone mix used for SDS-PAGE. After 16 hours at –20 °C, samples were centrifuged (14,000g, 10 min, 4 °C) and the resulting pellets were washed with 1 mL of cold acetone. After an additional centrifugation step (14,000g, 10 min, 4 °C), the obtained pellets were dried by placing the open tubes on a heating mantle set at 75 °C. Dry pellets were dissolved in 30 µL of Laemmli Sample

Buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing 50 mM dithiothreitol (Bio-Rad Laboratories) as a reducing agent. Samples were loaded on Mini-Protean TGX stain-free precast gels 8–16 % (Bio-Rad Laboratories). Precision Plus Protein Standards broad range (range 10–250 kDa, Bio-Rad Laboratories) were used to indicate molecular weight (MW). Proteins were stained with Pierce Imperial Protein Stain (Quantum Scientific, Sydney, NSW, Australia) according to the manufacturer's instructions. Images of the gels were acquired at 300 DPI resolution with a ChemiDoc™ XRS molecular imager (Bio-Rad Laboratories).

## 5. Statistical analysis

For each wine sample (biological replicates), proteins, polysaccharides and tannins analyses were conducted at least in triplicate. All data were processed, statistically analysed and visualised using the GraphPad Prism software version 7.05 (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) followed by a post hoc Tukey test was used to determine statistical significance using an alpha value of 0.05.

## RESULTS AND DISCUSSION

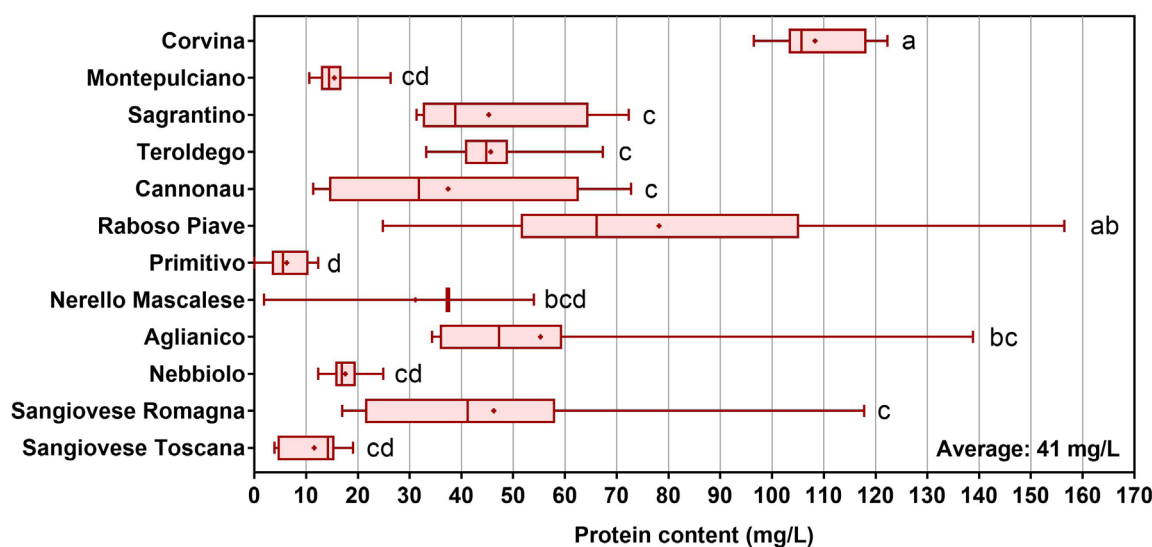
This article reports on the macromolecules of a large set of Italian red wines collected as part of the activities of a collaborative project named D-Wines (Diversity of the Italian Wines). Therefore, the here presented results are to be interpreted in the context of the previously published findings of the D-Wines project, which reported on the analytical and sensory composition of the set of wines

here analysed (Arapitsas *et al.*, 2020; Giacosa *et al.*, 2021; Parpinello *et al.*, 2019a; Piombino *et al.*, 2020).

The three classes of wine macromolecules—including proteins, polysaccharides and condensed tannins—are key elements in determining several wine organoleptic and technological characteristics. These macromolecules have been shown to interact with each other, thus determining the wine colloidal state that, in red wines, is a feature connected with both colour and stability (Marassi *et al.*, 2021; Mateus *et al.*, 2004; Pascotto *et al.*, 2021).

While the measurement of polyphenols is a routine analysis in red wines and several data are available in the literature for Italian wines so far (Arapitsas *et al.*, 2020; Gambacorta *et al.*, 2011; Giacosa *et al.*, 2021; Parpinello *et al.*, 2019a), a systematic quantification of proteins and polysaccharides for the main Italian red wine varieties has never been performed. Initially, the protein concentration was measured in 110 red wines from 11 Italian varieties (Figure 2 and Table 1). Protein quantification in red wines traditionally has been challenging as it is carried out with colorimetric methods whose results are negatively affected by the strong interferences of the phenolic compounds (Smith *et al.*, 2011; Vincenzi *et al.*, 2005).

Results presented in Figure 2 show that the protein contents of the wines here studied ranged between 0 (for a Primitivo sample) and 159 mg/L (for a Raboso Piave), with an overall average of 41 mg/L. The wines' protein contents did not correlate with any of the over 60 wine parameters reported, for these wines, by Giacosa and colleagues (Giacosa *et al.*, 2021) (data not shown). It is important



**FIGURE 2.** Boxplot analysis of the total quantitative distribution of proteins (expressed as mg/L of yeast invertase) as determined in 110 Italian red wines grouped by variety. For each wine type, the dot within the box represents the average value, while the overall average is reported within the graphs. Different letters represent statistically significant differences between treatments (post-hoc Tukey test).



to point out that these values are here used to highlight differences within the samples considered, even though the quantification method adopted has been validated against the results of the micro-Kjeldahl analysis (Smith *et al.*, 2011), that is the reference method to quantify proteins in different foods including wine. It is noteworthy that the here reported protein values are in line with those published by other authors on other wines and using other quantification methods on both red (Kassara *et al.*, 2022; Mainente *et al.*, 2014; Smith *et al.*, 2011; Vincenzi *et al.*, 2005; Wigand *et al.*, 2009) and white wines (Fukui and Yokotsuka, 2003; Van Sluyter *et al.*, 2015; Vincenzi *et al.*, 2011).

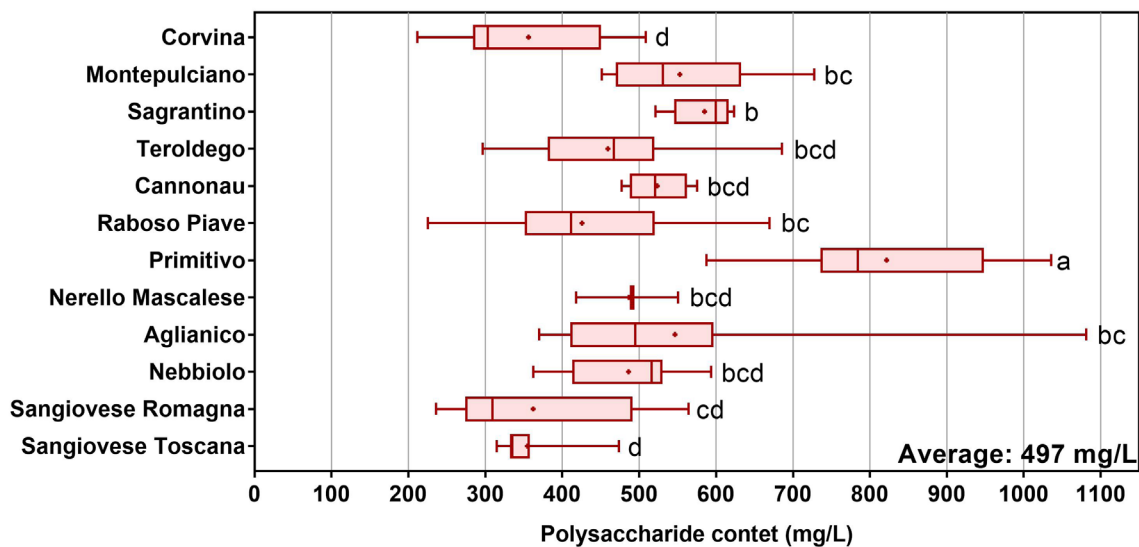
Data from Figure 2 allow us to make the following considerations. Although the statistical analysis does not allow one to discriminate the monovarietal wines clearly, nor this was an aim of this study, these latter could be divided into three groups: i) wines with average protein content well below the overall average (< 20 mg/L, Montepulciano, Primitivo, Nebbiolo and Sangiovese di Toscana); (ii) wines with average protein content grouped around the overall average (31–55 mg/L; Sagrantino, Teroldego, Cannonau, Nerello Mascalese, Aglianico and Sangiovese di Romagna) and (iii) wines with an average protein content above the overall average (Raboso Piave and Corvina).

An additional observation can be made regarding the intra-varietal variability that was very low for Montepulciano, Nebbiolo, Teroldego, Nerello Mascalese, Primitivo and Sangiovese di Toscana wines, and larger for the other origin wines.

Polysaccharides are the other class of wine macromolecules known to play key roles in different aspects related to red wine quality (Jones-Moore *et al.*, 2022). Therefore, the total polysaccharides' content was measured in the wines (Figure 3).

The concentration of wine polysaccharides ranged between 211 (for a Corvina sample) and 1081 mg/L (for an Aglianico sample). Most of the origin wines grouped around the overall average (497 mg/L), a value in line with those reported by other authors for red wines (Apolinar-Valiente *et al.*, 2013; Guadalupe *et al.*, 2012; Guadalupe & Ayestaran, 2007; Jones-Moore *et al.*, 2021), with the exceptions represented by Corvina (356 mg/L), Sangiovese Toscana (355 mg/L) and Sangiovese Romagna (362 mg/L) that showed lower values and Primitivo, which showed values (822 mg/L) well above the average being significantly different from all other wines. However, the here-reported concentrations include yeast-derived polysaccharides released during fermentation (mannoproteins), whose quantity depends on the yeast strain used and the fermentation conditions. Given the abundance of mannoproteins in the total polysaccharides' content, a distinction with the grape-derived polysaccharides cannot be made based on these results, which impairs the possibility to relate the polysaccharide content to the grape variety. Indeed, the wines' polysaccharides contents did not correlate with any of the wine parameters reported, for these wines, by Giacosa *et al.* (Giacosa *et al.*, 2021) (data not shown), with the unsurprising exception of dry extract ( $R^2 = 0.869$ ).

Despite the sample type and size, which does not allow for generalisation, nor for varietal discrimination,



**FIGURE 3.** Boxplot analysis of the total quantitative distribution of polysaccharides (expressed as mg/L of glucose) as determined in 110 Italian red wines grouped by variety. For each wine type, the dot within the box represents the average value, while the overall average is reported within the graphs. Different letters represent statistically significant differences between treatments (post-hoc Tukey test).

possible explanations of the differences observed for both proteins and polysaccharides of the origin wines of each variety could be attributed to different factors. For example, the grape maturity level at harvest has been reported to influence the amount of both proteins (Tian *et al.*, 2019) and polysaccharides (Dubourdiou *et al.*, 2006), even if, in this case, no correlations were found with the wines' general oenological parameters. Additionally, the extractability of these compounds increases during ripening due to the softening of the cell walls (Gil *et al.*, 2012; Vicens *et al.*, 2009). Other factors that could contribute to explaining the intra-varietal differences described here could be ascribed to differences in viticultural practices (e.g., irrigation, fertilisation) leading to modifications in the micro-environmental conditions between vineyards of a given area, leading to variations in the metabolism within each variety (Pasquier *et al.*, 2021). Obviously, differences in the maceration step (e.g., length, temperature, and modality, frequency and intensity of the cap management) also directly affect the extraction of proteins (Gómez-Plaza *et al.*, 2002) and polysaccharides (Gil *et al.*, 2015; Martínez-Lapuente *et al.*, 2021) from the skins, leading to different quantities of these macromolecules in wines. The above-mentioned reasons are only possible for the origin wines studied here as they did not undergo any post-fermentation treatment (e.g., fining treatment, filtration), leading to variations in macromolecular content.

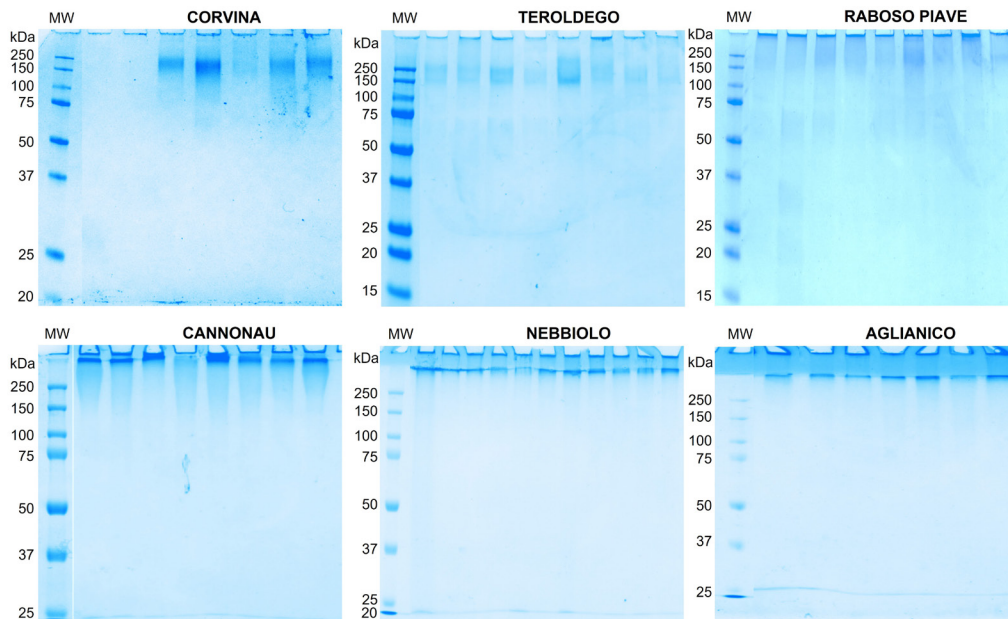
To complicate things further, the observed variability of soluble macromolecules in wines could also be partly ascribed

to the interactions occurring between macromolecules and the phenolic compounds specific to each grape variety, resulting in the formation of different proportions of soluble and insoluble complexes. It has been shown that the phenolic composition of the here investigated Italian origin wines greatly differs (Arapitsas *et al.*, 2020; Giacosa *et al.*, 2021). The quantity of total condensed tannins ( $T_{MCP}$ ) was measured by the MCP assay (Mercurio and Smith, 2008), while that of the portion of tannins reactive to BSA ( $T_{BSA}$ ) was measured with the method proposed by Harbertson (Harbertson *et al.*, 2003). These values were used to calculate the ratios between tannins and total protein content ( $T_{MCP}/P$  and  $T_{BSA}/P$ ) (Table 1).

Results show that Corvina was the origin wine with the lowest average  $T_{MCP}$  value (533 mg/L), whereas Sagrantino had the highest amount (2965 mg/L). The variability for the other 10 wine groups was lower, as they showed  $T_{MCP}$  contents between 1341 mg/L (Montepulciano) and 2043 mg/L (Aglianico). Besides  $T_{MCP}$  also tannins reactive to BSA ( $T_{BSA}$ ) were measured. The linear correlation between these two parameters showed a moderate fitting ( $R^2 = 0.654$ ;  $P = 0.0015$ ), thus indicating that different origin wines, although containing similar amounts of total tannins ( $T_{MCP}$ ), have different amounts of protein-reactive tannins ( $T_{BSA}$ ), a fact with potential implications for the assembly of colloids. In particular, Nebbiolo was the variety showing the highest percentage of protein-reactive tannins (> 89 %), while this percentage ranged between 39 and 57 % for all other varieties except for Montepulciano and Teroldego, for which this ratio was the lowest (29 and 24 %, respectively).

**TABLE 1.** Total proteins (P), total condensed tannins ( $T_{MCP}$ ) and total BSA-reactive tannins ( $T_{BSA}$ ) of the 110 monovarietal Italian red wines. Tannins were expressed as mg (+)-catechin/L. These values have been used to calculate the percentage contribution of  $T_{BSA}$  on the  $T_{MCP}$ , the ratios between condensed tannins and total proteins ( $T_{MCP}/P$ ), and that between BSA-reactive tannins and total proteins ( $T_{BSA}/P$ ). Wines shown in bold are those selected for follow up investigations by SDS-PAGE. Within each column, means followed by different letters are significantly different ( $P \leq 0.05$ ) according to the post-hoc Tukey test.

Wine	Total proteins (P, mg/L)	$T_{MCP}$ (mg/L)	$T_{BSA}$ (mg/L)	$T_{BSA}/T_{MCP}$ (%)	$T_{MCP}/P$	$T_{BSA}/P$
Sangiovese Toscana	11.6 cd	1723.4 bc	933.1 b	54.2	148.7	80.5
Sangiovese Romagna	46.2 c	1835.1 bc	956.9 b	52.1	39.7	20.7
Nebbiolo	17.6 cd	1716.0 bc	1534.9 a	89.4	97.5	87.2
Aglianico	55.3 bc	2043.0 b	1022.1 b	50.0	37.0	18.5
Nerello Mascalese	31.1 bcd	1519.3 bc	866.1 bc	57.0	48.9	27.8
Primitivo	6.2 d	1908.4 bc	752.9 bc	39.4	305.1	120.4
Raboso Piave	78.2 ab	1468.9 bc	736.4 bc	50.1	18.8	9.4
Cannonau	37.4 c	1615.2 bc	755.2 bc	46.8	43.1	20.2
Teroldego	45.7 c	1584.2 bc	384.8 cd	24.3	34.7	8.4
Sagrantino	45.3 c	2965.2 a	1749.7 a	59.0	65.5	38.6
Montepulciano	15.5 cd	1341.0 c	384.3 d	28.7	86.8	24.9
Corvina	108.3 a	533.4 d	212.2 d	39.8	4.9	2.0



**FIGURE 4.** SDS-PAGE visualization of proteins in representative wine samples of Corvina (n = 7), Teroldego (n = 9), Raboso Piave (n = 9), Cannonau (n = 8), Nebbiolo (n = 11) and Aglianico (n = 7) red wines. MW: Molecular weight.

From these data, it can be argued that differences in tannin content and protein reactivity affect the colloidal state of red wines. This assumption is consistent with the previously proposed model in which we hypothesised that the red wine colloids are constituted by associations of several protein-tannins subunits interacting with each other to form colloidal particles kept soluble by the association with polysaccharides (Marassi *et al.*, 2021). In this model, the red wine colloids are constituted by building blocks of covalently linked proteins-tannins subunits interacting by non-covalent forces with polysaccharides. Given that the proteins-tannins subunits can be detected in electrophoretic gels (Marassi *et al.*, 2021), SDS-PAGE was used to analyse a total of 51 samples from 6 origin wines representative of the three groups (high, medium and low protein content) identified from data shown in Table 1 (Figure 4).

As observed by other authors, the electrophoretic mobility of proteins in red wines is often hindered by aggregation phenomena with phenolic compounds (Vogt *et al.*, 2016; Wigand *et al.*, 2009). This fact has been recently attributed to the formation of covalent bonds between wine proteins and tannins (Marassi *et al.*, 2021), an occurrence resulting in the formation of complexes with MW much larger than that of free proteins as they can be found in white wines (Van Sluyter *et al.*, 2015) and occasionally also in reds (Wigand *et al.*, 2009). However, in the wines here analysed, bands with MW consistent with that of grape proteins (10–35 kDa) were never found. Conversely, the detectable protein material always showed low electrophoretic mobility, a fact that confirms the presence of tannin-protein complexes, as previously reported (Marassi *et al.*, 2021). Nevertheless, differences were visible between varieties. Indeed, for Corvina, Teroldego and Raboso Piave, proteins

partially entered the gels and migrated to an apparent MW of about 150 kDa, with some faint bands in the 50–75 kDa range also visible for Teroldego and Raboso Piave wines. For Cannonau, Nebbiolo and Aglianico, protein complexes did not enter the gel and remained blocked on the top of the stacking and resolving gels, indicating that the detectable protein-containing complexes had very high MW. An explanation for these different electrophoretic behaviours could be found by looking at the differences in the ratios between tannins and proteins' content (Table 1). Indeed, if the tannin/protein ratio was calculated using the total condensed tannins content ( $T_{MCP}$ ), results indicated that when this ratio was low (as in Raboso Piave and Corvina), protein bands with an MW sufficiently low to enter the gel pores were present. Conversely, if this ratio was high (as in Cannonau, Nebbiolo and Aglianico), the electrophoretic mobility of the protein material was hindered. However, this explanation does not fit with the data on Teroldego that, having a similar  $T_{MCP}/P$  ratio to Aglianico, should not have had protein material able to migrate in the gel. An explanation for this finding was obtained by calculating the ratio using the numerator of the protein-reactive tannins, as these are those involved in forming the protein-tannins subunits. Indeed, the relation between electrophoretic protein mobility and the tannin/protein ratio becomes stronger when considering the ratio calculated using only the tannins reactive to the BSA ( $T_{BSA}/P$ ). Among the six examined varieties, those in which proteins migrated in the gel (Corvina, Teroldego and Raboso Piave) had the lowest  $T_{BSA}/P$  ratios (2.0, 8.4, 9.4, respectively), while those with the largest complexes (Cannonau, Nebbiolo and Aglianico) had the highest ratios (20.2, 87.2, 18.5, respectively). Another element of diversity to be considered is that Teroldego



wines were shown to contain 3–4 times more anthocyanins than all the other wines in this study, while their tannins' content was close to the average (Giacosa *et al.*, 2021). Given the high reactivity between flavanols and anthocyanins, a different ratio of anthocyanins/flavanols is expected to lead to polymeric tannins with a different structure. Therefore, the electrophoretic behaviour of Teroldego could also be due to its different anthocyanins/flavanols ratio resulting in the formation of wine tannins having a different reactivity with the proteins.

These findings allow us to make important considerations. It is well established that different grape varieties, both red and white, share the same classes of proteins (Giribaldi and Giuffrida, 2010; Righetti and D'Amato, 2017; Wigand *et al.*, 2009) and that as a result of the vinification activities, only a few survive to be found in wines (Waters *et al.*, 2005). Conversely, as demonstrated here, the amount of proteins in wines differ greatly among varieties. On the other hand, a parameter that is strictly linked to the variety is the wine phenolic composition (Arapitsas *et al.*, 2020; Giacosa *et al.*, 2021; Parpinello *et al.*, 2019a). Therefore, the fact that origin wines sourced from different wineries showed, within varieties, a very similar electrophoretic profile while having very different compositions (see Figure 2, Figure 3, Table 1) (Arapitsas *et al.*, 2020; Giacosa *et al.*, 2021) suggests that the electrophoretic mobility of red wine proteins is modulated by their interaction with other wine components. Due to their protein-binding nature, the obvious candidate are tannins, in particular those reactive to proteins ( $T_{BSA}$ ). Therefore, it is suggested that it is the type of tannins present in a wine, a parameter strictly linked to the variety, the driver of the assembly of protein-tannins subunits in red wines. This in turn could result in the formation of tannin-protein complexes of different sizes, thus affecting the colloidal state of the wine that, as demonstrated here, is strictly dependent on the characteristics of individual varieties.

## CONCLUSIONS

The Italian red wines analysed display a large macromolecular diversity, with the content of proteins and tannins varying more than that of polysaccharides whose quantity is also affected by the presence of yeast mannoproteins.

For proteins, this diversity can be ascribed to a multitude of factors including genetic and environmental conditions. Additionally, the levels of proteins soluble in red wines are greatly affected by their interaction with tannins during vinification. Therefore, their final content can be modulated by the nature and protein-reactivity of the specific tannins which are extracted from a given grape variety. Protein-tannins complexes have been demonstrated to exist in all the here analysed origin wines. Given that it has previously been hypothesised that these complexes are the building blocks of wine colloids, it can be affirmed that the content and type of tannins, as well as the content of proteins present in a wine,

are mostly responsible for the type of colloids that will be formed in that wine.

In this context, wine polysaccharides also play an important role in binding to the protein-tannin subunits, thus maintaining their solubility over time. Conversely to proteins and tannins, red wine polysaccharides have shown low inter-varietal variability. While the variety should play a role in soluble polysaccharides content in grape berries at harvest, the observed variability can mostly be attributable to the different vinification and maceration techniques (e.g., yeast strain, fermentation conditions, length and temperature of maceration, modality of cap management) that are typically used to produce origin wines with a given style.

Among all varietal distinctive traits, the diversity in the colloidal state of red wines seems mostly attributable to differences in the quantity and reactivity of the molecules involved in the formation of protein-tannin complexes, which are the building blocks of the colloidal particles affecting wine quality. Therefore, more attention is needed to manage the macromolecules involved in colloids' assembly through their interactions. This, in turn, will allow for better management of colour and colloidal stability, therefore, improving key red wine sensory characteristics, such as its colour and limpidity.

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## REFERENCES

- Apolinar-Valiente, R., Williams, P., Romero-Cascales, I., Gómez-Plaza, E., López-Roca, J. M., Ros-García, J. M., & Doco, T. (2013). Polysaccharide composition of monastrell red wines from four different Spanish terroirs: Effect of wine-making techniques. *Journal of Agricultural and Food Chemistry*, 61(10), 2538–2547. <https://doi.org/10.1021/jf304987m>
- Arapitsas, P., Ugliano, M., Marangon, M., Piombino, P., Rolle, L., Gerbi, V., Versari, A., & Mattivi, F. (2020). Use of untargeted Liquid Chromatography-Mass Spectrometry metabolome to discriminate Italian monovarietal red wines, produced in their different terroirs. *Journal of Agricultural and Food Chemistry*, 68(47), 13353–13366. <https://doi.org/10.1021/acs.jafc.0c00879>
- Bavareseco, L., Pecile, M., & Zavaglia, C. (2014). Evolution of ampelographic platform in Italy. *Territoires Du Vin*, 6, 1–19.
- Bindon, K. A., Carew, A. L., Mierczynska-Vasilev, A., Kassara, S., Kerslake, F., & Smith, P. A. (2016). Characterization of macromolecular complexes in red wine: Composition, molecular mass distribution and particle size. *Food Chemistry*, 199, 838–846. <https://doi.org/10.1016/J.FOODCHEM.2015.12.079>



- Del Barrio-Galán, R., Medel-Marabolí, M., & Peña-Neira, Á. (2015). Effect of different aging techniques on the polysaccharide and phenolic composition and sensory characteristics of Syrah red wines fermented using different yeast strains. *Food Chemistry*, *179*, 116–126. <https://doi.org/10.1016/j.foodchem.2015.01.075>
- Dubois, M., Gilles, K., Hamilton, J., Rebers, P., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356. <https://doi.org/10.1021/ac60111a017>
- Dubourdieu, D., Glories, Y., & Maujean, A. (2006). *Handbook of Enology Volume 2 The Chemistry of Wine Stabilization and Treatments* (2nd ed., Vol. 2). John Wiley & Sons, Ltd.
- FederDOC (2020). *I vini Italiani a Denominazione d'Origine*. [https://www.federdoc.com/new/wp-content/uploads/2020/06/vini\\_italiani\\_denominazione\\_origine\\_2020.pdf](https://www.federdoc.com/new/wp-content/uploads/2020/06/vini_italiani_denominazione_origine_2020.pdf)
- Fukui, M., & Yokotsuka, K. (2003). Content and origin of protein in white and red wines: Changes during fermentation and maturation. *American Journal of Enology and Viticulture*, *54*(3), 178–188. <http://www.ajevonline.org/cgi/content/abstract/54/3/178>
- Gambacorta, G., Antonacci, D., Pati, S., la Gatta, M., Faccia, M., Coletta, A., & La Notte, E. (2011). Influence of winemaking technologies on phenolic composition of Italian red wines. *European Food Research and Technology*, *233*(6), 1057–1066. <https://doi.org/10.1007/S00217-011-1613-7>
- Gazzola, D., Van Sluyter, S. C., Curioni, A., Waters, E. J., & Marangon, M. (2012). Roles of proteins, polysaccharides, and phenolics in haze formation in white wine via reconstitution experiments. *Journal of Agricultural and Food Chemistry*, *60*(42), 10666–10673.
- Giacosa, S., Parpinello, G. P., Río Segade, S., Ricci, A., Paissoni, M. A., Curioni, A., Marangon, M., Mattivi, F., Arapitsas, P., Moio, L., Piombino, P., Ugliano, M., Slaghenaufi, D., Gerbi, V., Rolle, L., & Versari, A. (2021). Diversity of Italian red wines: A study by enological parameters, color, and phenolic indices. *Food Research International*, *143*, 110277. <https://doi.org/10.1016/j.foodres.2021.110277>
- Gil, M., Kontoudakis, N., González, E., Esteruelas, M., Fort, F., Canals, J. M., & Zamora, F. (2012). Influence of grape maturity and maceration length on color, polyphenolic composition, and polysaccharide content of Cabernet-Sauvignon and Tempranillo wines. *Journal of Agricultural and Food Chemistry*, *60*(32), 7988–8001. <https://doi.org/10.1021/jf302064n>
- Gil, M., Quirós, M., Fort, F., Morales, P., Gonzalez, R., Canals, J.-M., & Zamora, F. (2015). Influence of grape maturity and maceration length on polysaccharide composition of Cabernet-Sauvignon red wines. *American Journal of Enology and Viticulture*, *66*(3), 393–397. <https://doi.org/10.5344/AJEV.2014.14114>
- Giribaldi, M., & Giuffrida, M. G. (2010). Heard it through the grapevine: Proteomic perspective on grape and wine. *Journal of Proteomics*, *73*(9), 1647–1655. <https://doi.org/10.1016/j.jprot.2010.05.002>
- Gómez-Plaza, E., Gil-Muoz, R., López-Roca, J. M., Martínez-Cutillas, A., & Fernández-Fernández, J. I. (2002). Maintenance of colour composition of a red wine during storage. Influence of prefermentative practices, maceration time and storage. *LWT - Food Science and Technology*, *35*(1), 46–53. <https://doi.org/10.1006/FSTL.2001.0809>
- Guadalupe, Z., & Ayestaran, B. (2007). Polysaccharide profile and content during the vinification and aging of Tempranillo red wines. *Journal of Agricultural and Food Chemistry*, *55*(26), 10720–10728. <https://doi.org/10.1021/jf0716782>
- Guadalupe, Z., Martínez-Pinilla, O., Garrido, A., David Carrillo, J., & Ayestaran, B. (2012). Quantitative determination of wine polysaccharides by gas chromatography-mass spectrometry (GC-MS) and size exclusion chromatography (SEC). *Food Chemistry*, *131*(1), 367–374. <https://doi.org/10.1016/j.foodchem.2011.08.049>
- Harbertson, J. F., Picciotto, E. A., & Adams, D. O. (2003). Measurement of polymeric pigments in grape berry extracts and wines using a protein precipitation assay combined with bisulfite bleaching. *American Journal of Enology and Viticulture*, *54*, 301–306.
- International Organisation of Vine and Wine (2019). Statistical Report on World Vitiviniculture. In *Statistical Report on World Vitiviniculture*. <https://doi.org/64/19/6835> [pii]n10.1158/0008-5472.CAN-04-1678
- Jones-Moore, H. R., Jelley, R. E., Marangon, M., & Fedrizzi, B. (2021). The polysaccharides of winemaking: From grape to wine. *Trends in Food Science & Technology*, *111*, 731–740. <https://doi.org/10.1016/J.TIFS.2021.03.019>
- Jones-Moore, H. R., Jelley, R. E., Marangon, M., & Fedrizzi, B. (2022). The interactions of wine polysaccharides with aroma compounds, tannins, and proteins, and their importance to winemaking. *Food Hydrocolloids*, *123*, 107150. <https://doi.org/10.1016/j.foodhyd.2021.107150>
- Kassara, S., Li, S., Smith, P., Blando, F., & Bindon, K. (2019). Pectolytic enzyme reduces the concentration of colloidal particles in wine due to changes in polysaccharide structure and aggregation properties. *International Journal of Biological Macromolecules*, *140*, 546–555. <https://doi.org/10.1016/j.ijbiomac.2019.08.043>
- Kassara, S., Norton, E. L., Mierczynska-Vasilev, A., Lavi Sacks, G., & Bindon, K. A. (2022). Quantification of protein by acid hydrolysis reveals higher than expected concentrations in red wines: Implications for wine tannin concentration and colloidal stability. *Food Chemistry*, *385*, 132658. <https://doi.org/10.1016/J.FOODCHEM.2022.132658>
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685. <https://doi.org/10.1038/227680a0>
- Mainente, F., Zoccatelli, G., Lorenzini, M., Cecconi, D., Vincenzi, S., Rizzi, C., & Simonato, B. (2014). Red wine proteins: Two dimensional (2-D) electrophoresis and mass spectrometry analysis. *Food Chemistry*, *164*, 413–417. <https://doi.org/10.1016/j.foodchem.2014.05.051>
- Marassi, V., Marangon, M., Zattoni, A., Vincenzi, S., Versari, A., Reschiglian, P., Roda, B., & Curioni, A. (2021). Characterization of red wine native colloids by asymmetrical flow field-flow fractionation with online multidetection. *Food Hydrocolloids*, *110*, 106204. <https://doi.org/https://doi.org/10.1016/j.foodhyd.2020.106204>
- Martínez-Lapuente, L., Guadalupe, Z., & Ayestarán, B. (2020). Properties of wine polysaccharides. In *Pectins - Extraction, Purification, Characterization and Applications* (pp. 1–21). IntechOpen. <https://doi.org/10.5772/intechopen.85629>
- Martínez-Lapuente, L., Guadalupe, Z., Pérez-Porrás, P., Bautista-Ortín, A. B., Gómez-Plaza, E., & Ayestarán, B. (2021). Effect of sonication treatment and maceration time in the extraction of polysaccharide compounds during red wine vinification. *Molecules*, *26*(15), 4452. <https://doi.org/10.3390/MOLECULES26154452>
- Mateus, N., Carvalho, E., Luís, C., & De Freitas, V. (2004). Influence of the tannin structure on the disruption effect of carbohydrates on protein-tannin aggregates. *Analytica Chimica Acta*, *513*(1), 135–140. <https://doi.org/10.1016/j.aca.2003.08.072>

- Mercurio, M. D., Damberg, R. G., Herderich, M. J., & Smith, P. A. (2007). High throughput analysis of red wine and grape phenolics - Adaptation and validation of methyl cellulose precipitable tannin assay and modified somers color assay to a rapid 96 well plate format. *Journal of Agricultural and Food Chemistry*, 55(12), 4651–4657. <https://doi.org/10.1021/jf063674n>
- Mercurio, M. D., & Smith, P. A. (2008). Tannin quantification in red grapes and wine: comparison of polysaccharide- and protein-based tannin precipitation techniques and their ability to model wine astringency. *Journal of Agricultural and Food Chemistry*, 56(14), 5528–5537. <https://doi.org/10.1021/jf8008266>
- Parpinello, G. P., Ricci, A., Arapitsas, P., Curioni, A., Moio, L., Segade, S. R., Ugliano, M., & Versari, A. (2019a). Multivariate characterisation of Italian monovarietal red wines using MIR spectroscopy. *Oeno One*, 53(4), 741–751. <https://doi.org/10.20870/oeno-one.2019.53.4.2558>
- Parpinello, G. P., Ricci, A., Rombolà, A. D., Nigro, G., & Versari, A. (2019b). Comparison of Sangiovese wines obtained from stabilized organic and biodynamic vineyard management systems. *Food Chemistry*, 283, 499–507. <https://doi.org/10.1016/j.foodchem.2019.01.073>
- Pascotto, K., Leriche, C., Caillé, S., Violleau, F., Boulet, J. C., Geffroy, O., Levasseur-Garcia, C., & Cheynier, V. (2021). Study of the relationship between red wine colloidal fraction and astringency by asymmetrical flow field-flow fractionation coupled with multi-detection. *Food Chemistry*, 361, 130104. <https://doi.org/10.1016/J.FOODCHEM.2021.130104>
- Pasquier, G., Feilhes, C., Dufourcq, T., & Geffroy, O. (2021). Potential contribution of climate change to the protein haze of white wines from the French southwest region. *Foods* 2021, 10(6), 1355. <https://doi.org/10.3390/FOODS10061355>
- Piombino, P., Pittari, E., Gambuti, A., Curioni, A., Giacosa, S., Mattivi, F., Parpinello, G. P., Rolle, L., Ugliano, M., & Moio, L. (2020). Preliminary sensory characterisation of the diverse astringency of single cultivar Italian red wines and correlation of sub-qualities with chemical composition. *Australian Journal of Grape and Wine Research*, 26(3), 233–246. <https://doi.org/10.1111/ajgw.12431>
- Quijada-Morín, N., Williams, P., Rivas-Gonzalo, J. C., Doco, T., & Escribano-Bailón, M. T. (2014). Polyphenolic, polysaccharide and oligosaccharide composition of Tempranillo red wines and their relationship with the perceived astringency. *Food Chemistry*, 154, 44–51. <https://doi.org/10.1016/j.foodchem.2013.12.101>
- Righetti, P. G., & D'Amato, A. (2017). Proteomics of grapevines and wines. In M. L. Colgrave (Ed.), *Proteomics in Food Science: From Farm to Fork* (pp. 405–414). Academic Press Inc. <https://doi.org/10.1016/B978-0-12-804007-2.00024-2>
- Segarra, I., Lao, C., López-Tamames, E., & De La Torre-Boronat, M. C. (1995). Spectrophotometric methods for the analysis of polysaccharide levels in winemaking products. *American Journal of Enology and Viticulture*, 46(4), 564–570. <http://www.ajevonline.org/content/46/4/564.short>
- Smith, M. R., Penner, M. H., Bennett, S. E., & Bakalinsky, A. T. (2011). Quantitative colorimetric assay for total protein applied to the red wine Pinot noir. *Journal of Agricultural and Food Chemistry*, 59(13), 6871–6876. <https://doi.org/10.1021/jf200547u>
- Tian, B., Harrison, R., Morton, J., & Jaspers, M. (2019). Changes in pathogenesis-related proteins and phenolics in *Vitis vinifera* L. cv. 'Sauvignon blanc' grape skin and pulp during ripening. *Scientia Horticulturae*, 243, 78–83. <https://doi.org/10.1016/J.SCIEN.2018.08.018>
- Van Sluyter, S. C., McRae, J. M., Falconer, R. J., Smith, P. A., Bacic, A., Waters, E. J., & Marangon, M. (2015). Wine protein haze: mechanisms of formation and advances in prevention. *Journal of Agricultural and Food Chemistry*, 63(16), 4020–4030. <https://doi.org/10.1021/acs.jafc.5b00047>
- Vernhet, A. (2019). Red wine clarification and stabilization. In A. Morata (Ed.), *Red wine technology* (pp. 237–251). Academic Press. <https://doi.org/10.1016/B978-0-12-814399-5.00016-5>
- Vicens, A., Fournand, D., Williams, P., Sidhoum, L., Moutounet, M., & Doco, T. (2009). Changes in polysaccharide and protein composition of cell walls in grape berry skin (cv. Shiraz) during ripening and over-ripening. *Journal of Agricultural and Food Chemistry*, 57, 2955–2960. <https://doi.org/10.1021/jf803416w>
- Vidal, S., Williams, P., Doco, T., Moutounet, M., & Pellerin, P. (2003). The polysaccharides of red wine: total fractionation and characterization. *Carbohydrate Polymers*, 54(4), 439–447. [https://doi.org/10.1016/S0144-8617\(03\)00152-8](https://doi.org/10.1016/S0144-8617(03)00152-8)
- Vincenzi, S., Marangon, M., Tolin, S., & Curioni, A. (2011). Protein evolution during the early stages of white winemaking and its relations with wine stability. *Australian Journal Of Grape And Wine Research*, 17(1), 20–27. <http://doi.wiley.com/10.1111/j.1755-0238.2010.00113.x>
- Vincenzi, S., Mosconi, S., Zoccatelli, G., Pellegrina, C. D., Veneri, G., Chignola, R., Peruffo, A., Curioni, A., & Rizzi, C. (2005). Development of a new procedure for protein recovery and quantification in wine. *American Journal of Enology and Viticulture*, 56(2), 182–187. <http://www.ajevonline.org/content/56/2/182.abstract>
- Vogt, E. I., Kupfer, V. M., Vogel, R. F., & Niessen, L. (2016). A novel preparation technique of red (sparkling) wine for protein analysis. *EuPA Open Proteomics*, 11, 16–19. <https://doi.org/10.1016/J.EUPROT.2016.03.001>
- Waters, E. J., Alexander, G., Muhlack, R., Pocock, K. F., Colby, C., O'Neill, B. K., Høj, P. B., & Jones, P. (2005). Preventing protein haze in bottled white wine. *Australian Journal of Grape and Wine Research*, 11(2), 215–225. <https://doi.org/10.1111/j.1755-0238.2005.tb00289.x>
- Wigand, P., Tenzer, S., Schild, H., & Decker, H. (2009). Analysis of protein composition of red wine in comparison with rosé and white wines by electrophoresis and high-pressure liquid chromatography-mass spectrometry (HPLC-MS). *Journal of Agricultural and Food Chemistry*, 57(10), 4328–4333. <https://doi.org/10.1021/jf8034836>
- Wine Australia (2018). Uncovering Australia's emerging varieties. *Market Bulletin*, 132, 1. <https://www.wineaustralia.com/news/market-bulletin/issue-132>