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SPECIAL ISSUE

The role of protein-phenolic interactions in the formation of red wine colloidal particles

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ABSTRACT

Colloids play a crucial role in red wine quality and stability, yet their composition and formation mechanisms remain poorly understood. Recent studies from the D-Wines (Diversity of the Italian Wines) project aimed to elucidate the structure, composition, and formation mechanisms of red wine colloids by analysing monovarietal wines from 10 Italian red grape varieties. Colloid-forming molecules, specifically proteins, polysaccharides, and tannins, were examined in over 100 wines, showing a wide diversity across the samples. Electrophoretic analysis demonstrated that all proteins in the wines exist as high molecular weight aggregates, likely including tannins. Moreover, the wines could be categorised into two groups based on the electrophoretic mobility of the protein aggregates, which appeared to be related to the quantity of protein-reactive tannins in each variety. Asymmetrical Flow-Field Flow Fractionation (AF4) with online multidetection was used to isolate and characterise red wine colloids in their native state, revealing diverse colloidal populations across wines. This diversity was attributed to the varying proportions of proteins, polysaccharides, and phenolics present in the colloidal particles. These latter were coloured, indicating the presence of red pigments in the colloids. A correlation analysis of the compositional data of the wines and their colloidal particles indicated that the association of proteins with polymeric pigments should be important for red wine colour. Overall, the findings led to the proposal of an updated model for colloidal particles in red wines, suggesting that the process for their formation occurs through the assembly of protein-tannin sub-aggregates, followed by their interaction with polysaccharides. The compactness of these colloidal particles has been linked to the wine's protein content, with colloidal particles containing higher protein levels being less compact. These findings suggest that proteins likely play a role in determining the structure and properties of red wine colloidal particles. Moreover, this study provides an updated framework for understanding how compositional differences among grape varieties, particularly the content of protein-reactive tannins, shape colloidal structures, ultimately impacting key wine quality parameters such as colloidal stability and colour.

KEYWORDS: red wine colloids, protein-tannin interactions, Asymmetrical Flow-Field Flow Fractionation (AF4), colour stability, Macrowine 2025

INTRODUCTION

Wine contains compounds that can be categorised by size, including small soluble molecules like alcohols, organic acids, sugars, and monomeric phenolics, and larger entities classified as colloids. This latter category includes macromolecules and colloidal particles that can remain stable over time or aggregate, producing haze and/or sediments (Mierczynska-Vasilev & Smith, 2015). Most of the wine colloidal particles are the result of the association of wine macromolecules, including polysaccharides (which are known to comprise mainly rhamnogalacturonan II, arabinogalactan proteins, and mannoproteins), tannins, and proteins (Marangon *et al.,* 2022; Marassi *et al.,* 2021; Pascotto *et al.,* 2021; Vernhet *et al.,* 1996). These macromolecules, either alone or in colloidal assemblies, significantly affect many wine quality parameters, including stability, sensory attributes, and colour (Jones-Moore *et al.,* 2022; Vernhet, 2019). In red wines, polysaccharides and phenolics are the primary colloid-forming molecules studied (Jones-Moore *et al.,* 2021), while the role of proteins has been overlooked due to analytical challenges and the belief that their reactivity with tannins leads to the formation of insoluble complexes that precipitate during winemaking (Siebert *et al.,* 1996). However, recent studies have shown that red wines contain significant amounts of proteins (Kassara *et al.,* 2022; Smith *et al.,* 2011), which may remain stable due to inclusion in colloidal particles (Marassi *et al.,* 2021). The weak interactions among wine macromolecules contributing to the formation of colloidal particles can be easily disrupted during analysis, impairing the ability to study these particles as they exist in the wine (Le Bourvellec & Renard, 2012). This challenge has long prevented a comprehensive understanding of the native colloidal structure, despite the importance that this can have for red wine quality.

Asymmetrical Flow-Field Flow Fractionation (AF4) is a technique that permits overcoming the above-mentioned challenges by allowing the separation of colloidal particles according to their size (hydrodynamic radius) while maintaining them in native conditions. Moreover, when AF4 is coupled with specific detection methods, information on the quantity and shape (size and compactness) of colloidal particles can be obtained. Leveraging these capabilities, these techniques have been successfully applied in recent years to study red (Marassi *et al.,* 2021; Osorio-Macías *et al.,* 2020; Pascotto *et al.,* 2020; Pascotto *et al.,* 2021) and white (Coelho *et al.,* 2017; Figué *et al.,* 2024; Osorio-Macías *et al.,* 2022) wine colloidal particles, enabling their characterisation under conditions that closely mimic the wine environment.

This paper aims to present our latest findings (Marangon *et al.,* 2022; Marangon *et al.,* 2024; Marassi *et al.,* 2021) on the structure, composition, and formation mechanisms of red wine colloids, so to propose an updated model for colloidal aggregation based on the relationship between colloids' structural features and their macromolecular content. While detailed data are discussed in previous publications, this paper focuses on synthesising key insights and providing a conceptual framework for understanding colloidal behaviour in red wines.

MATERIALS AND METHODS

1. Monovarietal wines

A total of 110 red wines were sourced directly from various Italian commercial wineries located in typical and homogeneous production areas. The wines were produced using single Italian varieties and sampled from winery tanks six months after alcoholic fermentation. Fermentations were carried out with the yeast(s) of choice for each winery, without filtration, oak contact, fining treatments, or ageing on yeast lees. Wines were clarified by static settling and racking only, adjusted to 50 mg/L free SO_2 before bottling, and stored at 13–15 °C in glass bottles sealed with Select Green 500 corks until analysis. Eleven grape varieties were selected based on their regional importance: Sangiovese (n = 19), 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 wines were extensively characterised as part of the research activities of the D-Wines group (Arapitsas *et al.,* 2020; Arapitsas *et al.,* 2022; Giacosa *et al.,* 2021; Parpinello *et al.,* 2019; Piombino *et al.,* 2020).

2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

A total of 51 red wines from 6 varieties were analysed by SDS-PAGE as described previously (Marangon *et al.,* 2022). Briefly, wine samples were treated with PVPP (5 mg/mL) and shaken for one hour before centrifugation (3500 g, 5 min, 4 °C). The supernatants were filtered (0.45 µm, PES syringe filters, Sartorius) and mixed at a 1:2 ratio with a 10 % (w/v) trichloroacetic acid (TCA; Scharlau, Barcelona, Spain) solution in acetone, then incubated at –20 °C for 16 hours. After centrifugation (14,000 g, 10 min, 4 °C), the resulting pellets were washed with cold acetone, dried at 75 °C, and dissolved in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) with 50 mM dithiothreitol (Bio-Rad Laboratories) as a reducing agent. The samples were run 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), and gels were stained with Pierce Imperial Protein Stain (Quantum Scientific, Sydney, NSW, Australia). Images were captured at 300 DPI using a ChemiDoc™ XRS molecular imager (Bio-Rad Laboratories).

3. Protein content determination

The determination of protein content was performed colorimetrically, as detailed in Marangon *et al.* (2022), using a modified version of the method proposed by Smith *et al.* (2011). Briefly, after the removal of phenolic

compounds by PVPP (Polyclar, Ashland, 5 mg/mL), wine samples (500 µL) were added with 1 mL of cold acetone containing 10 % TCA. Samples were stored at –18 °C for 16 hours, and insoluble proteins were recovered by centrifugation (14,000 g, 15 min, 4 °C). The protein pellets were washed with acetone, dried at 65 °C for 30 minutes, and dissolved in 500 µL distilled water. Protein content was determined using the Bradford method, measuring absorbance at 595 nm with yeast invertase as the calibration standard.

4. Polysaccharide content determination

The determination of polysaccharide content was performed colorimetrically, as detailed in Marangon *et al.* (2022), using an adaptation of the method proposed by Segarra *et al.* (1995). Briefly, filtered and PVPP-treated wine (20 µL) was mixed with 500 µL of absolute ethanol, stored at 4 °C for 16 hours, and centrifuged at 14,000 g for 30 minutes. The pellets were dried at 65 °C for 30 minutes and then solubilized in 1 mL of a 2 % (v/v) phenol solution in water. After adding 1 mL of pure sulphuric acid to 400 µL of the sample, the absorbance at 490 nm was measured after 30 minutes. A calibration curve was created using glucose dilutions (0–100 mg/L) in the same water/phenol solution.

5. Tannin content determination

Total wine tannin contents were analysed by the methylcellulose precipitable (T_{MCP}) tannin assay (Mercurio & Smith, 2008). Briefly, 1 mL of the filtered wine sample was mixed with 0.5 mL of 0.5 % (w/v) methyl cellulose solution in distilled water. The mixture was incubated at 4 °C for 24 hours. After incubation, the precipitate was separated by centrifugation at 3000 g for 10 minutes. The supernatant was removed, and the precipitate was washed twice with 1 mL of cold acetone. The washed pellet was dissolved in 1 mL of 1 % (w/v) sodium carbonate solution and mixed thoroughly. The absorbance of the resulting solution was measured at 750 nm using a spectrophotometer.

The iron/BSA reactive tannins (T_{BSA}) were determined according to the method proposed by Harbertson *et al.* (2003). Briefly, 1 mL of the filtered wine sample was incubated with 1 mL of 1 % (w/v) bovine serum albumin (BSA) solution in 50 mM phosphate buffer (pH 7.0) for 30 minutes at room temperature. After incubation, 0.5 mL of 0.1 % (w/v) $FeCl_3$ in 50 mM phosphate buffer was added to the reaction mixture. The solution was then incubated for an additional 30 minutes at room temperature. Absorbance was measured at 510 nm using a spectrophotometer.

6. Asymmetrical Flow Field-Flow Fractionation (AF4) for colloids' characterisation

Red wines were analysed by AF4 as described by Marassi *et al.* (2021) using an Agilent 1100 system equipped with a DAD UV/Vis spectrophotometer and a MALS detector. The mobile phase was model wine (12.5 % ethanol, 2.5 g/L L-tartaric acid, pH 3.5). Prior to separation, the total colloidal content was assessed using Flow-Injection Analysis (FIA) and Focus-FIA. In FIA, the sample was injected without cross or focus flow, enabling the detection of all species present in the wine. Conversely, Focus-FIA included a preliminary focusing step that filtered out components smaller than 5 kDa, isolating the colloidal fraction. The ratio of signal areas between FIA and Focus-FIA provided the percentage recovery of colloids.

7. Statistical analysis

For each wine sample, analyses were conducted with at least three replicates. Data processing, statistical analysis, and visualisation were performed using GraphPad Prism software version 7.05 (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

1. Colloid-forming molecules in red wines

An examination of 110 monovarietal red wines revealed significant variability in the concentration of molecules potentially involved in colloid formations, including proteins, polysaccharides, and condensed tannins (Marangon *et al*., 2022). The protein content of the wines ranged from 0 to 159 mg/L, while polysaccharide levels ranged from 211 to 1081 mg/L. It should be noted that the PVPP pre-treatment may have partially removed covalently bound tannin–protein complexes, potentially leading to an underestimation of their levels: however, this does not affect the validity of the results, as all samples were treated in the same way and the study aimed to assess variability across wines rather than absolute concentrations. Among the different types of tannins measured (Giacosa *et al*., 2021), those which were found to be more interesting in relation to the colloidal characteristics of the wines were the tannins reactive to BSA (T_{BSA}), which ranged, among wines, from 212 to 1749 mg/L. These values showed only a moderate correlation with the total tannin content ($R^2 = 0.654$), suggesting that wines with similar total tannin levels can have different percentages of protein-reactive tannins, which, in our case, ranged from 24 % to 89 %. These compositional differences explain the results obtained by SDS-PAGE analysis of over 50 red wines.

While none of these latter showed protein bands compatible with the presence of free proteins (which are known to have a MW around 20–30 KDa) (Van Sluyter *et al*., 2015), all showed the presence of protein aggregates (boxed in Figure 1). However, among wines, two distinct electrophoretic patterns could be distinguished: in one group (Group 1), the aggregates could enter the gel and migrated to an apparent MW around 250 KDa, whereas in the second group (Group 2), the aggregates remained blocked at the top of the gel, indicating much larger dimensions (Figure 1). Interestingly, this different behaviour of the two groups could be related to the ratio between the quantity of protein-reactive tannins (T_{BSA}) with that of proteins (T_{BSA}/protein). Wines with protein aggregates that successfully migrated in the gel (panels at top of Figure 1) had the lowest T_{BSA}/protein ratios (2.0, 8.4, and 9.4), whereas those with the largest aggregates (panels at bottom of Figure 1) exhibited

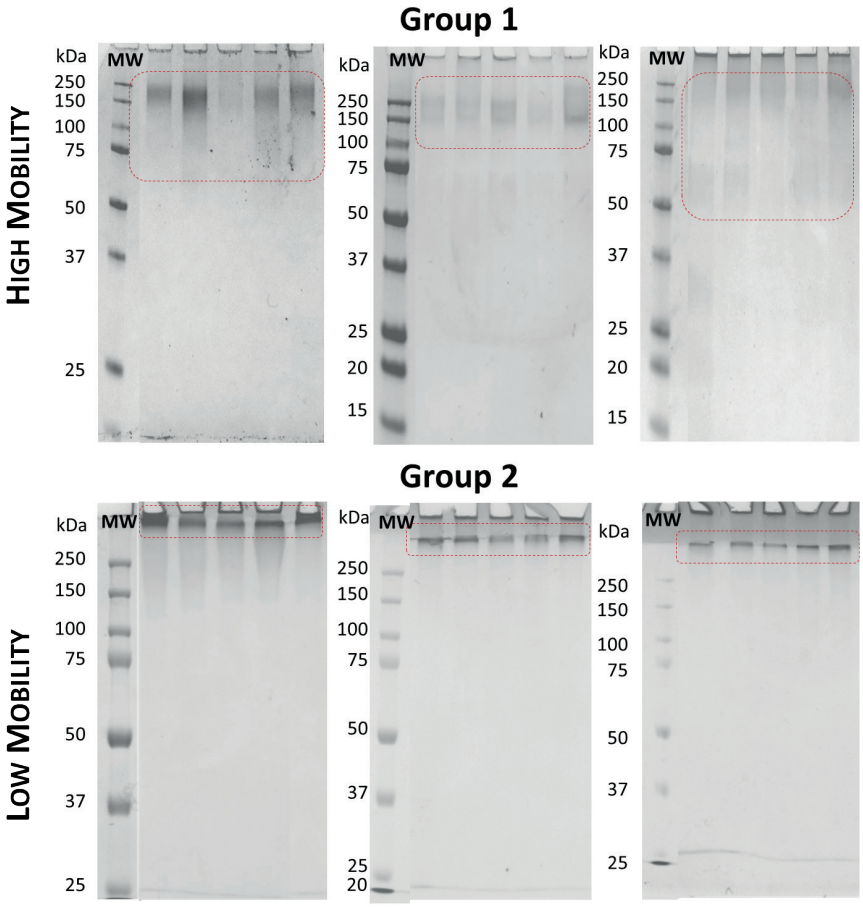

FIGURE 1. SDS-PAGE analysis of proteins in 5 representative wines (lanes) for 6 varieties (panels). MW: apparent molecular weight. For each panel, MW standard proteins are in the first lane of each panel.

higher ratios (20.2, 87.2, and 18.5). This suggests that the amount of T_{BSA}, which has been shown to be related to the grape variety (Giacosa *et al.*, 2021), determines the size of protein-phenolic aggregates. These aggregates, stabilised by strong interactions, can be considered as the "building blocks" for more complex colloidal structures. While the exact timing for their formation remains to be determined, unpublished data from our group indicate that these can be detected by SDS-PAGE immediately after crushing red grapes, suggesting a strong affinity between T_{BSA} and grape proteins that results in the presence of aggregates since the beginning of vinification.

2. AF4 to study red wine colloidal particles

To study the mechanisms of formation of colloidal particles in red wines, Asymmetric Flow Field-Flow Fractionation (AF4) combined with multiple detectors was employed (Marangon *et al.*, 2024; Marassi *et al.*, 2021). Initially, AF4 was used on two wines of the same variety presenting the same electrophoretic behaviour (that of Group 1, low T_{BSA}/protein, Figure 1) but different colloidal content, as measured by AF4 with A_{280} nm detection (Figure 2, blue line).

The fractions collected after AF4 separation (Marassi *et al.*, 2021) were analysed to determine their composition, revealing that the two wines had colloidal particles with different contents of macromolecules (Figure 2, bars). In particular, the wine with the highest quantity of colloidal particles (wine B) showed a higher proportion of phenolics in every fraction, while polysaccharides were predominant in most fractions of wine A. However, in both wines, proteins were found only in the first eluting fractions, namely those in which most of the colloidal material was present.

3. Colloidal particle formation in red wines

This new information, along with SDS-PAGE data (see Figure 1), enabled the formulation of an initial hypothesis regarding how these macromolecules assemble to form red wine colloidal particles of different type (Figure 3).

Given that proteins were not present in all fractions, while phenolics and polysaccharides were, we hypothesised that the interaction of proteins, polysaccharides, and phenolics to form colloidal particles in red wines involves two key steps. First, all the proteins form covalently linked aggregates with phenolics, creating the "building blocks" (sub-aggregates) visible in SDS-PAGE (Figure 1). Then, these sub-aggregates

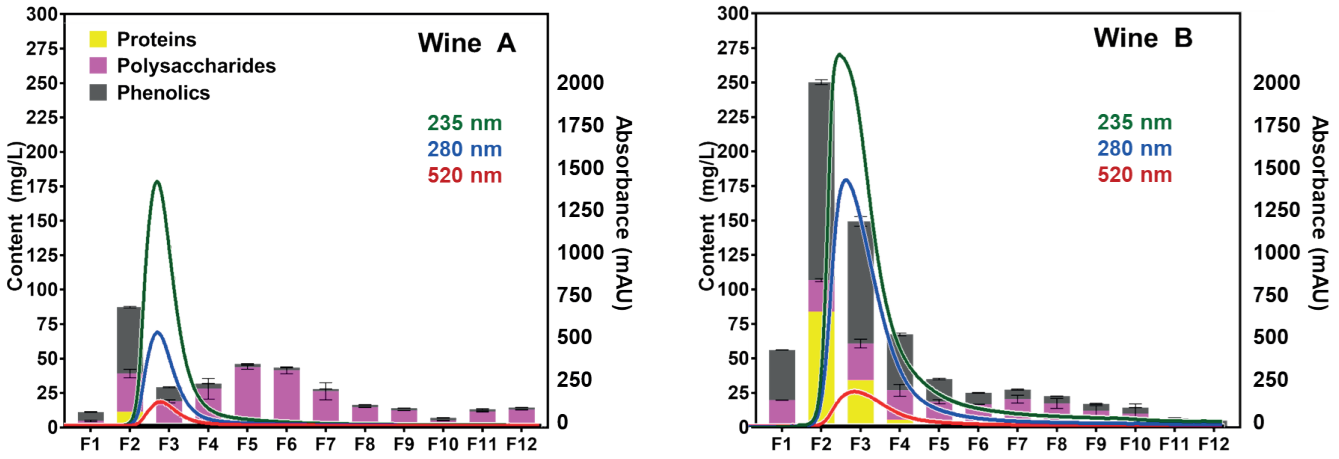

FIGURE 2. Lines: absorbance at 235, 280, and 520 nm of wine colloids during AF4 separation. Bars: proteins, polysaccharides, and phenolic content of the AF4-Fractions.

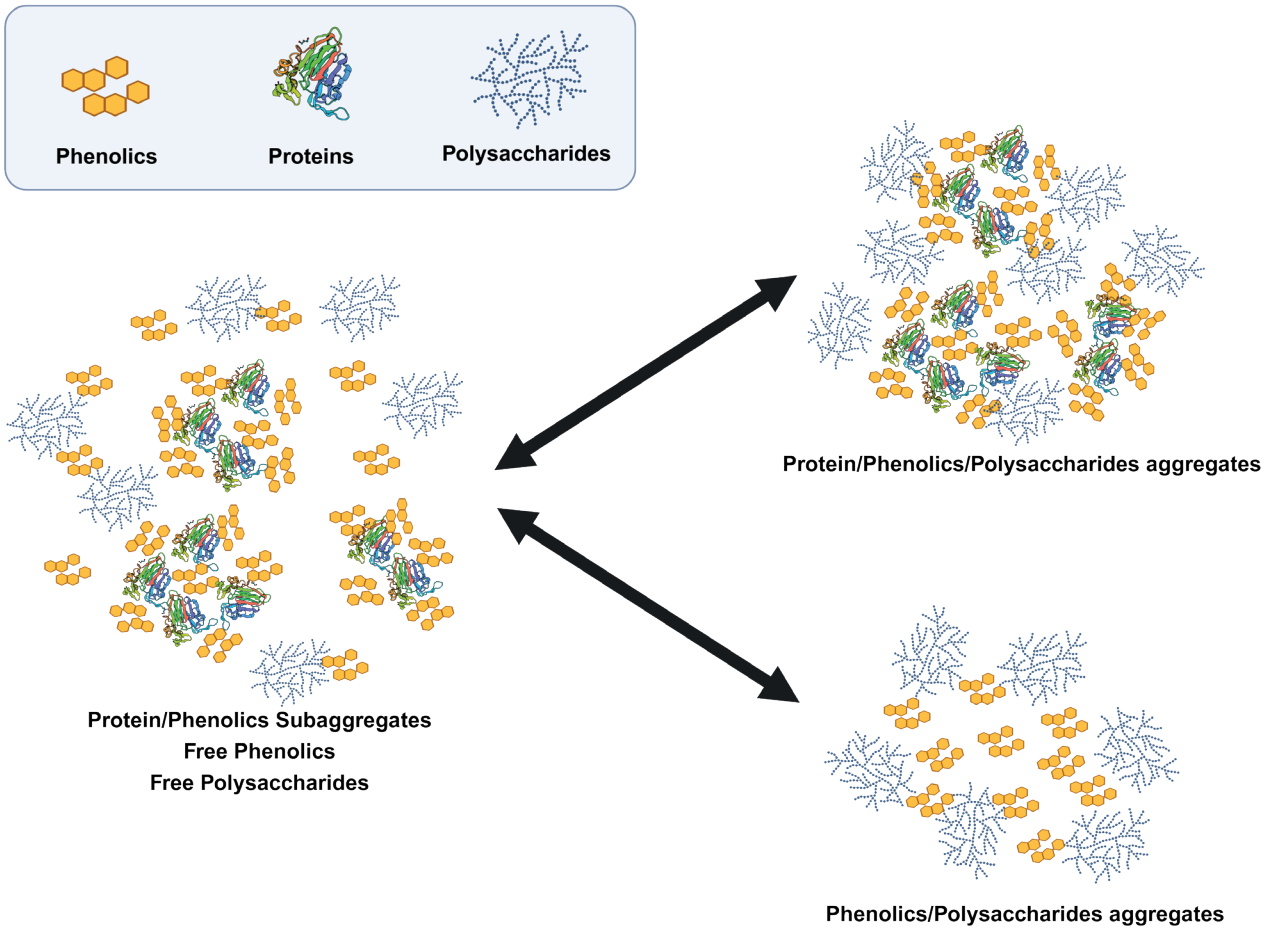

FIGURE 3. Preliminary proposed mechanism for colloidal aggregation in red wine (redrawn according to the model published by Marassi *et al.* (2021).

interact with polysaccharides through non-covalent forces, resulting in the formation of stable ternary colloidal particles, a finding that aligns with the ternary complex model proposed by Mateus *et al.* (2004). The particles found in the initial fractions (F1 to F4), which account for most wine colloids, likely adhere to this model. Additionally, as seen in the later AF4 fractions (F5 to F12), red wine also contains aggregates formed solely by phenolics and polysaccharides. Among the latter, mannoproteins appear to play a key role in the formation of aggregates, as previously demonstrated (Marassi *et al.*, 2021), supporting observations already reported in model systems (Mateus *et al.*, 2004; Riou *et al.*, 2002).

The mechanism of colloidal particle formation in red wines was further elucidated by using the data obtained and analysed by AF4, with 24 wines belonging to both groups 1 and 2 (see Figure 1) (Marangon *et al.*, 2024). For each wine, the percentage of colloids was calculated by comparing the A_{280} nm signal of the material with dimensions > 5 kDa (colloids), as obtained by the AF4 focusing step (Focus-FIA), with that of unfractionated wine as obtained after AF4 in FIA mode. Moreover, the same percentage was calculated also from the A_{520} nm absorbance, which is indicative of the quantity of red-coloured colloids, which is potentially important in relation to the problems of colour stability of red wines. The different wines exhibited great variability in colloidal content, ranging from 1.1 to 43.4 % of the total wine species absorbing at A_{280} and 5.1 to 58.7 % of the total coloured species absorbing at A_{520}. By considering the correlations between the A_{280} and A_{520} signals of the colloids and the concentration of colloid-forming molecules (proteins, polysaccharides, phenolics) (Marangon *et al.*, 2024), it can be hypothesised that while the phenolic compounds are the primary contributors to the A_{280} signal, the A_{520} signal is due to the presence of polymeric pigments associated with other colloid-forming molecules. Additionally, important differences in the percentage of A_{280} absorbing material in the colloidal fractions of the 24 wines were observed, with Group 2 wines (high T_{BSA}/protein ratio) showing values much higher than Group 1 wines (low T_{BSA}/protein ratio) (Marangon *et al.*, 2024). These differences likely stem from the different structure of the "building blocks" (protein/phenolics sub-aggregates), which, as mentioned above, depend on the grape variety, although the vinification process can also play a role. Therefore, the quantity of colloids in red wines appears to be more influenced by the phenolic content and type of the grape variety rather than by the protein content, which does not correlate with the differences detected among wines. However, the potential impact of winemaking practices, such as maceration conditions, the use of exogenous protein-based fining agents, and the addition of stabilising polysaccharides or tannins on the proposed model warrants further investigation.

Given that it was demonstrated that different varieties possess different colloidal particles (Marassi *et al.*, 2021), and that these differences are likely to be related mainly to the early reactivity of tannins with grape proteins, it is important to try to understand how these differences can affect the structure of the colloidal particles, as this information can be relevant also to explain the mechanism of colloidal stability. To this aim, the morphology (structural compactness and shape) of wine colloidal particles, separated by AF4, was determined by Multiangle Light Scattering (MALS) measurements (Marangon *et al.*, 2024). In this way, it was revealed that all 24 examined wines contained 4 populations (peaks) of particles with different size and compactness (Figure 4).

It is noteworthy that all peaks contained populations of colloidal particles with size significantly larger than individual macromolecules (Marangon *et al.*, 2024), indicating that these colloids are assemblies of various macromolecular species. While in AF4, the size of the particles (hydrodynamic radius, r_H) increases with the separation time, the particles' compactness (Rg) decreased from peak 1 to peak 3, to increase again in the final peak 4 (Figure 4B), that, however, contains negligible amounts of particles (Figure 4A). Indeed, four distinct populations with similar sizes (r_H) but with varying degrees of compactness (Rg) were detected across all wines (Figure 4B). In particular, peak 1, containing most colloids (see UV signal in Figure 4A), showed Rg values ranging from 24 nm (most compact) to 55 nm (least compact), whereas peaks 2 and 3 showed higher size and compactness compared to peak 1.

Taken together, the results here reported indicate that red wines contain not only different quantities of colloids, but also colloids differing in structure and composition. Based on the wines' composition, AF4 data, and previous studies (Giacosa *et al.*, 2021; Marangon *et al.*, 2022; Marangon *et al.*, 2024; Marassi *et al.*, 2021), it seems that differences in colloidal particle structure are modulated by the type and proportion of the interacting macromolecules.

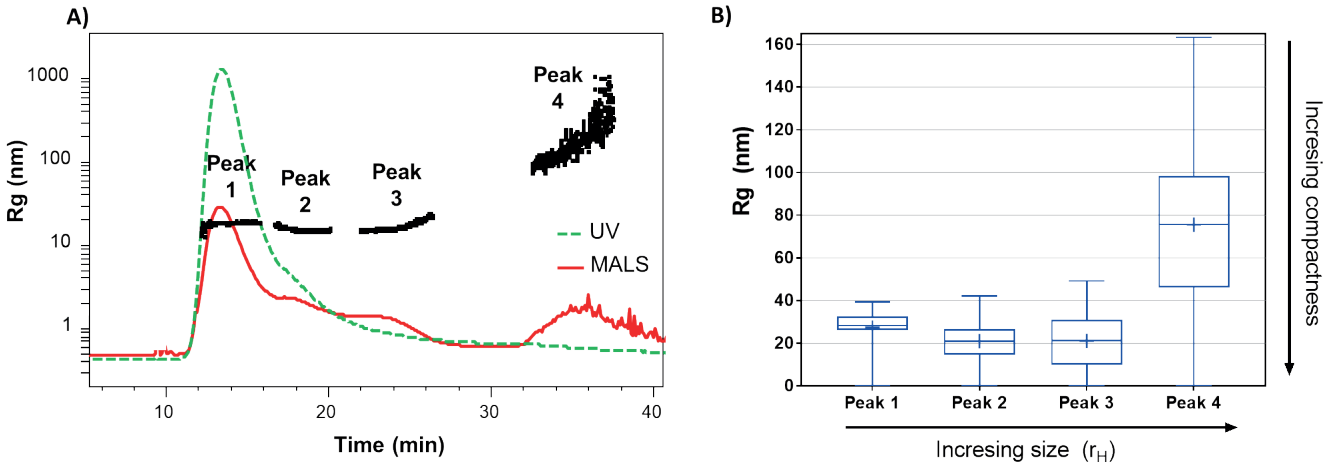

FIGURE 4. A) Representative fractogram for the sizing of red wine colloids. Dashed green line: A_{280} signal. Solid red line: MALS signal @90°. Black dots clouds are used to calculate the compactness (Gyration radius, Rg, in nm) for the 4 peaks as in B), which shows the boxplot analysis of the total compactness distribution of wine colloidal particles of the four MALS peaks for the 24 red wines. r_H: hydrodynamic radius.

While phenolics and polysaccharides were present in all the particles, proteins were mainly found in those with the smallest size and compactness (Figures 2 and 4). This suggests that the varying compactness of the particles may be linked to their protein content, as these are essential components of the protein/phenolics sub-aggregates (introduced in Figure 3), which are hypothesised to be the "building blocks" of the entire structure.

Therefore, a more detailed and updated model of colloidal aggregation is proposed (Figure 5).

Another aspect that is considered by the model is related to the fact that the particles are coloured, as shown by their capacity to absorb at A_{520} (Figure 2). It is well known that during vinification, polymeric pigments (PP) are formed through the reaction of anthocyanins with tannins (Oliveira *et al.*, 2019), which should also include those reactive to BSA (T_{BSA}). During the crushing and maceration phases, proteins are also extracted and, due to their affinity for tannins, it is likely that they interact with the PP to form coloured protein-PP sub-aggregates. According to the model proposed above, these protein-PP sub-aggregates then interact with each other and with polysaccharides, which, thanks to their water affinity, help stabilise the colloidal system (Zhai *et al.*, 2025).

The mechanism proposed in Figure 5 also considers the observed variability in particles' compactness. The presence of proteins strongly bound to tannins to constitute protein-PP sub-aggregates ("building blocks" of the particles) seems to be the key factor to modulate particles' compactness: colloid populations with high protein content are less compact than those with fewer proteins (Figure 4B). A third type of large colloid was also detected, characterised by a very loose structure and entirely lacking proteins. However, this peak showed negligible amounts of particles, as indicated by the absence of A_{280} and A_{520} signals.

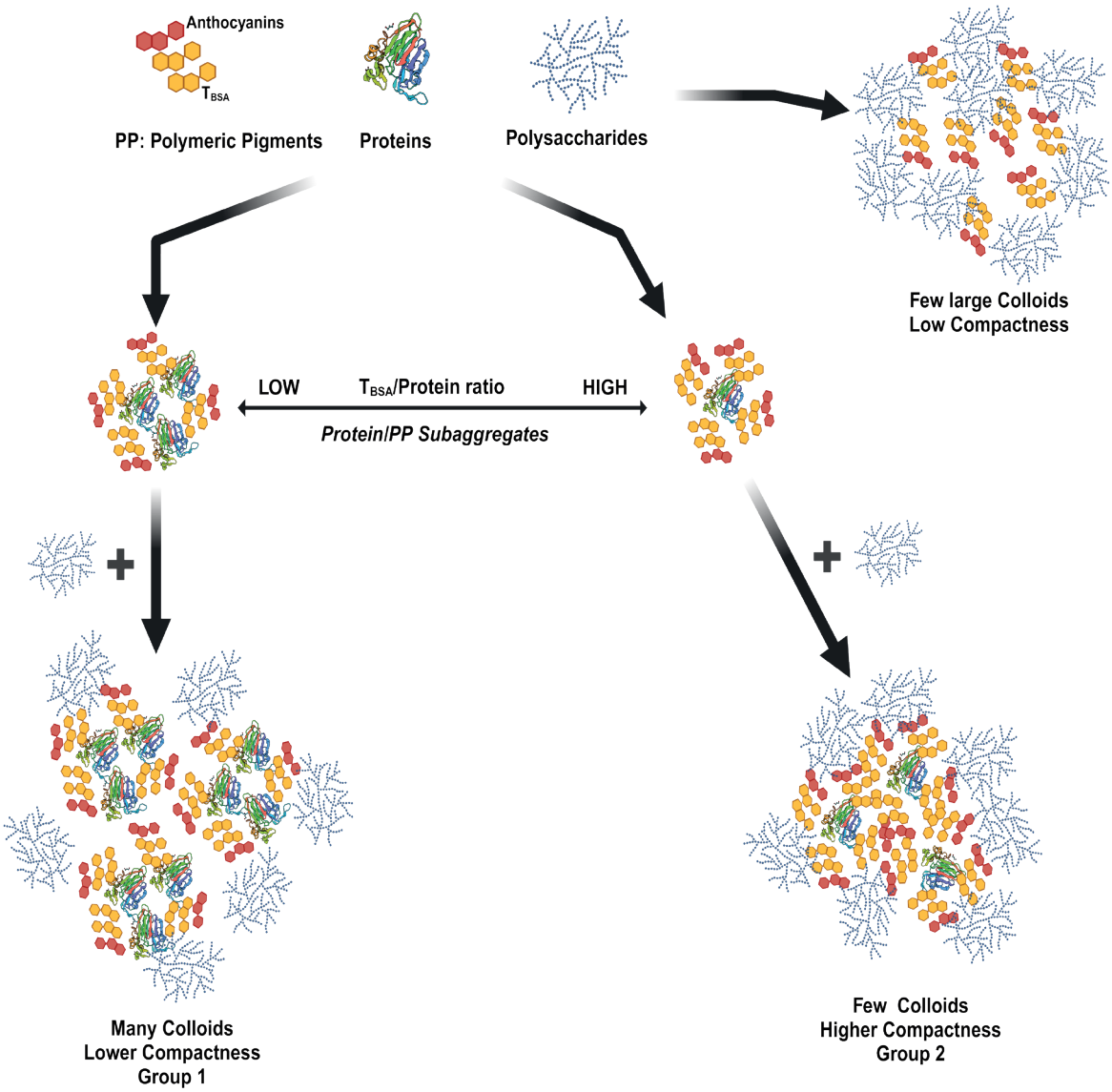

FIGURE 5. Updated mechanism for colloidal aggregation in red wine to incorporate the concept of colloids compactness as well as the concept of protein/polymeric pigments (PP) sub-aggregates and TBSA/proteins ratios (Marangon *et al.*, 2022). Redrawn according to the model published by Marangon *et al.* (2024).

According to the well-known structure/functionality relationship of molecules, the effects of the structural differences described for the colloidal particles of red wines deserve further investigation. However, since the structure and functionality are also influenced by the environmental conditions, the roles of important parameters such as wine pH, ethanol concentration, and ionic strength should also be studied in this context.

CONCLUSIONS

The findings presented here enhance the understanding of the complex role that macromolecules play in shaping the structural characteristics of the colloidal particles in red wines. The application of the AF4 technique and the large set of analytical data determined for many monovarietal red wines revealed that these latter wines contain colloidal particles, also coloured, that vary significantly in quantity, size, and shape. Notably, it seems that these differences are primarily determined by the type and amount of tannins, as well as by their reactivity with proteins, which determine the presence of protein-tannin sub-aggregates, where tannins are strongly bound to a small amount of protein. In our model, these basic structures can be seen as the "building blocks" for the formation of most colloidal particles present in red wines. These particles are stabilised by the inclusion of wine polysaccharides, deriving from yeast and/or extracted from the grape skins during maceration. If these are involved, this can be a major reason in explaining the different colloidal behaviour between red and white wines, together with the obvious differences in phenolic content. Indeed, white wines, with their very low T_{BSA}/protein ratio, lack the protein-phenolics sub-aggregates that form the core of red wine colloidal particles, which, in turn, are stabilised by grape-derived polysaccharides that are present only in very small quantities in white wines.

An additional important aspect of red wine quality is colour stability. Our findings showed that red wine colour is not only due to the exclusive presence of phenolics, *i.e.*, polymeric pigments and free anthocyanins, but is also due to coloured colloidal particles where polymeric pigments are bound to proteins. This highlights the critical role of proteins and suggests that a better understanding of proteins, polysaccharides, and tannins interactions is essential for fully elucidating the mechanisms behind the stability of the colloidal systems in red wines. Indeed, these interactions significantly impact colloid size and morphology, which in turn can affect both the technological and sensory properties of the wine, such as stability and colour.

Given the importance of the colloid-forming molecules in terms of quantity and proportion, winemakers should manipulate these components during vinification, considering the grape variety and the winemaking techniques adopted.

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