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Comprehensive analysis of colloid formation, distribution, and properties of monovarietal red wines using asymmetrical flow field-flow fractionation with online multidetection

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A R T I C L E I N F O

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A B S T R A C T

Red wine colloids, crucial in determining wine quality and stability, are understudied due to inadequate techniques for studying them effectively in the natural wine environment. Recently, Asymmetrical Flow Field-flow Fractionation (AF4) with online multidetection has emerged as a novel analytical tool for quantifying, fractionating, and characterizing red wine colloids in their native state. This study aimed to characterize the colloidal composition of 24 monovarietal Italian wines produced without filtration, oak contact, fining treatments, malolactic fermentation, macerating enzymes or ageing on yeast lees. AF4 analysis allowed quantification and characterization of wine colloids based on light scattering signal (MALS; gyration radius – Rg), size (hydrodynamic radius – Rh) and absorbance (A_{280} & A_{520} nm).

The results showed that each wine contained up to five distinct colloids' populations, varying in size and gyration radii. Despite possessing very similar Rh, most colloids exhibited great differences in compactness, as indicated by their varying Rg values. Comparing the A_{280} signal of whole wines to those of wines containing only species larger than 5 kDa (considered colloids) allowed to calculate the percentage of molecules involved in colloidal particles assembly, ranging from 1 to 44 % of the total A_{280} absorbing compounds, reflecting the diversity among wines. The A_{520} signal indicated the presence of polymeric pigments in the colloidal fraction. Notably, colored colloids all had Rg > 20 nm, indicating their association with other colloidal-forming compounds. This observation led to the conclusion that, apart from free anthocyanins and polymeric pigments, the color of red wines is also due to colloidal particles formed by the latter bound to proteins, with their quantity being highly variable across wines of different origin. These findings, which highlight the fundamental role of proteins in shaping the colloidal status of red wines, were utilized to propose an updated hypothetical model for colloidal aggregation in red wine.

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1. Introduction

Wine contains several classes of compounds that can be categorized based on their size, including small soluble molecules (<1 nm) such as alcohols, organic acids, sugars, and monomeric phenolics, and larger entities which can be classified as colloids. This last category, in addition to macromolecules, comprises also colloidal particles that can remain stable over time or can aggregate producing haze and/or sediments. These colloidal particles seem to be the result of the association of wine macromolecules, including polysaccharides, phenolics and proteins (Marangon et al., 2022; Marassi et al., 2021; Pascotto et al., 2021; Phillips & Williams, 2009). While in white wines the effects of proteins are well known as they determine wine instability and haze formation (Van Sluyter et al., 2015), in red wines their role on wine quality is not clear. Indeed, conversely to a common belief, several studies have shown that red wines contain significant amounts of proteins (Kassara et al., 2022; Marangon et al., 2022), which apparently remain in red wines also for long times (Smith et al., 2011). This is possibly due to their inclusion in colloidal particles (Marassi et al., 2021) showing stability over time. However, despite their importance as red wine constituents, the nature of these colloidal particles is poorly understood. One reason for this is probably the weakness of the interactions occurring among wine macromolecules that can be easily disrupted during the analysis (Le Bourvellec & Renard, 2012), impairing the possibility to study wine colloids as they are present in the wine.

Several techniques have been used to analyze the size and distribution of colloidal particles in wines, including dynamic light scattering (Nguela et al., 2016; Riou et al., 2002), electron (Vernhet et al., 2003) and atomic force microscopy (Vaquero et al., 2022), size exclusion chromatography (Coelho et al., 2018), scanning ion occlusion sensing (Gazzola et al., 2012), fluorescence correlation spectroscopy (Mierczynska-Vasilev et al., 2021) and nanoparticle tracking analysis (NTA) (Bindon et al., 2016; Li et al., 2019), all techniques showing some limitations towards the integrity of the studied objects and /or the definition of their composition in the native state. To overcome these problems, the asymmetrical flow-free flow fractionation (AF4) technique recently applied to the study of red (Marassi et al., 2021; Osorio-Macías et al., 2020; Pascotto et al., 2020, 2021) and white (Coelho et al., 2017; Osorio-Macías et al., 2022) wine colloids, proved to be a powerful tool to study their size, morphological properties, behavior and composition. Indeed, AF4 coupled with different on-line detection systems can be applied to analyze shape-dependent properties such as size along with mass-based parameters, thus being a powerful tool in the selective characterization of complex colloidal systems (Contado, 2017; Lie-Piang et al., 2021; López-Sanz et al., 2019; Marassi, Casolari, et al., 2022; Marassi, Mattarozzi, et al., 2022; Zappi et al., 2023). In particular, carrier fluids, pH, and salinity can be freely adjusted to mimic the key characteristics of the required environment, such as those existing in wines, allowing a soft separation (empty channel) to study associated and labile macromolecular systems (Marassi, Mattarozzi, et al., 2022; Ventouri et al., 2022).

Building upon previous findings (Marassi et al., 2021), this study seeks to comprehensively characterize, by AF4 technique, the structure, morphological composition, and the relative sizes of colloidal particles present in 24 monovarietal Italian red wines, and to establish meaningful connections between these particle properties and the chemical composition of the wines, so to enhance our understanding of the role and characteristics of colloidal particles in red wines.

2. Materials and methods

2.1. Wine samples

A total of 24 red wines (vintage 2016) were sourced directly from several Italian commercial wineries. The wines were sampled from winery tanks in early year 2017. In order to avoid the impact of some processing factors on wine composition (other than grapes), the wine-making protocol did not include any filtration, oak contact, fining treatments, malolactic fermentation, macerating enzymes or ageing on yeast lees. Wines were clarified by settling and racking only and were adjusted to 50 mg/L free SO_2 prior to bottling. Wines were stored at 13–15 °C in glass bottles sealed with Select Green 500 corks (Nomacorc, Rivesaltes, France) until analysis. All wines were produced using a single grape variety. A total of ten grape varieties were selected according to their importance for each Italian region. These were: Sangiovese (n = 4, of which 2 samples from Toscana – SAT – and 2 from Romagna – SAR – regions), Nebbiolo (n = 4, NEB), Primitivo (n = 2, PRI), Teroldego (n = 2, TER), Aglianico (n = 2, AGL), Raboso Piave (n = 4, RAB), Sagrantino (n = 2, SAG), Cannonau (n = 1, CAN), Corvina (n = 2, COR), and Nerello Mascalese (n = 1, NER). These wines have been extensively characterized as part of the research activities of the D-Wines group, and data have been published in several previous articles (Arapitsas et al., 2020, 2022; Giacosa et al., 2021; Marangon et al., 2022; Parpinello et al., 2019; Piombino et al., 2020).

2.2. Protein and polysaccharide content determination

The content of proteins and polysaccharides was determined colorimetrically. Briefly, wines were added with polyvinylpolypyrrolidone (PVPP, Polyclar, Ashland) at 5 mg/mL. After 1 h, PVPP was removed via centrifugation (3500 x g, 5 min, 4 °C, Mikro 200, Hettich) and the supernatants were filtered (0.45 μm, PES syringe filters, Sartorius). Total protein quantification was performed using the protocol proposed by Smith et al. (Smith et al., 2011) and modified as described by Marangon et al. (Marangon et al., 2022). Proteins were precipitated by using 2 volumes of cold acetone (Sigma-Aldrich) containing 10 % (w/v) of trichloroacetic acid (TCA; Scharlau, Barcelona, Spain). After 16 h at −18 °C, proteins were recovered by centrifugation (14000 x g, 15 min, 4 °C). The obtained pellets were washed with 1 mL of acetone, vigorously mixed and centrifuged again (14000 x g, 10 min, 4 °C). Then, pellets were air-dried before being dissolved in 500 μL of distilled water. One hundred μL of sample was added with 1 mL of Bradford solution (AppliChem, Darmstadt, Germany), mixed vigorously and the absorbance (595 nm) was measured spectrophotometrically (Jasco 7800, Jasco Europe S.r.l., Cremella, LC, Italy) using yeast invertase (0–1000 mg/L, Sigma-Aldrich, Milan, Italy) as standard for the calibration curve.

The content of polysaccharides was determined as reported previously (Marassi et al., 2021). Briefly, polysaccharides were precipitated by adding 500 μL of absolute ethanol (Sigma-Aldrich) to 20 μL of filtered and PVPP-treated wine. After 16 h at 4 °C, insoluble polysaccharides were recovered by centrifugation (14000 x g for 30 min). The obtained pellets were air-dried, and 1 mL of a water/phenol solution prepared by dissolving phenol (Fluka, Buchs, Switzerland) at 2 % (v/v) in distilled water was added. Then, 400 μL of the samples were added with 1 mL of pure sulphuric acid (Sigma-Aldrich), and 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, Sigma-Aldrich) prepared in the water/phenol solution.

The protein and polysaccharides data here presented are reported, in aggregated form, in a previous article published by the D-Wines group (Marangon et al., 2022).

2.3. Phenolic compounds quantification assays

Total wine phenolics' contents were determined using the Folin-Ciocalteu method (Singleton & Rossi, 1965) and expressed as mg (+)-catechin/L. The iron/bovine serum albumin (BSA) reactive tannins (T_{BSA}) were determined according to the method proposed by Harbertson and colleagues (Harbertson et al., 2003) and expressed as mg (+)-catechin/L (mg/L CE). The mean degree of tannins' polymerization (mDP) was determined as recently proposed as reported by Arapitsas et al. (Arapitsas et al., 2021). Total anthocyanins were determined

following the method proposed by Di Stefano (Di Stefano et al., 1989) and expressed as mg malvidin-3-O-glucoside chloride/L. The phenolic compounds' data here presented are reported, in aggregated form, in a previous article published by the D-Wines group (Giacosa et al., 2021).

2.4. Asymmetrical flow field-flow fractionation

Red wines were analyzed by AF4 as previously described (Marassi et al., 2021). AF4 analysis was performed using an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) combined with an Eclipse 3 Separation System (Wyatt Technology Europe, Dernbach, Germany). The channel was 152 mm long, 16 mm wide, and 350 μm thick. Regenerated cellulose membranes with 5 kDa cut-off (Microdyn-Nadir, Wiesbaden, Germany) were used. The channel outlet flow rate was set to 0.5 mL/min. The focusing step was performed for 1 min with a focusing flow rate of 2 mL/min to equilibrate the flows and then for 8 min in focus-injection mode to allow for the complete sample injection and focusing. For the separation step, an initial crossflow rate of 1.5 mL/min was set, and then lowered to 0.00 mL/min in 28 min using a linear gradient. The crossflow rate was then maintained to 0.00 mL/min in elution mode for 5 min to ensure complete elution of the largest aggregates (in the 90–300 nm range). The mobile phase was model wine (12.5 % (v/v) ethanol, 2.5 g/L L-tartaric acid adjusted to pH 3.5 with KOH). The software package Wyatt Eclipse @ ChemStation Version B.03.01 (Wyatt Technology Europe) was used to set and control the flow rate values. On-line detection of the eluted species was performed with an Agilent 1100 DAD UV/Vis spectrophotometer, and a multi angle light scattering (MALS) detector (MALS DAWN HELEOS, Wyatt Technology Corporation, Santa Barbara, CA). Carrier solutions were degassed using an on-line vacuum degasser Agilent, 1100 series (Agilent Technologies). Prior to separation, the total colloidal content of the wines was evaluated with a Flow-Injection Analysis (FIA) and a Focus-FIA (Fig. 1).

A Flow Injection Analysis (FIA) is a shortened, non-separative method: the sample is injected into the channel in absence of cross/focus flow, and it reaches the detector without separation. It allows evaluating the signal related to 100 % recovery of sample. A Focus-FIA is a FIA with an added preliminary focusing step, where the sample is subject to the focus flow and narrowed in a thin band at the beginning of the channel. In FIA, the entirety of the sample reaches the detector, while in Focus-FIA the sample components smaller than the membrane cutoff (5 kDa) are filtered out, and only the colloidal portion of the sample goes through the detectors. The ratio between the areas under signal curve obtained during the Separation method and in Focus-FIA (% Separation/Focus-FIA) gives the relative recovery of colloids after fractionation.

The detector flow and the focus flow were kept identical to the separation method. An injection volume of 100 μL was employed for FIA and Focus-FIA analyses and of 400 μL for MALS characterization.

2.5. Statistical analysis

For each wine (biological replicates), analyses were conducted at least in triplicate. All data were processed, statistically analyzed and visualized 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. Two-tailed Pearson's correlation coefficients among all compositional and AF4 data were calculated using an alpha value of 0.05.

3. Results and discussion

The 24 monovarietal red wines investigated in this study have been selected from 110 red wines previously analyzed in the context of the D-Wines project (Arapitsas et al., 2020, 2022; Giacosa et al., 2021; Marangon et al., 2022; Parpinello et al., 2019; Piombino et al., 2020). This subset was selected to have representative wines in terms of macromolecular variability (e.g., high, medium, low protein, phenolics and polysaccharides' content), and to include samples from all the varieties studied within the D-Wines project. Some analytical parameters relevant to interpret the following data on colloid formation and composition are reported in Table 1.

The values for the key analytical parameters mostly involved in the formation of colloids such as proteins, polysaccharides and phenolics (Giacosa et al., 2021; Marangon et al., 2022) show great variability among the considered wines. Indeed, protein content ranged between 0.1 to 156.5 mg/L (average 50.8 mg/L), polysaccharides between 211.5 to 1035.7 mg/L (average 492.3 mg/L), total phenolics between 734.8 to 4114.1 mg/L (average 2533.8 mg/L), protein (BSA)-reactive tannins (T_{BSA}) between 54.0 to 2326.9 mg/L (average 1020.1 mg/L) and total anthocyanins between 61.0 to 907.1 mg/L (average 267.9 mg/L). The mean degree of polymerization (mDP) of the tannins of the wine samples ranged between 8.8 and 27.5 units, with an average of 16.9 units. The relevance of these values for the colloidal status of the wines will be discussed in relation to the AF4 results.

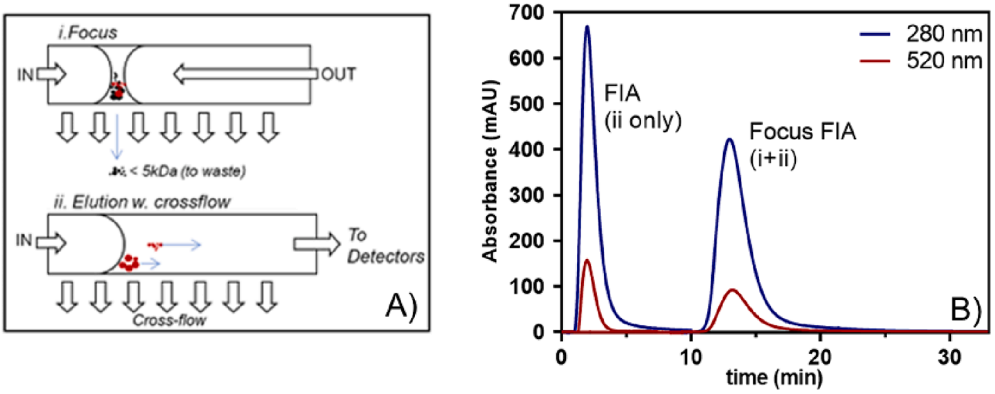

Fig. 1. Schematization and representative fractogram of red wine samples in FIA and Focus-FIA mode. A) Different steps involved in FIA and Focus-FIA analyses. Top (*i*): focusing step; the sample is narrowed in a band at the beginning of the channel by two opposing flows of mobile phase, while species < 5 kDa are filtered away. Bottom (*ii*): elution step; in the absence of a separative force (crossflow), the injected sample (unmodified, FIA, or prefiltered, Focus-FIA) is carried to the detectors. B) FIA and Focus-FIA representative profiles for red wine; the difference in peak retention time is due to the addition of the focusing time, while the differences in peak height/area in the Focus-FIA are due to the removal of species non partaking in the colloid formation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Analytical parameters of the 24 selected wines. For a full analytical characterisation of the starting 110 wine samples please refer to previously published works (Arapitsas et al., 2020, 2022; Giacosa et al., 2021; Marangon et al., 2022; Parpinello et al., 2019; Piombino et al., 2020).

Wine sample	Total proteins (mg/L)	Total polysaccharides (mg/L)	Total phenolics (mg/L)	T_{BSA} [1] (mg/L)	mDP *	Total anthocyanins (mg/L)	A_{280} signal (x1000)			A_{520} signal (x1000)		
							Whole wine [2]	Colloids [3]	Decrease (%)	Whole wine [4]	Colloids [5]	Decrease (%)
AGL1604	63.8 ± 4.0	587.0 ± 31.8	3251.1 ± 39.2	1169.5 ± 42.1	17.2	373.9 ± 25.7	289.3	27.9	90.3	25.5	5.0	80.4
AGL1609	34.7 ± 1.7	413.8 ± 50.4	2173.2 ± 4.9	400.8 ± 17.8	14.5	285.4 ± 9.1	220.4	2.4	98.9	11.6	0.6	94.9
CAN1608	13.5 ± 1.8	477.2 ± 23.7	2270.2 ± 132.3	813.5 ± 40.6	20.4	289.0 ± 4.0	243.1	17.4	92.8	16.3	2.8	82.6
COR1605	103.2 ± 9.5	211.5 ± 7.5	734.8 ± 0.1	155.1 ± 4.7	8.8	61.0 ± 4.0	115.4	1.7	98.5	5.5	0.4	93.1
COR1606	122.3 ± 25.3	508.3 ± 30.2	1698.3 ± 58.8	54.0 ± 1.6	12.1	172.2 ± 4.6	222.0	3.7	98.3	14.0	0.9	93.6
NEB1602	15.6 ± 0.1	405.4 ± 12.3	2287.6 ± 137.2	1558.8 ± 43.2	19.6	93.0 ± 0.1	260.6	49.7	80.9	11.5	4.2	63.4
NEB1604	15.6 ± 2.8	362.4 ± 22.3	2433.1 ± 29.4	1146.6 ± 23.0	27.5	112.0 ± 2.9	246.3	39.4	84.0	10.6	3.8	64.0
NEB1605	16.9 ± 6.1	412.9 ± 30.5	3036.2 ± 9.8	1435.0 ± 31.4	17.5	120.5 ± 2.3	269.4	96.8	64.0	16.4	7.9	52.0
NEB1610	19.6 ± 2.8	593.5 ± 10.5	3320.4 ± 147.0	1903.8 ± 99.5	19.7	106.7 ± 0.1	301.7	130.8	56.6	16.9	9.9	41.3
NER1603	1.9 ± 0.6	490.6 ± 69.9	1930.6 ± 63.7	934.7 ± 66.3	13.6	124.9 ± 2.9	221.6	9.8	95.6	7.6	1.6	79.4
PRI1604	0.1 ± 0.1	587.4 ± 79.6	2516.3 ± 39.2	761.4 ± 17.7	13.1	386.9 ± 12.0	292.8	60.1	79.5	22.9	8.6	62.3
PRI1607	9.4 ± 5.0	1035.7 ± 29.3	2526.7 ± 152.0	832.1 ± 3.8	9.2	132.2 ± 6.3	269.8	68.7	74.5	15.5	7.2	53.7
RAB1609	97.3 ± 5.2	401.7 ± 7.1	2925.3 ± 49.0	1389.1 ± 56.8	17.3	423.2 ± 9.7	302.5	65.0	78.5	25.4	9.2	63.8
RAB1613	89.0 ± 11.5	376.2 ± 29.4	2703.5 ± 0.1	970.6 ± 20.0	13.9	348.9 ± 10.9	268.0	48.6	81.9	26.9	8.8	67.3
RAB1615	129.0 ± 21.4	669.5 ± 61.9	3500.7 ± 58.8	964.0 ± 20.2	12.0	578.5 ± 14.3	466.9	64.5	86.2	39.3	12.3	68.5
RAB1619	156.5 ± 11.5	464.7 ± 64.4	3140.2 ± 78.4	1050.0 ± 0	15.7	167.0 ± 4.0	281.1	47.9	82.9	24.8	8.7	64.9
SAG1612	72.3 ± 7.6	623.3 ± 27.6	4114.1 ± 122.5	2326.9 ± 47.5	13.6	180.7 ± 8.6	539.8	126.0	76.7	25.0	11.3	54.7
SAG1614	41.3 ± 10.4	553.5 ± 35.1	3999.8 ± 88.2	1855.2 ± 20.7	19.0	257.5 ± 6.3	420.5	104.7	75.1	26.4	11.5	56.3
SAR1604	23.9 ± 1.4	286.6 ± 52.9	1823.1 ± 107.8	1113.6 ± 67.6	18.7	216.3 ± 1.7	257.2	60.7	76.4	18.0	7.8	56.7
SAR1608	78.3 ± 7.7	564.1 ± 28.2	2523.2 ± 127.4	919.4 ± 20.2	20.4	219.9 ± 8.0	246.4	39.9	83.8	16.1	5.7	64.7
SAT1603	3.9 ± 1.9	334.6 ± 44.3	1868.2 ± 34.3	794.5 ± 46.2	23.2	216.7 ± 2.3	246.4	40.2	83.7	16.1	5.2	67.6
SAT1607	15.5 ± 3.9	473.8 ± 12.5	2031.1 ± 39.2	1214.0 ± 84.9	25.1	285.4 ± 5.7	242.9	45.8	81.2	14.8	6.1	58.9
TER1617	40.7 ± 2.9	296.5 ± 6.9	1389.9 ± 34.3	227.0 ± 0.8	15.7	369.7 ± 12.3	209.9	5.5	97.4	23.6	2.0	91.5
TER1620	54.0 ± 3.5	685.7 ± 15.5	2613.4 ± 107.8	493.5 ± 1.3	18.6	907.1 ± 24.0	395.0	24.3	93.9	40.7	6.9	83.0

[1] T_{BSA}, iron/BSA reactive tannins.
[2] Absorbance at 280 nm of whole wine (FIA) and of.
[3] Wine compounds > 5 kDa (Focus-FIA) expressed as peak area.
[4] Absorbance at 520 nm of whole wine (FIA) and of
[5] Wine compounds > 5 kDa (Focus-FIA) expressed as peak area.
* The expected standard error for mDP values is < 20 % thanks to the accuracy and repeatability of the method used.

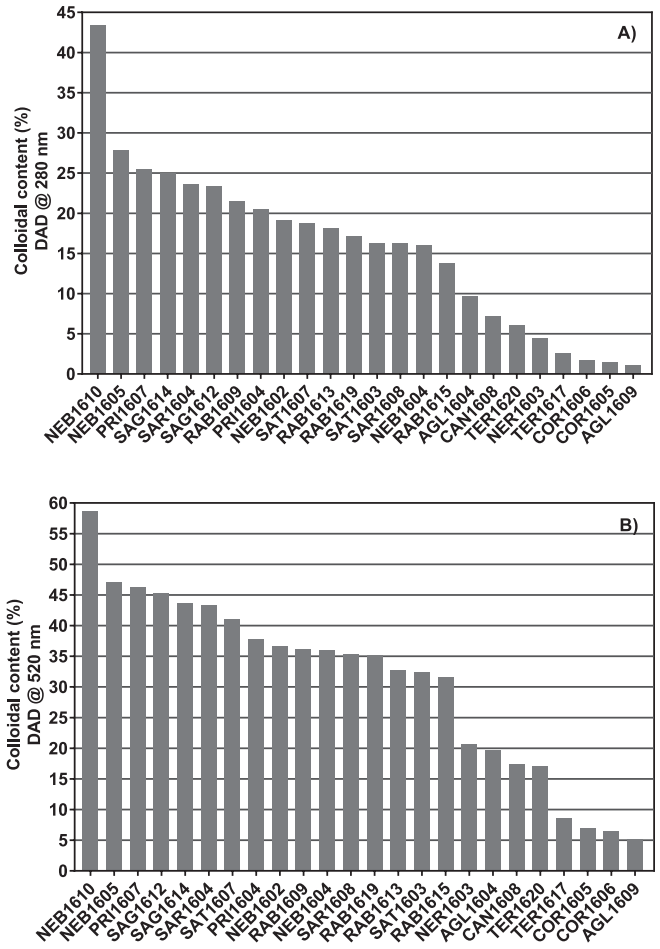

Fig. 2. A) Ratio between the A_{280} nm signals of Focus-FIA / FIA chromatograms expressed in percentage. B) Ratio between the A_{520} nm signals of Focus-FIA / FIA chromatograms expressed in percentage. The individual A_{280} & A_{520} values are shown in Table 1.

3.1. Colored and non-colored red wine colloids

FIA and Focus-FIA analyses were performed on the 24 wines to gather information on the amount (%) of material in colloidal state and on the amount of color associated with wine colloids. The first analysis applied to all the 24 wines was performed in FIA mode. With this approach, injected samples reach the detectors without any separation, and therefore the absorbance signals (A_{280}, A_{520}, the two relative absorption maxima recorded for red wines (Marassi et al., 2021)) are due to the wine constituents, without distinctions based on the molecular size of the absorbing species. Then, samples were also analyzed in Focus-FIA mode, which includes an initial focusing step during which the compounds with a molecular weight < 5 kDa were eliminated from the samples (see Fig. 1A). Therefore, the obtained A_{280} and A_{520} signals were solely due to entities > 5 kDa, that were considered as the wine colloids. So, the differences between the signals obtained with the two approaches (FIA and Focus-FIA) allowed to calculate the percentage of absorbing entities that were present in wines in colloidal state (Fig. 2). The 24 wines differed in the quantity of the total A_{280} absorbing species (FIA mode) (Table 1), but more interestingly, also in the proportion between this quantity and that of the A_{280} absorbing species present as colloidal entities (Focus-FIA mode) (Table 1 and Fig. 2A).

It is well known that the A_{280} value is mainly due to molecules containing aromatic rings (Airado-Rodríguez et al., 2011). In wines, the main molecules involved are proteins and, at larger extent, phenolic compounds (Marangon et al., 2022). Therefore, the differences detected for the different wines by FIA (Table 1) are likely to be due to a different content of these classes of compounds.

Based on the data from Table 1, and on Pearson's correlation coefficients shown in Fig. 3, it can be excluded that proteins are main drivers of these differences. Indeed, the protein content of the wines does not correlate with their A_{280} value after both FIA ($r = 0.182$; $P = 0.395$) and Focus-FIA ($r = -0.107$; $P = 0.620$). This indicates that the primary contributor to the A_{280} FIA signal must be the phenolic compounds, as their quantity exhibits a significant positive correlation ($r = 0.843$; $P < 0.0001$) with this parameter. Indeed, the wines with a A_{280} signal above average were those with the highest total phenolic content, whereas the low signal for other samples could be explained by their lower phenolic content (Table 1). For example, the two Sagrantino (SAG1612 and SAG 1614), one Raboso (RAB1615) and one Teroldego (TER1620) samples displayed A_{280} values above the average (284.5 x1000), while one Corvina sample (COR1605) showed the lowest absorbance values.

Compared to the FIA results, every wine showed an important decrease in A_{280} signals after Focus-FIA (Table 1). This decrease is obviously due to the removal of A_{280} absorbing molecules with a molecular weight (MW) smaller than the membrane cut-off (5 kDa), which occurred during the focusing step. These molecules certainly included some phenolic compounds, the most important UV absorbing wine components, in particular those in monomeric form and/or polymerized up to about a mDP of 18 (Santos-Buelga & Freitas, 2009). Indeed, most of the wines contained polyphenols with mDPs around or below this value (Table 1).

Interestingly, the results obtained in the absence of the molecules with a MW < 5 KDa (as occurs in the Focus-FIA mode) reveal a correlation that is statistically significant but not particularly strong ($r = 0.651$, $P = 0.001$; see Fig. 3) when compared to those obtained for the whole wines (FIA mode), indicating that the quantity of colloids (MW > 5 KDa) absorbing at 280 nm contributes at different extents to the total absorbance of the wines.

Indeed, when looking at the percentage of A_{280} absorbing material in the colloidal fraction of the 24 wines there are clear differences, with Nebbiolo, Sagrantino, Primitivo and Sangiovese showing the largest values, while Teroldego, Aglianico and Corvina showing the lowest percentage of A_{280} absorbing colloids (Fig. 2A). These differences should be attributable to the mode of assembly of the molecules involved in colloid formation, which have been previously identified as polyphenols, proteins (both absorbing at 280 nm), and polysaccharides (Marassi et al., 2021). These modes must be related to the grape variety, and in particular to the compounds deriving from the grapes (Giacosa et al., 2021; Marangon et al., 2022), but also to the vinification processes adopted in order to reach the desired red wine style. Indeed, some of the varieties studied here are typically vinified to produce wines suitable for ageing, with a consequent need to maximize the phenolic extraction during processing. This is the case of Nebbiolo, Sagrantino, Primitivo and Sangiovese, all varieties showing the highest percentage of colloids. Conversely, fresh grapes from varieties like Corvina and Teroldego are typically vinified to produce ready-to-drink wines, employing less extractive winemaking methods. This aligns well the observation of a very low percentage of colloids found in wines from these varieties (Fig. 2A). If this is the case, phenolics and proteins (both absorbing at 280 nm) should be both present in colloidal particles (Marassi et al., 2021). Therefore, it would be confirmed that the relative quantity of colloids present in a given red wine is modulated by the quantity and type of phenolic compounds it contains (Marangon et al., 2022), rather than by the grape proteins, whose quantity is unrelated to the colloidal content (Fig. 3). Indeed, it is well documented that the quantities of these compounds in wines are not necessarily linearly related (Springer et al., 2016), and that the content of colloids in wine depends also on other physico-chemical factors that can affect their reactivity and stability over time (Chursina & Zagorouiko, 2021).

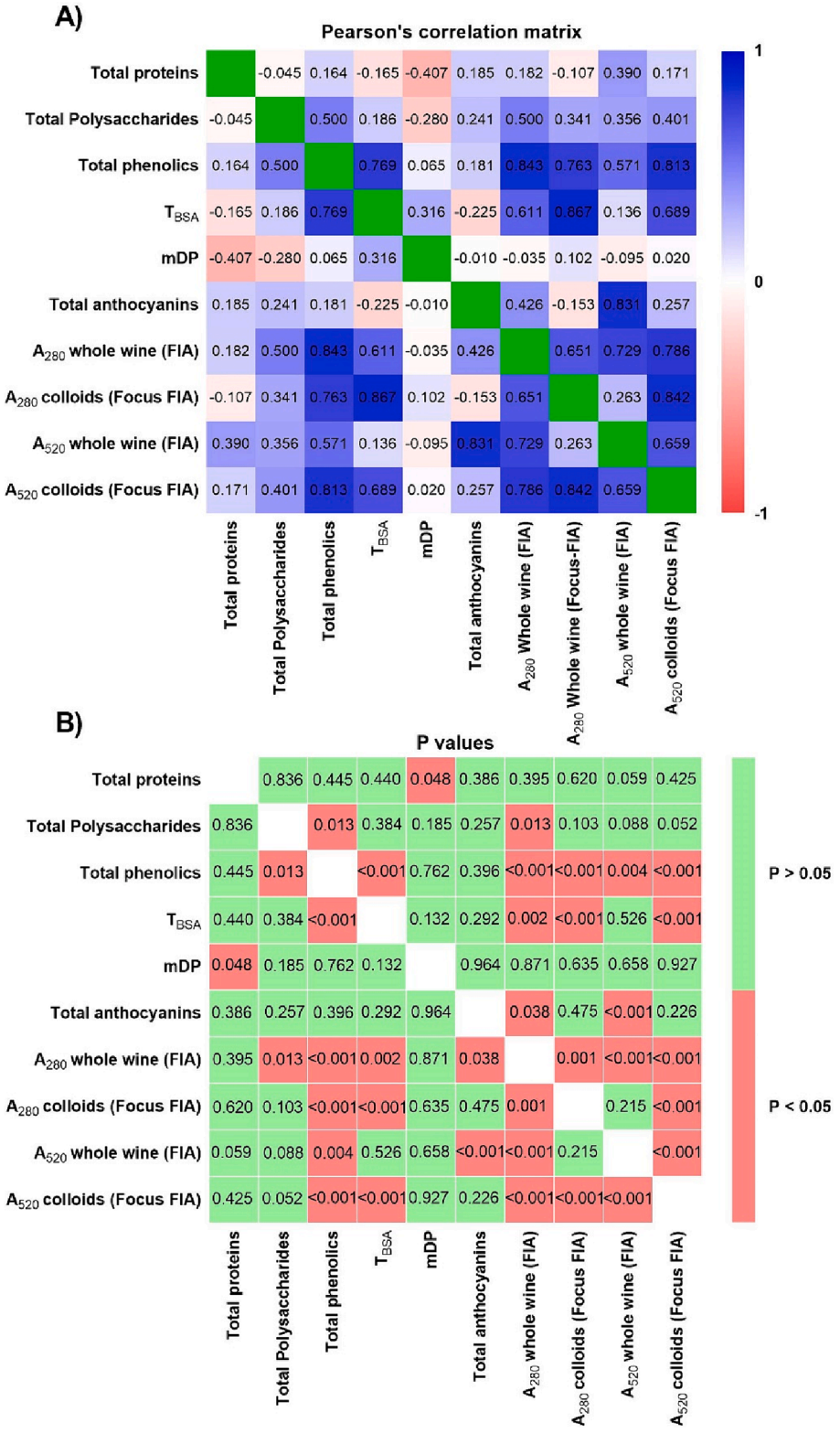

Fig. 3. Two-tailed Pearson's correlation matrix (A) and relative P values of significance (B) of data presented in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Another interesting point is that the peak detected at 280 nm after Focus-FIA, corresponding to wine colloids, is overlapped by a peak at 520 nm (see Fig. 1B), and this occurs for all the examined wine samples (not shown). This indicates that part of the color detected in the whole wine (i.e., at 520 nm after FIA) is present in entities with a MW > 5 kDa, confirming the idea that red wines contain colored colloids. This behavior, among other things, strongly contributes to the so-called color stability/instability (Oliveira et al., 2019). It is well known that the pigments responsible for the color of red wines are anthocyanins, which are low MW polyphenols of about 300–350 Da characterized by being easily modified during winemaking (Waterhouse et al., 2016). Indeed, the amount of free anthocyanins greatly decreases during winemaking

and wine ageing as these bind to tannins to form polymeric pigments (PP), which are more stable compounds (Oliveira et al., 2019). The MW of these PP is relatively small; typically they have an average mDP between 3 and 10, which means a size of 840–2800 Da (Santos-Buelga & Freitas, 2009). If this was the case, all these compounds would have been lost during the focusing step of Focus-FIA. However, part of the color components remained in the colloidal fraction detected after Focus-FIA (Table 1 and Fig. 2B). This clearly indicates that pigments are associated with other colloidal-forming compounds, the obvious candidates being proteins as they are known to strongly interact with tannins, which probably maintain their protein binding capacity also when part of the PP. However, the role of proteins in such complexes remains underexplored, despite evidence suggesting their presence in red wine colloidal fractions that absorb at ≈ 520 nm and also contain polysaccharides and tannins (Marassi et al., 2021). Interestingly, the percentage of color components present in the colloids of the different wines is significantly correlated ($r = 0.689$; $P = 0.0002$) with their content of T_{BSA} (Fig. 3). If this type of BSA-reactive tannins participates in the formation of PP by reacting with anthocyanins, it would be possible to assume that the PP originating from them would be able to maintain the capacity to bind proteins, thus explaining the presence of proteins in the colored colloids. Indeed, tannins incorporate anthocyanins into the terminal subunit of the PP; consequently, the extension subunits of the PP are likely available for potential hydrophobic interactions and hydrogen bonding with proteins (Campbell et al., 2021).

The FIA and Focus-FIA A_{520} data for the 24 red wines exhibit significant variations (Table 1). This diversity was detected for both the whole wines (FIA mode) and for their colloidal fraction (Focus-FIA mode). Concerning the whole sample set, the highest A_{520} values were those of the 2 samples with the highest content of total anthocyanins (RAB1615 and TER1620), while the lowest value was for a Corvina sample (COR1605) that contained only 61 mg/L of total anthocyanins. Indeed, a significant correlation was determined between the A_{520} signals of the whole wines (FIA mode) and the total anthocyanins ($r = 0.831$; $P < 0.0001$; Fig. 3).

When looking at the A_{520} signal of wine colloids (Focus-FIA mode, Table 1), it is evident that the loss in color due to the focusing step (which removes the low MW compounds including free anthocyanins, but also PP) is small for some wines (e.g., Nebbiolo, Primitivo, Sangiovese and Sagrantino) and very high for others (e.g., Corvina, Aglianico, Teroldego, Cannonau). Therefore, it seems that the wine total color components are differently distributed between small (<5 KDa) entities and colored colloids (>5 KDa) in the different wines. This fact is clearer when examining the percentage of colored species present in colloidal form (Fig. 2B), and looking at the lack of significant correlation between the A_{520} colloids signal (Focus-FIA mode) and total anthocyanins ($r = 0.257$; $P = 0.226$; Fig. 3). Therefore, the capacity of colored material (PP) to be part of the colloidal matter would depend on the variety, although the effects of the vinification style cannot be excluded. In particular, the role of the quantity of protein-reactive tannins (T_{BSA}) typical of each variety must be considered. These tannins, after interacting with anthocyanins to form PP, are likely to bind to wine proteins, thus becoming part of the colored colloidal matter. This idea is supported by the finding that wines with low quantity of protein-reactive tannins (T_{BSA}) (e.g., Corvina and Teroldego) have a low percentage of colored colloidal matter, whereas the quantity of T_{BSA} in samples with a high percentage of colored colloids (e.g., Nebbiolo, Sagrantino, Sangiovese) is high (Table 1, Fig. 2B). In this context, it is interesting to note the significant correlation ($r = 0.842$; $P < 0.0001$; Fig. 3) between the A_{520} and the A_{280} signals obtained for the colloids (Focus-FIA mode) of the different wines. Given that the A_{280} signal is due to phenolics and proteins, most of the red wine colloids containing proteins and tannins are colored, although at different extent. Therefore, also considering the above-mentioned possibility of an interaction between proteins and PP, the role of proteins in the formation of the colored colloidal matter and on red wines' color stability deserves further attention.

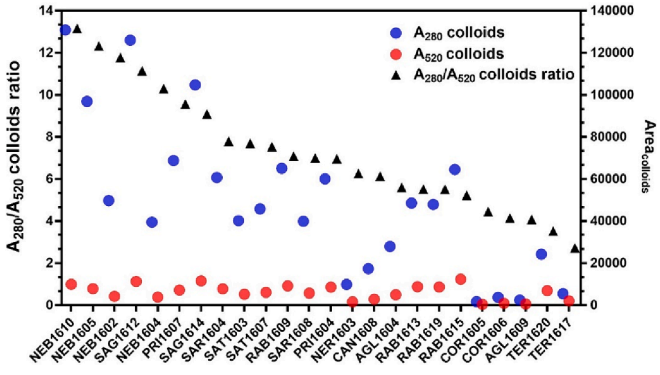

Fig. 4. Focus-FIA signals at 280 nm (A_{280} colloids), at 520 nm (A_{520} colloids) and ratio between the A_{280} and A_{520} Focus-FIA signals for the 24 wine samples (see also data in Table 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Whether for wine color stability it would be better to have the polymeric pigments in free form (i.e., not associated with proteins) or as part of colloidal particles (i.e., associated with proteins and/or polysaccharides) cannot be inferred here. However, colored colloidal particles were found in all 24 wines, although in different proportion compared to the total colloidal content (Fig. 4).

Besides the grape variety composition, the type of colloids in a given wine is certainly also influenced by the vinification methods adopted typical for each Italian grape variety here investigated. As above mentioned, some of these typically undergo a long ageing period (e.g., Nebbiolo, Sagrantino, Sangiovese), and therefore contain a higher proportion of colloids compared to others made in a ready-to-drink style (e.g., Teroldego, fresh Corvina), a fact that is related to different maceration regimes (Fig. 4, blue dots). However, the quantity of colloidal color components (Fig. 4, red dots) seems to be less variable than that of A_{280} absorbing colloids. It can be hypothesized that this variability is related to the composition of the colloids showing variable amounts of PP included in them. Therefore, the vinification style seems to be less impacting for the colored colloidal matter.

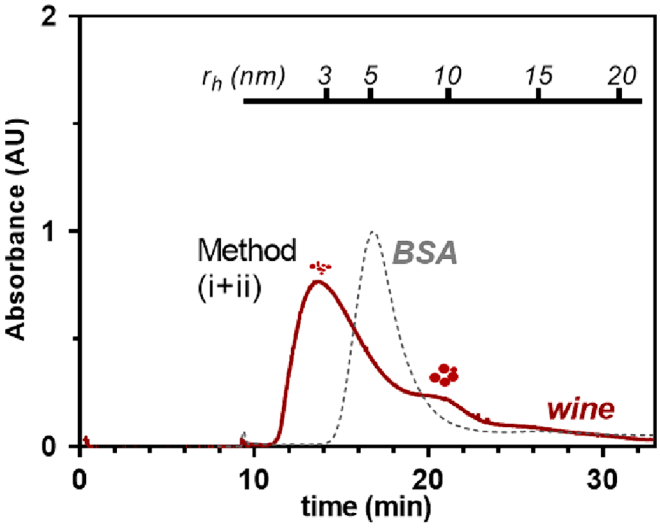

Fig. 5. A_{280} profile of a standard protein (BSA, dotted line) and of a representative wine sample (red line), including the hydrodynamic radius (r_h) values as a function of elution time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Size and morphology of red wine colloids

The 24 red wines were analyzed by AF4, a technique already employed in a different setup in the previous section, with a separation method able to detect the different populations of colloids present in them. In addition to UV detection, light scattering (MALS) was employed to directly measure the gyration radius (Rg) of the eluting species, thus obtaining information on the structural compactness of the detected particles, which could be used to make assumptions on their shape and, possibly, behavior and composition.

As previously observed by UV detection, AF4 fractionation shows a single broad peak with retention time of about 14 min (Marassi et al., 2021) (Fig. 1B). In addition to this, MALS detection, which is sensitive to particle concentration and size, allowed to identify a series of particles' populations eluting between 14 and 30 min, plus a residual one at field release corresponding to totally retained particles. Following the focusing step (removal of species < 5 kDa, see Fig. 1A), AF4 separates the particles according to their hydrodynamic radius (Rh) which is correlated, for particles with known composition, with the elution time (Fig. 5).

Using BSA as a standard, in our experiments a single UV peak was detected at 17 min, corresponding to a Rh of about 5 nm. This finding is coherent with an estimated molar mass of a low-order BSA aggregate (dimer/trimer) (Fig. 5, dotted line). In contrast, wine colloids eluted at a time corresponding to a Rh radius < 3 nm (Fig. 5, red line), confirming the results of others (Osorio-Macías et al., 2020). However, MALS results described below are not compatible with this Rh radius. This unexpected result highlights how BSA, a globular non-glycoprotein with a molecular weight of 66.430 Da (Carter & Ho, 1994), behaves differently than wine particles and indicates that wine colloids must have some characteristics which confer them a different retention behavior in AF4, probably due to the presence of non-protein components such as tannins and polysaccharides (Marassi et al., 2021).

With MALS detection, all wines showed to contain colloids with Rgs larger than that of individual macromolecules (Smilgies & Folta-Stogniew, 2015; Zanchi et al., 2008). Considering also the unsuitability of BSA as a calibration standard for the size (Rh) of this type of particles, this is an indication that colloids of all red wines must result from an assembly of different species, including not only proteins, as demonstrated previously (Marassi et al., 2021).

In details, MALS detection of the colloids of the 24 red wines showed a total of 4 light scattering peaks eluting at different times after AF4 separation (Fig. 6). However, an additional small peak between peak 2 and 3 was observed for the two Teroldego wines (TER1617 and TER1620) (data not shown).

The presence of 4 MALS peaks agrees with previously reported results (Pascotto et al., 2020), and 3 MALS peaks (before the field release of peak 4, Fig. 6) were obtained for young Merlot and Tempranillo wines (Osorio-Macias, 2022), but not with those described for aged red wines produced in Argentina, in which only 2 MALS peaks were detected (Osorio-Macías et al., 2020). In most of the cases, an increase of the structural compactness of the colloidal particles from peak 1 to 3 was noted (decreasing Rg), whereas the last eluting peak 4 was the least compact (high Rg) (Fig. 7 and Fig. 8). Although a calibration was not possible for the hydrodynamic radius (Rh), this parameter increases with the retention time (see Fig. 5). The UV profiles of the same AF4

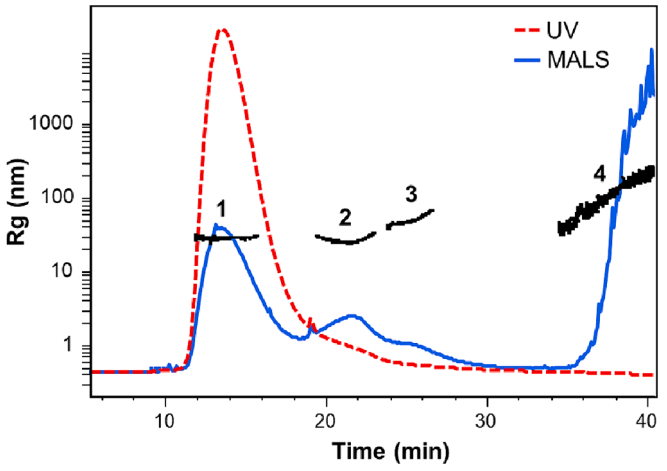

Fig. 6. Representative fractogram (NEB1604 sample) for the sizing of red wine colloids. Dashed red line: A_{280} absorption. Solid blue line: Light Scattering (LS) signal @90°. Black dots clouds are used to calculate the Rg values (in nm) for the 4 peaks (see Fig. 7 for individual, and Fig. 8 for aggregated, Rg values).

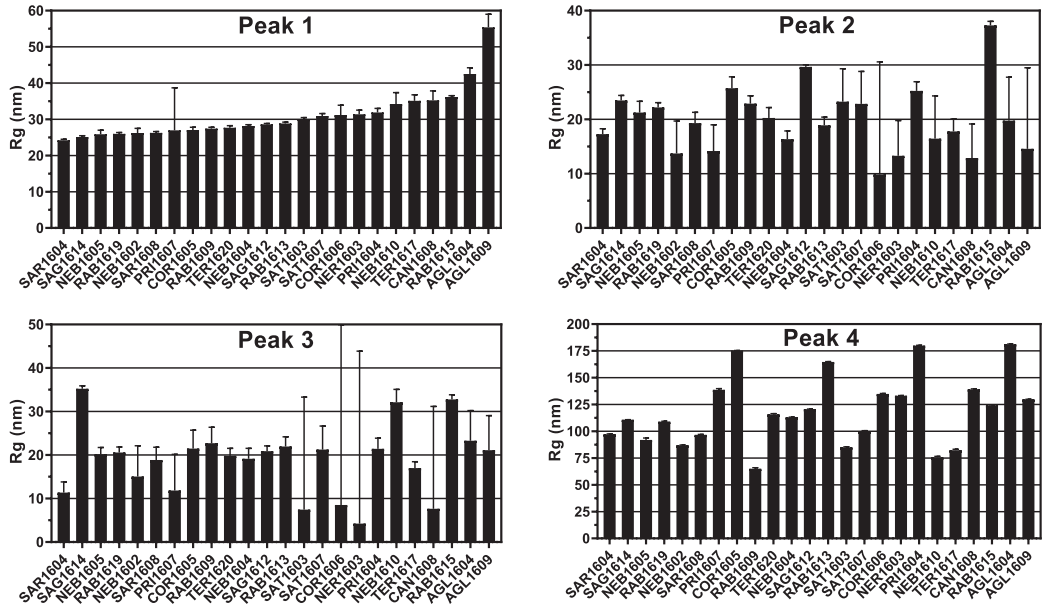

Fig. 7. Gyration radius (Rg in nm) values for the 4 main peaks detected by MALS after AF4 separation of the colloids (Focus-FIA) of the 24 wine samples. The retention times for the peaks were: 12–16 min for peak 1,18–22 min for peak 2, 24–28 min for peak 3, and 36–42 min for peak 4.

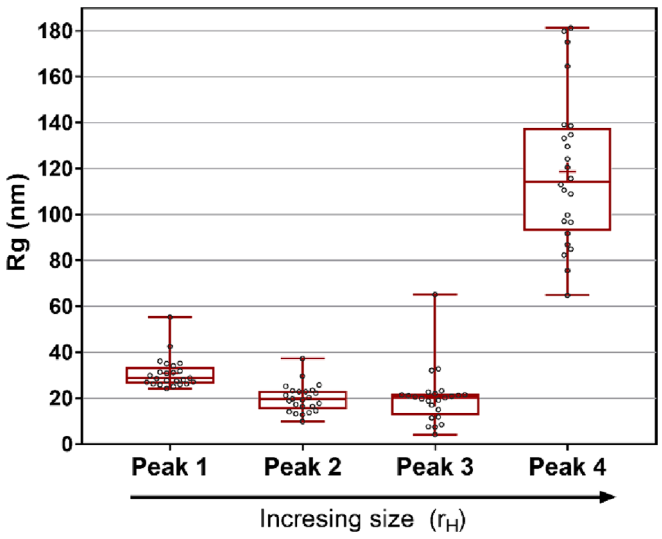

Fig. 8. Boxplot analysis of the total Rg distribution of wine colloids (in nm) as determined in the 24 Italian monovarietal red wines grouped by MALS peaks.

separations (see Fig. 6, UV signal) indicate that the major peaks, i.e. those eluted at the beginning of the fractogram, represent the vast majority of all eluted species, confirming previous findings (Marassi et al., 2021; Osorio-Macias, 2022; Osorio-Macías et al., 2020; Pascotto et al., 2020).

The different Rg populations (peaks 1 to 4) detected by MALS are eluted within the same retention time range and therefore their sizes (Rh) can be assumed to be similar for all wines. However, the differences detected for the Rgs of those populations indicate that their compactness differ among wines (Fig. 7). Indeed, for MALS peak 1, that is the peak in which most of the colloids are found, colloids' Rg ranged between 24 (for SAR1604, the most compact) to 55 nm (for AGL1609, the least compact) nm, with an average of 31 nm. Interestingly, the two wines with very low percentage of A_{280} absorbing material in colloidal form (AGL1609 and COR1605, see Fig. 2A) showed a very weak signal for MALS peak 1, indicating that these particles were present in minimal quantities in those wines, in line with the data presented in Table 1 and Fig. 2A. Peak 2 showed Rg values ranging from 10 (for COR1606) to about 37 nm (for RAB1615), with an average Rg of 20 nm, whereas for peaks 3 and 4 the ranges were broader (peak 3: 4–65 nm; average 20 nm; peak 4: 65–181 nm; average 120 nm) (Fig. 7). Peaks 2 and 3, at least on average, show populations of colloids with a higher size (Rh) (higher retention time) but, with lower Rg (higher compactness) compared to the population present in peak 1 (Fig. 8). This indicates that colloids of different size, as deduced by the retention time of the peaks in which they are found, are structurally different.

Considering the wines' composition (Table 1), the AF4 data and the findings from our previous studies (Marangon et al., 2022; Marassi et al., 2021), it is therefore possible to hypothesize that the differences in colloid structural characteristics are governed by the type and proportion of colloid-forming macromolecules involved. Our previous findings (Marassi et al., 2021) showed that, while phenolics and polysaccharides were distributed along the whole fractogram, proteins were only found in peak 1 and, at less extent, in peak 2. Therefore, it is possible that the different degree of compactness of the 4 peaks is related to the quantity of proteins present in the colloidal assemblies. Again, it can be stated that wine proteins need to be studied for their relevance and role in affecting the structure and behavior of red wine colloids. In this framework, the information on the compactness of wine colloids (Fig. 8) was used to revise the model for colloidal aggregation in red wines previously proposed (Marassi et al., 2021), and this, together with the

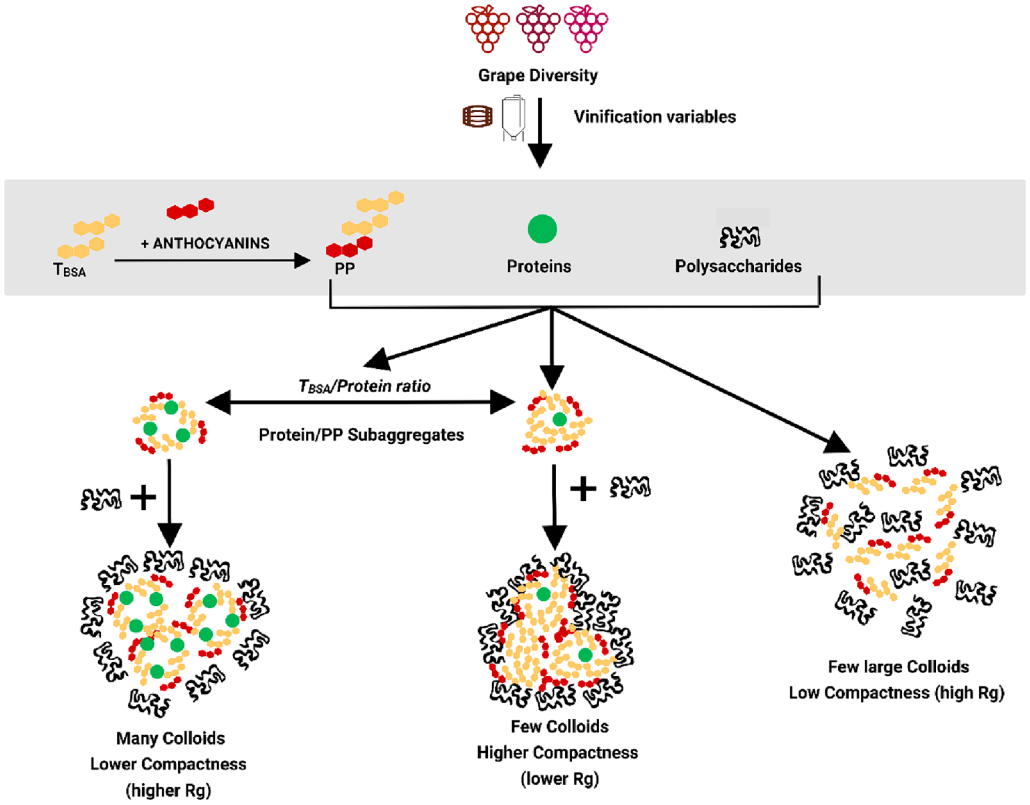

Fig. 9. Revised mechanism for colloidal aggregation in red wine to incorporate the concept of colloids compactness here presented as well as the concept of protein/polymeric pigments (PP) sub-aggregates and T_{BSA}/proteins ratios (Marangon et al., 2022). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

data on the macromolecular composition of Italian red wines (Marangon et al., 2022), allowed for the proposal of the improved model shown in Fig. 9.

During vinification, PP are formed via the reaction of anthocyanins with tannins (Oliveira et al., 2019), including those reactive to the BSA (T_{BSA}). After the individual macromolecules have been extracted from grapes during crushing and maceration, protein-PP sub-aggregates are formed thanks to the protein-binding capacity of tannins. Experimental evidence for the presence of these sub-aggregates was given in our previous work by determining the presence of proteins and tannins in red wine colloidal fractions that showed to be colored (Marassi et al., 2021). Additionally, SDS-PAGE results (Marangon et al., 2022) indicated that all proteins in red wines are bound to phenolic compounds, as visible from the lack of bands with the apparent MW expected for wine proteins (Van Sluyter et al., 2015). Then, protein-PP sub-aggregates interact with each other to form colored colloidal particles in which also polysaccharides are present and are likely to stabilize the colloidal dispersion (Jones-Moore et al., 2022).

The great variability of Rgs (compactness) observed for colloids of the different wines allowed to further elaborate on the proposed aggregation mechanism. Indeed, particles of different size (Rh) showed also different average compactness (Fig. 8). Compactness can be governed by the type and proportion of colloid-forming macromolecules involved, and this can also explain the differences detected for each particle size in the different wines. It seems that protein-tannin sub-aggregates containing many phenolic compounds covalently bound to a limited amount of proteins (Marangon et al., 2022), allow for sub-aggregates to bind together in a more compact structure, as shown by looking at the average Rg value of colloids in peaks 2 and 3. Indeed, these two peaks contain very low amounts of proteins compared to the less compact peak 1 (Fig. 8), as previously shown (Marassi et al., 2021).

A third type of large colloids (peak 4) in which proteins were not found (Marassi et al., 2021), was also detected by MALS, being characterized by a very loose structure (Fig. 8). However, both A_{280} and A_{520} signals were absent for this MALS peak, indicating that the quantity of colloids present in it is negligible.

4. Conclusions

The application of AF4 allowed to discover that different monovarietal red wines contain colloids differing in quantity, size (Rh) and compactness (Rg), and these differences seem to be influenced by the type and quantity of tannins in a wine and by their reactivity with other colloid-forming molecules, characteristics being potentially linked to the variety. The information collected on the type of compounds present in wine colloids allowed for the formulation of an updated hypothetical model for colloidal aggregation in red wine, in which also the color was considered, leading to the conclusion that, besides polymeric pigments and free anthocyanins, red wine color is also due to colloidal particles containing pigments bound to proteins. This further reinforces the idea that proteins play a fundamental role in the colloidal status of red wines. However, for the elucidation of the mechanisms governing the stability of the colloidal system of red wines, which is a main issue in enology, the precise role of proteins needs to be further explored in the context of their interaction with other wine components, such as polysaccharides and tannins. These interactions can lead to differences in size and morphology of the colloidal particles which, in turn, are likely to affect technological (e.g., stability) and sensory (e.g., color, astringency) properties of red wines. Indeed, various types of tannins (condensed or hydrolysable) and polysaccharides (e.g., mannoproteins, arabic gum) are often used in red winemaking to modulate wine characteristics such as color stability. Conversely, colloid-forming compounds can also be managed by subtractive techniques aimed at removing some of the components that have been shown to participate in colloidal assemblies. In this context it is then important to consider both the grape-derived compounds and the extraction procedures used during winemaking, as all these factors can affect the relative content of the different components responsible for the structural characteristics of wine colloids.

CRediT authorship contribution statement

Matteo Marangon: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Valentina Marassi:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Barbara Roda:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology. **Andrea Zattoni:** Writing – review & editing, Supervision, Resources, Project administration, Methodology. **Pierluigi Reschiglian:** Writing – review & editing, Supervision, Resources, Project administration. **Fulvio Mattivi:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Luigi Moio:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Arianna Ricci:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Paola Piombino:** Writing – review & editing, Resources, Project administration, Funding acquisition, Data curation. **Susana Río Segade:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Simone Giacosa:** Writing – review & editing, Project administration, Investigation, Formal analysis, Data curation. **Davide Slaghenaufi:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Andrea Versari:** Writing – review & editing, Supervision, Resources, Project administration, Methodology. **Urska Vrhovsek:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Maurizio Ugliano:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Alberto De Iseppi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Christine Mayr Marangon:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Andrea Curioni:** Writing – review & editing, Supervision, Resources, Project administration, Methodology.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andrea Zattoni, Barbara Roda, Pierluigi Reschiglian and Valentina Marassi are associates of the academic spinoff company byFlow Srl (Bologna, Italy). The company mission includes know-how transfer, development, and application of novel technologies and methodologies for the analysis and characterization of samples of nano-biotechnological interest.

Data availability

Data will be made available on request.

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