Asymmetrical Flow Field-Flow Fractionation in the Separation, Characterization and Application of Wine Particle Matter

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DOCTORAL DISSERTATION

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Abstract

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These compounds exhibit fundamental properties in relation to the stability of the wine (color, polymerization, haze formation and precipitation) with impact in sensory properties i.e., bitterness, astringency and lastly towards the final quality of the product.

However, there is a lack of understanding of the dynamics of the properties of these compounds during distinct stages of the winemaking process and during storage. This could possibly be due to the lack of methodologies that allow the separation and characterization of these colloidal and macromolecular compounds and their properties at their native state.

These compounds, especially the phenols that are the major part of the colloidal fraction, are highly reactive and tend to undergo changes. Hence, it is of vital importance to develop methodologies for the characterization of these compounds and their properties, and therefore generate a useful knowledge of the dynamics in which these compounds are involved.

This thesis presents a methodology for the separation and characterization of these colloidal and macromolecular compounds using Asymmetrical flow field fractionation coupled with multiple detectors (AF4-UV-MALS-dRI) determining the main macromolecular properties, such as molar mass (M_W), hydrodynamic radius (r_H), apparent density ($\hat{\rho}$), concentration (c) and specific absorptivity (ε) in relation to the processes that occur during vinification.

The understanding and monitoring of these properties along with the effects and changes that occur at colloidal and macromolecular level during the production process chain may enable the control and monitoring of these compounds. In addition, knowledge of their different properties will assist decision making in the manufacturing of wine with a desired profile.

Key words: AF4, wine colloids, wine macromolecules, phenolic compounds, polysaccharides, winemaking

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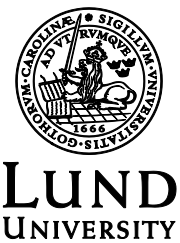

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To all my professors in chemistry…

Popular Scientific Summary

Wine is a fermented alcoholic beverage made from grape juice (Vitis vinifera) that contains ethyl alcohol. However, there are also components with some potential health benefits such as the polyphenols. Numerous investigations have been reported linking the moderate consumption of red wines and the consequent high intake of antioxidants with a possible reduction of cardiovascular disease. A well-known French paradox supports these arguments, since it has been documented that a French diet rich in fats displays low negative effects on public health.

In general, the diversity of compounds that constitute grapes, and therefore wines, can be affected by various factors, from the geography of the mountains where the vineyards are located, the climate, the type of soil, the latitude and the altitude above sea level. All these factors play an important role in the composition and in the final quality of the wine. In addition, it has been reported that the type of grape variety and the techniques used during the winemaking process can affect the chemical grape constituents in the wine.

Of all the above-mentioned factors, it can be emphasized that the temperature variations between night and day (in some regions up to 30-degrees during spring and summer) cause delays in the ripening of the grape, which is smaller with more skin than pulp. Several molecules with antioxidant activity (polyphenols) are present in the skin of the fruit, which favor important properties such as color, bitterness, flavor, etc.

A potential key factor is the altitude above sea level of the vineyards in wine districts located at low latitudes, e.g., northern Argentina and southern Bolivia. Grapes exposed to ultraviolet (UV) rays, favor the formation of antioxidants with a dry astringent character. In addition, the abundance of sunlight dehydrates the grapes, producing a more concentrated juice and thus a different wine.

These antioxidants, phenolic compounds, reduce the wine stability as they tend to aggregate and precipitate. This instability phenomena becomes a slightly unpleasant element for consumers, resulting in loss of value for the producer.

However, this topic within wine research has particular challenges as this material may occur as poorly defined, very small particles, referred to as wine colloids, or as complex, extremely large molecules, wine macromolecules.

In this thesis, methods have been developed to assess various properties, such as the size, structure mass and others, of the wine colloids and wine macromolecules. A separation technique based on the Asymmetrical Flow Field-Flow Fractionation method has been used for this purpose.

Moreover, this project intends to evaluate how the methodology can be used to understand the way in which these colloidal and macromolecular components are affected by different process operations during winemaking, e.g., the role of clarifier agents and how these affects colloidal structure. Another interesting application for this developed methodology has been to monitor particle formation throughout the vinification process (alcoholic fermentations, malolactic fermentations, clarification, bottling and storage).

It has been observed that particles in wine undergo many changes throughout vinification and may eventually influence the properties of the wine itself. Therefore, the knowledge developed in this thesis (AF4 on wine particle matter) provides many insights into the field and should be of considerable interest in the scientific and technology field, as well as in the industrial and oenological areas.

Resumen Científico Popular

El vino es una bebida alcohólica fermentada hecha de jugo de uva (*Vitis vinifera*), que contiene alcohol etílico. Asimismo, contiene componentes con algunos beneficios potenciales para la salud, como los polifenoles. Numerosas investigaciones reportadas han relacionado el consumo moderado de vinos tintos y la consecuente alta ingesta de antioxidantes, con una posible reducción de enfermedades cardiovasculares. Una conocida paradoja francesa apoya estos argumentos, ya que se ha documentado que una dieta francesa rica en grasas tiene pocos efectos negativos sobre la salud pública.

En general, la diversidad de compuestos que constituyen la uva, y por tanto los vinos, puede verse afectada por diversos factores, desde la geografía de las montañas donde se ubican los viñedos, el clima, el tipo de suelo, la latitud y la altitud sobre el nivel del mar. Todos estos factores juegan un papel importante en la composición y en la calidad final del vino. Además, se ha reportado que el tipo de variedad de uvas y las técnicas utilizadas durante el proceso de vinificación, afectan los componentes químicos de la uva en el vino.

De todos los factores mencionados anteriormente, se destaca que las variaciones de temperatura entre la noche y el día (en algunas regiones hasta 30 grados durante la primavera y el verano) provocan un retraso en la maduración de la uva, que es más pequeña y con más piel que pulpa.

Varias moléculas con actividad antioxidante (polifenoles) están presentes en la piel de la fruta, las cuales favorecen importantes propiedades como color, amargor, sabor, etc.

Un factor potencial clave es la altitud sobre el nivel del mar de los viñedos en distritos vitivinícolas ubicados en latitudes bajas, por ejemplo, el norte de Argentina y el sur de Bolivia. En tales localidades, las uvas expuestas a los rayos ultravioleta (UV), favorecen la formación de antioxidantes con carácter astringente seco. Además, la abundancia de luz solar deshidrata las uvas, produciendo un jugo más concentrado y por lo tanto un vino diferente.

Estos antioxidantes, compuestos fenólicos, reducen la estabilidad del vino ya que tienden a agregarse y precipitarse. La inestabilidad se convierte en un elemento levemente desagradable para los consumidores, resultando en pérdida de valor para el productor.

Sin embargo, este tema dentro de la investigación del vino tiene desafíos particulares, ya que este material puede presentarse como partículas muy pequeñas a veces mal definidas, denominadas coloides del vino, o como moléculas complejas extremadamente grandes, llamadas macromoléculas del vino.

En esta tesis se han desarrollado métodos para evaluar diversas propiedades, como el tamaño, la estructura, la masa y otras relacionadas a los coloides y macromoléculas del vino. Para ello se ha utilizado una técnica de separación basada en el método de fraccionamiento de flujo de campo de flujo asimétrico.

Este proyecto tiene la intención de evaluar cómo se puede utilizar el enfoque y la metodología desarrollada para comprender la forma en que estos componentes coloidales y macromoleculares se ven afectados por diferentes operaciones de proceso durante la elaboración del vino, por ejemplo, el papel de los agentes clarificantes y cómo estos afectan la estructura coloidal. Otra aplicación interesante de esta metodología desarrollada ha sido el seguimiento de la formación de partículas a lo largo del proceso de vinificación (fermentaciones alcohólicas, fermentaciones malolácticas, clarificación, embotellado y almacenamiento).

Se ha observado que las partículas en el vino sufren muchos cambios a lo largo de la vinificación y eventualmente pueden influir en las propiedades del vino mismo. Por lo tanto, el conocimiento desarrollado en esta tesis (AF4 sobre partículas en el vino) proporciona nuevas perspectivas, ideas y conocimientos en el campo científico, tecnológico, industrial y enológico.

Abstract

Wine particle matter is mainly constituted by wine colloids and wine macromolecules. These wine colloidal and macromolecular compounds are barely studied, possibly due to the difficulty in facing the challenges that their study merits. These compounds are mainly formed by polyphenols, proteins and polysaccharides that come from the grape seeds, skins and pulp and even, to a lesser extent, from the winemaking itself, since many of them are released during the process.

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List of Papers

This thesis is based on the following papers, referred to in the summary by their corresponding Roman numerals.

I. **Resveratrol, phenolic antioxidants, and saccharides in South American red wines.**
Daniel E. Osorio-Macías, Vásquez P, Carrasco C, Bergenståhl B, Peñarrieta JM.
International Journal of Wine Research. **2018**; 10:1-11. https://doi.org/10.2147/IJWR.S152026.

II. **Fractionation of Nanoparticle Matter in Red Wines Using Asymmetrical Flow Field-Flow Fractionation**.
Daniel E. Osorio-Macías, Dongsup Song, Johan Thuvander, Raúl Ferrer-Gallego, Jaeyeong Choi, J. Mauricio Peñarrieta, Lars Nilsson, Seungho Lee, and Björn Bergenståhl.
Journal of Agricultural and Food Chemistry 2020 68 (49), 14564-14576 https://DOI: 10.1021/acs.jafc.9b07251

III. **Characterization on the impact of different clarifiers on the white wine colloids using Asymmetrical Flow Field-Flow Fractionation.**
Daniel E. Osorio-Macías, Hans Bolinsson, Javier A. Linares-Pastén, Raúl Ferrer-Gallego, Jaeyeong Choi, J. Mauricio Peñarrieta, Björn Bergenståhl.
Food Chemistry, Volume 381, 2022, 132123, ISSN 0308-8146, https://doi.org/10.1016/j.foodchem.2022.132123.

IV. **Characterization and monitoring wine colloids and macromolecules during vinification process using Asymmetrical Flow Field-Flow Fractionation.**
Daniel E. Osorio-Macías, Raúl Ferrer-Gallego, Jaeyeong Choi, Mauricio Peñarrieta, Björn Bergenståhl.
Manuscript.

The Author's contributions to the Papers

Paper I. The author performed a major part of the experiments with the exception of the sugar determination. The authors performed the planning, the data evaluation and the writing of the paper in collaboration with the co-authors.

Paper II. The author performed a major part of the experiments except the HPAEC analysis. The authors performed the planning, the data evaluation and the writing of the paper in collaboration with the co-authors.

Paper III. The author performed a major part of the experiments except the clarification experiments and the gel electrophoresis. The authors performed the planning, the data evaluation and the writing of the paper in collaboration with the co-authors.

Paper IV. The author performed all the experiments except the sample preparation from the vinification process. The authors performed the planning, the data evaluation and the writing of the paper in collaboration with the co-authors.

Abbreviations and Symbols

FFF	Field Flow Fractionation
AF4	Asymmetrical Flow Field-Flow Fractionation
UV	Ultraviolet
MALS	Multi Angle Light Scattering
dRI	Differential Refractive Index
r_H^*	Hydrodynamic Radius (nm)
M_W^*	Molecular Mass (g/mol)
$\hat{\rho}^*$	Apparent Density (kg/m^3)
ε^*	Specific Absorptivity (mL/mg · 1/cm)
dn/dc	Refractive index increment (mL/g)
$(dn/dc)_{TS}$	Refractive index increment of total solids (mL/g)
$(dn/dc)_{WC}$	Refractive index increment of wine colloids fraction (mL/g)
$(dn/dc)_{WM}$	Refractive index increment of wine macromolecules fraction (mL/g)
c	Concentration (mL/mg)
TPH	Total Phenols (mmol GAE/L)

*	Calculated as Mass Weighted Average

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1. Introduction

Wine is one of the oldest documented beverages that has been consumed throughout history [1]. Nowadays, the grapevine is one of the world's most important agricultural fruit crop. However, it is impossible to compare modern wine with ancient wine, as today's winemaking process can be barely equated with what it used to be, as it has been a continuously growing trend throughout history and has evolved to become one of the most appreciated drinks in large parts of the world. Today, winemaking is practiced in a more specialized way. The art of winemaking goes hand-in-hand with the art of cooking and of fine cuisine. Few other foods and beverages have had as significant a success story as wine.

Wine is made by fermenting the fruits of the vine grape (*Vitus vinifera, V. labrusca, V. riparia, V. rotundifolia, V. aestivalis, V. mustangensis* and hybrids), and its various components show different effects for human health benefits and for the wine properties during manufacture. The moderate consumption of wine (red wine mostly) involves important biological activities that have been related to the grape constituents specifically for their protective effects; these include various phenolic compounds, as well as alcohol content, such as antioxidant, anticarcinogenic, immunomodulatory, antidiabetic, antiatherogenic, neuroprotective, anti-obesity and antiaging activity [2], [3], [4], [5].

The basic composition of wine can be classified in different ways, since values may vary according to the wine type analyzed and the extraction technique. However, a common classification of wine composition [6], [7], [8], [9], [10], [11] is shown in percentage and mg/L in table 1.

It is mainly the compounds that are soluble, or at least partially soluble, in water and ethanol that play an important role for the properties of wine, specifically phenolics, which are essential for wine quality and influence wine characteristics such as color and taste.

Furthermore, during the fermentation processes, pH balance and other factors increase solubility [12], [13] when extracting different compounds such as flavonoids, anthocyanins, catechins, proanthocyanidins, proteins and soluble polysaccharides (AGP). In addition, it is during fermentation that the content of these soluble compounds can increase the most, decrease or fluctuate significantly, depending on the crop or fermentation conditions [14], [15].

Table 1. Chemical composition of red wine and average quantities of major wine components [6-11].

Compounds	Percentage (%)	Characteristics
Water	~85	Water is an essential component in many of the chemical reactions involved in grape growth, juice fermentation, and wine aging.
Ethanol*	~12	Ethanol comes mainly from yeast fermentation and can accumulate to up to about 14-15%.
Trace compounds	~1	Sugars: (0.6 %), Minerals: Ca (0.08 %), Fe (0.5%), Mg (120 ppm), P (230 ppm), K (1270 ppm), Na (0.04%), Zn (14 ppm), Cu (0.11 ppm). Vitamins: Thiamin (0.05 ppm), Riboflavin (0.31 ppm), Niacin (2.2 ppm), Vitamin B6 (0.6 ppm) and nitrogenous compounds, anions, and cations
Volatiles	~0.5	Fusel alcohols (0.25 %), esters (0.2 %), ketones (75 ppm), C13 nor isoprenoids (70 ppm) fatty acids (65 ppm), phenols, amides & others (100 ppm)
Acids	~0.5	Mainly Tartaric Acids followed by other organic acids like malic, citric and ascorbic.
Larger compounds	**Range mg/mL**	
Polysaccahrides	100 - 250	White wines
Polysaccahrides	100 - 600	Red wines
Proteins	15 - 230	Red and White wines
Tannins	30 - 1895	Red and White wines

* Ethanol is represented as weight per volume (w/v), as are all other components, and it is equivalent to 14 % (v/v).

Wine colloids (condensed tannins) and wine macromolecules (wine polysaccharides) are the two large groups that constitute wine particle matter [16], regardless of whether wine macromolecules behave as wine colloids. Wine colloids, together with proteins, can be considered as part of the macromolecules, in addition to those complex molecules arising from their interactions. These condensed tannins (polymeric forms) have a strong propensity to interact with proteins and are made up mainly of flavanol units; the degree of polymerization may vary according to the tannin source, consequently showing a diversity of structures and molecular weights [17]. However, colloid formation represents a very small part (<5%) of total tannins in solution which are available for protein binding [18].

On the other hand, condensed tannins (proanthocyanidins) are derived from the grape berry, contributing to color and aging stability, and to the organoleptic properties of the wine. They represent the most abundant class of phenols in wine, accounting for about 25-50% of the total phenolics in young red wines, equivalent to 0.3 g/L depending on the variety [19]. This proportion may be higher in older wines [20], while hydrolysable tannin content rarely exceeds 20 mg/L.

From sensory perception involving phenols, bitterness is restricted to small molecules that activate the sensory signal detection process, while astringency is related to the number of protein interaction sites in the molecule. That is, the astringency increases with the concentration of tannins, the molecular size and the degree of galloylation [21], in addition to its ability to form complexes with

peptides and proteins [22], [23]. However, parameters such as the wine matrix, ethanol content and ionic strength play a crucial role in tannin self-aggregation [24]. However, it is worth noting that aggregation and stringency are not directly related, since tannin aggregation reaches its maximum at a certain size, and astringency increases with the molar mass of tannin [25].

Therefore, condensed tannins play an important role in bitterness or mouth-feel perception, and this sensory perception defines red wine quality [26], [27]. Furthermore, the characterization of condensed tannin chemistry and astringency in grapes and wines depends on the variety of grape, and is affected during the manufacturing process by many factors, such as fermentation management, pH, and wine age, among others [26].

Wine macromolecules are primarily constituted from polysaccharides, present in wine as part of endogenous materials [28] because of the release of extraction and maceration during vinification, from microorganism, or from plant cell walls [29], [30]. Mainly during alcoholic fermentation, parietal polysaccharides are released by *Saccharomyces cerevisiae* [31] and wine aging on lees [32], [33], [34]. These polysaccharides are mannoproteins with a high content of mannose (higher than 80%), existing as covalent complexes with proteins. They have different functions during winemaking, including improving the malolactic growth of bacteria and protecting against the formation of turbidity; they can also be complexed with tannins to modify the sensation in the mouth, can prevent the precipitation of tartrate salts, and modify the volatility of aromatic compounds [30], [31], [33], [35], [36]. In addition, it has been reported that wine polysaccharides may interact and aggregate with phenolic compounds [37], [38], affecting both tannin aggregation and tannin astringency, presumably through competition with salivary proteins in the formation of tannin complexes. It has also been reported that some polysaccharides increase the color stability of wines [33].

Wine proteins derived from grape pulp are present in low concentrations (15-230 mg/L) (see table 1). These proteins can survive the winemaking process as they are resistant to proteolysis and to the characteristics of the low pH of wines [7]. Most of these proteins have been identified as pathogenesis proteins, (PR) specifically thaumatin-like (TL) proteins and chitinases [39], [40]. Despite their low concentration, they are essential because they can affect the stability and clarity of the wine since they are likely to coagulate, aggregate and precipitate and/or flocculate, resulting in light-dispersing particles that produce visible haziness or turbidity [41], [42].

On the other hand, depending on the vintage of the wine, compounds that tend to polymerize, such as proanthocyanidins, vary in their solubility; if they are young, they tend to remain soluble but as they age, they tend to increase in size and become increasingly more insoluble, causing precipitation reactions with other compounds such as proteins[43], [44].

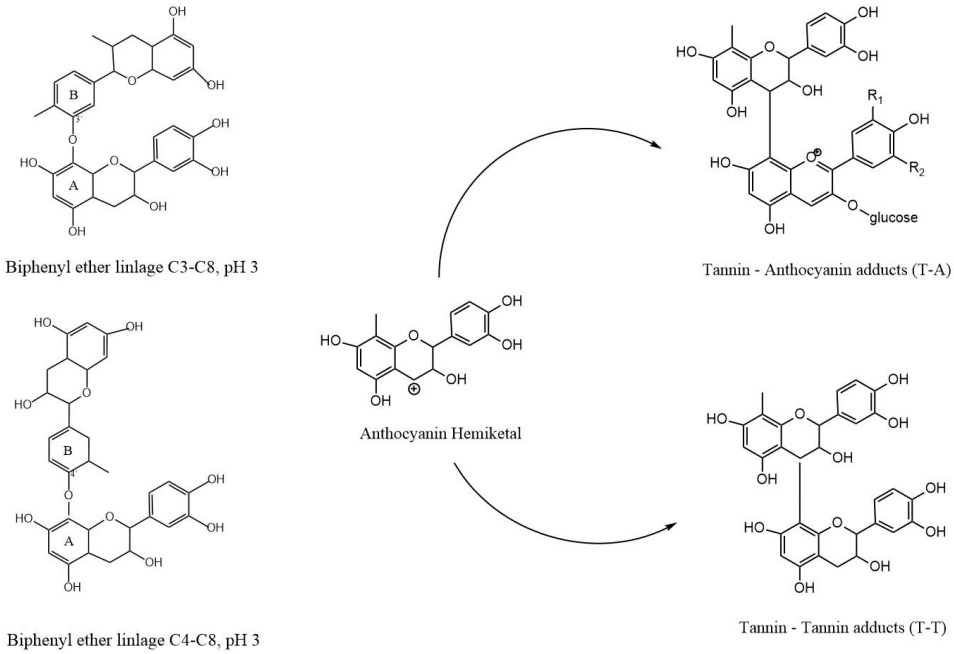

Figure 1. Sturctures characterized in catechin oxidation shoing the biphenyl ether linkage performed at pH 3 (left) and in non oxidative condition a propsed mechanis of the formation of T-A and T-T adducts [45].

Furthermore, wine flavonols are prone to autooxidation, exhibiting specific linkages (C3-C8 and C4-C8) in their structures, and at pH around 3 the biphenyl ether linkage is located between ring B of the upper unit and ring A of the lower unit, where the O is the linking oxygen atom (Figure 1, left). These interflavanyl linkages yielded by oxidation are totally resistant to acid cleavage, which by further oxidation leads to yellow pigments, inducing a further polymerization that finally can be highly dependent upon wine composition, and the different classes and content of polyphenols. However, if an anthocyanin is involved (in a hydrated or colorless form), this provides an electric charged molecule, carbocation, generating C4-C8 covalent bonds between two molecules with more uniform structure (Figure 1, right). Therefore, if the reaction is with another tannin, a much longer oligomer or polymer will be formed with stronger interactions between the tannin molecules.

In general, the polymerization of these compounds, anthocyanin-tannin and tannin-tannin polymers, occurs during wine maturation but they usually remain in suspension until they can form complexes with soluble proteins and tartrate salts. However, once the aggregates are large enough to protect hydrophobic centers and exclude water molecules, they tend to lose the ability to stay soluble and will precipitate from the solution.

In recent decades, these reactions between anthocyanins and tannins have been extensively studied and the main types of products have been characterized [17], [45].

However, the molecules identified in such cases do not reflect the wine composition since these monomeric and oligomeric compounds analyzed by HPLC do not represent the heterogeneous fraction of flavonols and tannins (higher polymers). Furthermore, these larger polymers, which tend to increase in chain length, lead to poor resolution of their components in all separation techniques. In addition, these wine macromolecules and colloids are found in low quantities in wines and, due to their low solubility, they are normally eliminated during the winemaking processes, whether in the clarification, pressing or fermentation stages. Hence, there is more qualitative data regarding these compounds, and a significant lack of quantitative data. For this reason, the quantitative analysis of wine tannins and determining the constituents formed during the vinification process (derived tannins) is one of the main challenges to be addressed in the coming years.

The isolation and characterization of these colloidal and macromolecular compounds in wines, as well as the determining of its physicochemical properties, has been a constant challenge due to the instability of these compounds present in the wine matrix. Hence the complexity in the separation and, therefore, the lack suitable analytical techniques and methodologies of characterization in situ. Different techniques have been used for the evaluation of the average particle size of the interactions of condensed tannins, such as Dynamic Light Scattering (DLS) [38], [46], [47]; gel permeation chromatography (GPL) has also been used to evaluate the size distribution of polymeric pigments [17], in addition to Nanoparticle Tracking Analysis (NTA) to evaluate the average particle size in tannin fractions [48], [49], and wine macromolecular aggregation using fluorescence correlation spectroscopy [50].

Although several techniques have been tried to characterize these colloidal aggregates, much information is still lacking, especially regarding the structure and functionality of these complexes in the wine dispersion, and how they contribute to wine characteristics. Thus, the importance of the characterization of these colloidal and macromolecular compounds lies in the fact that their properties play a major role during the colloidal and macromolecular changes that occurs throughout manufacturing practices. These include effects on the stability of the wine, such as tartaric precipitations, color stability, haziness and precipitate formation [38], [51], [52]. Other factors during the winemaking process include the transfer of the must, sedimentation of the lees, filtration and fouling, and aging potential [32], [53]–[55], which influence the sense of taste, the organoleptic and sensory attributes of the wine [13], as well as its quality. All these properties vary according to the concentration of colloidal and macromolecular compounds, as well as the characteristics of the grapes and the winemaking process [56].

The suitability of the AF4 technique, coupled with multiple detectors during the separation and characterization of food [57], food beverages [58], [59] and biomacromolecules [60], has been demonstrated with high levels of resolution and, through the use of multiple detectors, a complete analysis of the characterization of the colloidal macromolecules can be obtained. Table 2 summarizes some of the properties characterized by the AF4.

The advantages of using the AF4 over other separation techniques are that it provides low cut rates, minimizing shear degradation, as well as low sample loss without altering the particle structure that may occur due to possible interactions during separation. Thereby, AF4 appears to be an efficient technique for separating and characterizing wine colloids and macromolecules according to the hydrodynamic size.

The AF4 technique, coupled with multiple detectors (online detection applying UV-MALS-dRI detectors), may have a pivotal role in the fractionation of wine particle matter, thus addressing the limitation challenges of previous analytical techniques. This allows for separation and a higher level of characterization of the colloidal and macromolecular compounds present in the particle matter of the wine, thereby determining fundamental and specific macromolecular properties. In addition, the use of the AF4 methodology allows for easy analysis of wine samples at any stage of the vinification process, without prior sample treatment.

Therefore, the application of this technique to the wine field in research or industry can be very broad, either by providing new data on the fundamental properties of colloids and macromolecules of the wine, such as size, molar mass, apparent density, concentration, and specific absorptivity. It can also provide new knowledge about, and insight into, what can occur in these properties due to the effects of the winemaking process and similarly in relation to the oenological parameters (grape variety, stability, color, phenol content, sugars, proteins, and metals, among others).

Table 2. Example of properteis determined by the AF4 in different matrix [57], [60]–[65].

Type of Polysaccahrides	Information obtained	Range r_{rms}/ r_H and M_W (kDa)
Starch	r_{rms}, r_H, Mw	1.0-1.8 nm and 70 - 100000 kDa
Amylose	r_{rms}, r_H, Mw	1.64-2.20 nm and 20 – 1000 kDa
Amylopectin	r_{rms}, r_H, Mw	1.02-1.29 nm and 78000 - 270000 kDa
Glucogen	r_{rms}, r_H, Mw	0.4-1.5 nm and 1900-35900 kDa
Gum Arabic	r_{rms}, r_H, Mw	1.0-3.0 nm and 150 – 30000 kDa
Mesquite Gum	r_{rms}, r_H, Mw	1.7-3.0 nm and 1500 -5000 kDa

Other type of Samples	Information obtained	Range
Oxidez tannins	Mw and DP.	37 – 350 kDa, and 6-37.
Wine colloids and wine macro-molecules	r_H, Mw, C and ε	2,9-20 nm; 10 -2000 kDa; 30-1200 kg/m^3; 0.01 – 13 mL/mg cm^{-1}.
Casein Micells	r_{rms}, r_H, Mw,	121-217 nm; 95-132 nm; 4.4 x 10^5 - 1.7 x 10 ^{6} kDa

Natural inorganic nanoparticles	Information obtained	Size
Humic Acid, clay colloid	r_{rms}, r_H	200 nm
Nanosized minerals	r_{rms}, r_H	100 - 1000 nm
Natural clay minerals*	r_{rms}, r_H	10 - 350 nm
Natural colloids (Soil leachates)	Mw	< 450 kDa

Natural organic nanoparticles/macromolecules		Size
Virus-like particles	r_{rms}, r_H	20-80 nm
Fe organin complex	Mw	< 500 kDa
Polyethylene imine	Mw	25 kDa
Fat globules (Milk Suspension)*	r_{rms}, r_H	300 - 550 nm
Complex of extracellular metal with polymeric substances	Mw	52-737 kDa

** Determined using Sedimentation FFF.*

In parallel studies using the AF4 technique in wines, distinct separation methodologies were developed. Additionally, the AF4 technique was compared with other separation methodologies for macromolecular compounds, such as Size Exclusion Chromatography (SEC). It was found that using the AF4 technique, separation and characterization is more efficient than with the use of SEC, since there are no interactions with the stationary phases, thereby characterization results are more relevant [51]. Furthermore, it was shown that the colloidal fraction of wine is rich in polyphenols (red wine) and rich in proteins (white wine), while the macromolecular fraction is richer in polysaccharides such as mannoproteins and arabinogalactans [62]. Results also reported on the relationship between colloidal fraction and sensory properties, whereby astringency is directly related to colloidal phenols bigger than 5 kDa, and that astringency increases as the molar mass of the wine colloids increases [25]. Likewise, the ratio proportions of the phenolic, protein and polysaccharide contents that exist within each colloidal and macromolecular fraction were evaluated, thus demonstrating the existence, relationship, and interdependence of these compounds, which in turn enabled the hypothesis of possible mechanisms of aggregation in which at least two different types of particles coexist bound by different types of forces, covalent and non-covalent [56].

All these studies using the AF4 technique required complementary (offline) techniques (spectrophotometric, chromatographic, HPLC, and DLS, GC-LC-MS among others) to assist during the separation, characterization, quantification, and interpretation of the nature of the colloidal and macromolecular fractions. However, great efforts are required to analyze and interpret large number of samples. As the results indicate that the different content and properties of the particles in the colloidal and macromolecular fractions depend on the characteristics of the grape and the winemaking process, it is necessary to apply this methodology and technique in a further practical and efficient way in the characterization of the properties. For example, to evaluate qualitative and quantitative changes in the macromolecular properties, i.e., average tannin molecular size and mass at any stage of the winemaking process (after the fermentations, clarification, aging), and if possible, to the variation in the properties by using different grape varieties and different modulations during the winemaking techniques.

As the analysis using the AF4-UV-MALS-dRI allows the nano particle characterization of the colloidal and macromolecular fractions after the separation with minimal sample preparations, and by using the online detection, it is possible to analyze the sample during the wine manufacturing process, determining specific properties, such as the concentration, apparent density and specific absorptivity, which may help define the nature of the particles present in the colloidal and macromolecular fraction of the grape juice or wine. In addition, AF4 allows for the analysis of wine colloids and macromolecules in their native state with minimal

sample preparation, which means that samples taken at any point of the vinification process can be analyzed with no requirement for additional sample preparation steps.

In this thesis, the AF4 separation technique is proposed as a more appealing method of separation than the standard separation techniques. In addition, a qualitative and quantitative approach, followed by an interpretative analysis of the data, is designed and conducted along with different studies (papers II, III and IV) that complement each other to broadly describe the nature and behavior of the colloidal and macromolecular compounds at various stages of the winemaking process, and in relation to wine properties and oenological parameters. Thereby, the present approach can serve as the basis for future research considering the AF4 technique in relation to the colloidal and macromolecular compounds in wines.

2. Objective

The present study aims to evaluate the suitability of the Asymmetrical Flow Field-Flow Fractionation (AF4) technique as a tool to fractionate the wine particle matter in wines followed by an online specific and macromolecular characterization of the separated fractions.

Specific Objectives

- Characterize bottled red wines in relation to the factors that can affect their phenolic content and possible particle formation, antioxidant activities, and saccharides by using different wines (grape varieties), vintages and origin (Paper I).
- Use commercial bottled wines to develop a methodology of separation for the wine particle matter, applying different parameters such as cross flow (constant, lineal, decaying with variations in the half-life time), detector flow, injection volume, spacer thickness and membrane cut-off (Paper II).
- By using online detection (UV-MALS-dRI) together with the elution profiles (retention times), provide a macromolecular and specific characterization (Molar mass, Hydrodynamic Radii, Apparent density, Concentration, and Specific Absorptivity) of the fractionated populations, identifying the fundamental differences and properties of the colloidal and macromolecular fractions (Paper II).
- Utilize the methodological approach for evaluating the effects and efficiency of the use of different types of clarifiers on the colloidal and macromolecular fraction of a raw white wine during the clarification process, and to correlate with basic oenological parameters, such as turbidity, stability, color, protein concentration, metal content, absorptivity, and phenolic content (Paper III).
- Utilize the methodological approach for monitoring colloidal and macromolecular changes at different stages (fermentation, clarification, bottling and aging) of the red wine vinification process. Identifying distinctive characteristics of the unit operations used during the winemaking process in

relation to the characterization of the wine macromolecular properties, providing knowledge, understanding, and insights into the production line (Paper IV).

3. Method Section

FFF and Asymmetrical Flow Field-Flow Fractionation (AF4)

Field-flow Fractionation (FFF) is a family of high-resolution separation techniques with special application to colloids, particles, and macromolecules. In general, the FFF technique is based on a series of eluting techniques, such as chromatography, but with the difference that it does not utilize a stationary phase for the separation.

The FFF technique was introduced in the 1960s by J.C. Giddings [66]–[68]. The technique has been widely used in many application areas, such as the Environmental Sciences, Pharma and Biotechnology, Food-Agro-Cosmetics, Nanotechnology and Polymer Molecules, due to its ability to separate proteins, polymers and particles.

Years after its introduction, the FFF was modified and evolved into different techniques, i.e., Sedimentation or Gravitational FFF, Thermal FFF, Electric FFF and AF4. AF4 (Asymmetrical Flow Field-Flow Fractionation) is a sub-technique introduced in 1986 [69], [70], which and continued to develop around the early nineties [71]–[75].

AF4 is the most instrumentally developed type of FFF and can be used in combination with different detectors as a powerful method of polymer characterization. In addition, it has several advantages over other FFF techniques; for instance, its extensive applicability in a wide range of sizes (from approximately 10 nm to 50 x 10^3 nm, equivalent to 1 x 10^{-2} µm to 50 µm), gentle separation mechanism, low shear forces, accessibility of fraction collection for further analysis, possibility of using different buffer or carrier solutions similar to the sample analyte conditions and environment being studied.

Separation process, channel, the UV-MALS-dRI detectors and determined properties using AF4

Figure 2 shows the scheme of a channel where the separation process occurs. The gentle separation process is performed according to the differences in the diffusion coefficient of the components, thus the separation happens according to the size of the components, where the smaller molecules are eluted earlier and the larger after.

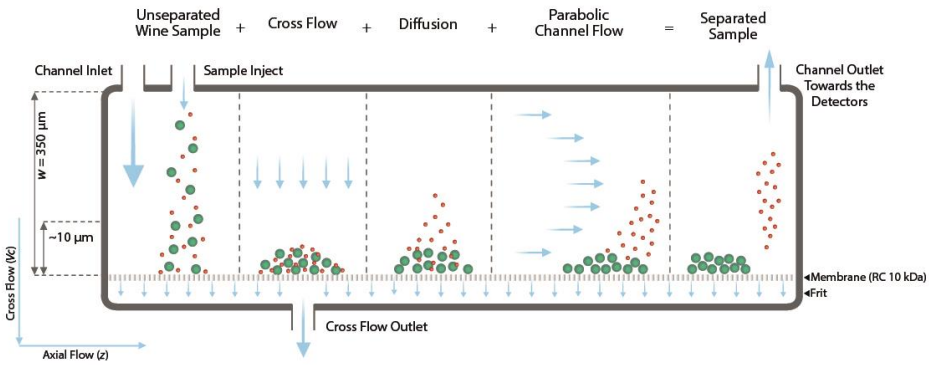

Figure 2. Schematic illustration of the Separation process including the separation steps, the separated compounds in red (•) the wine colloids, in green (●) the wine macromolecules, the axial (z) and perpendicular (Vc) flow direction, the channel thickness (w) and the cut-off of the membrane (modified after Wyatt Technology, 2021).

The separation is achieved in a special open channel with no stationary phase that separates the sample components under the influence of a perpendicular force; a crossflow flow field (*Vc*) that is precisely controlled and goes through the ultrafiltration membrane [70]. This *Vc* field interacts with the sample components and concentrates the sample against the lower channel wall where the ultrafiltration membrane takes place.

Hence, after the sample is injected into the separation channel and as a result of the perpendicular force of the crossflow, the sample is pushed towards the membrane, generating a relaxation of the sample that is automatically counteracted by a diffusional transport by a Brownian motion of the sample analytes. After this relaxation state, the sample has been accurately positioned in the channel for optimum resolution, and when the focus flow is reduced to zero, the sample can be eluted by the main eluent pump.

Consequently, the characteristic retention times after sample separation are based on size and, by using the UV-MALS-dRI, different macromolecular and

physicochemical properties can be determined, such as the Hydrodynamic Radius (r_H), Molar mass (M_W), Apparent density ($\hat{\rho}$), concentration and specific absorptivity ($\mathcal{E}$).

After separation, a fractogram is obtained and the data are treated in the Astra Software (v. 6.1.5.22, Wyatt Technology) to be analyzed and quantified.

UV-MALS-dRI online detectors

In addition to the separation of the colloidal and macromolecular fractions that constitute the wine particle matter, this thesis aims to develop a methodology using online UV-MALS-dRI detection for the macromolecular characterization of its physicochemical properties. Since these colloidal and macromolecular properties differ in composition, they may play a crucial role on diverse wine properties. Table 3 shows some specific properties of the colloidal and macromolecular components in wines.

Table 3. Principal properties of wine colloidal/macromolecular components [39], [40].

Fraction	Compound	Example	Mw	dn/dc	Specific Absorptivity (ε)
			(g/mol) · 10^3	(mL/g)	(mL/mg · 1/cm)
WC	Flavan-3-ols Proanthocyanidins	Epicatechins, Cyanidins	<2	> 0.2	>2
WC/WM	Proteins	LPT, Chitinases, PRs	10< Mw <50	0.18	0.66
WM	Carobohydrate macromolecules	Pectins	>50	0.14	<0.01

WC**,** Refers to Wine colloids.
WM, Refers to Wine macromolecules.

The AF4 coupled with the ultraviolet (UV) detector allows us to monitor the presence of UV absorbing chromophoric species in the separated fractions eluted through the AF4 channel. The wavelength used is 280 nm to monitor the presence of certain types of polyphenols in addition to the presence of proteins. However, since the UV absorbance does not provide the properties, such as concentration, the refractive index, and their specific absorptivity (ε) of the fractionated populations, it is necessary to acquire more information to provide additional valuable data regarding the properties and nature of fractionated populations.

By using the AF4 coupled with Multi Angle Light Scattering (MALS), it is possible to determine the size distribution (M_W) of the fractions present and possibly the shape and structure (r_{rms}) of the colloidal and macromolecular compounds directly, without the necessity for standard curves or calibrations. However, the information obtained using AF4-MALS depends on the relationship between the amount of the light scattering angle over the shape of the scattering molecule and other sample characteristic properties (concentration of the

scattering molecules, *Mw* – average molar mass, *n* – refractive index of the solvent and refractive index increment of the population, among other coefficients and constants).

To be more precise, if the scattering molecule is smaller than approximately <2 nm (or when the wavelength is large or the scattering angle is low) over the whole angular interval, the molecule is considered as an isotropic scatterer [76], indicating that the scatter light is equal in all directions. Thereby, the determination of the shape and structure (r_{rms}) of the colloidal fraction for wine samples is challenging (the r_{rms} is the average of distance from every mass object, monomer unit, to the central gravity mass) in a reproducible manner. This phenomenon can also be associated during the determination of the *Mw* of the wine colloids, where samples with too small r_H cause high fitting errors in the MALS detector, even though the estimation of the *Mw* is less sensitive to size and can be determined with much lower and smaller sizes.

In addition, to provide accurate values of size and molar masses, the MALS detector requires a concentration detector to detect the polymer fraction. Thus, the differential refractive index (dRI) detector is used because of its ability to determine (for light experiments) the refractive index increment of a substance.

Hydrodynamic radii

As an alternative to the r_{rms} measurement, the hydrodynamic radii of the populations are determined. The hydrodynamic radius is calculated using the Stokes-Einstein equation 1 [77]. The values of the hydrodynamic radii are obtained from the elution time by a numerical integration using the retention theory, and were evaluated using MATLAB script as described previously in literature in equation 2 [78]. The MATLAB script is also used for the determination of channel thickness.

$$r_{H,i} = \frac{k_B T}{6 \pi D_i} \quad (1)$$

where k_B is the Boltzmann constant, T temperature in Kelvin, η is the viscosity of the continuous phase, and D_i is the diffusion coefficient. The hydrodynamic radius (r_H) for a perfect sphere is equal to the radius r of the sphere. However, for a non-spherical object (i.e., a polymer, rod, random coil or a hyperbranched structure), the r_H refers to an average comparable to the outer periphery of the body structure.

The diffusion coefficient is obtained from

$$\frac{dz_i}{dt} = k\, V_c\, (t, z) D_i \quad (2)$$

Where z_i is the position of fraxtion *i* along the channel, *t* is the time, *k* is a constant including flow conditions and geometrical parameters, and V_C is the crossflow rate (which is a function of both *t* and *z*). Equation 2 is a simplified version of a detailed differential equation previously proposed [79]. Where the solution for z_i = Z gives $D_i = f(t_i)$ where t_i is the elution time of fraction *i*.

Apparent density

The apparent density provides information on the density and conformation. It can be interpreted as the average concentration of the particle (WC) or polymer (WM) within a spherical shape of the same hydrodynamic radius [80].

Thus, the measurement of the apparent density ($\hat{\rho}$), which considers the hydrodynamic radius, refers to a somewhat spherical object (could be an aggregate) which contains water, but the $\hat{\rho}$ calculation does not consider the water; thereby, the apparent density refers to a non-aqueous material property measurement.

The values of apparent densities are calculated from the molar mass and Hydrodynamic radius (r_H) distributions using homogeneous distribution of mass and a spherical shape. As the radii give only an approximate description of the volume of possible shapes, the density obtained should be considered as an apparent property.

The apparent density, $\hat{\rho}_i$, for component *i* of the sample is calculated from

$$\hat{\rho}_i = \frac{m_i}{v_i\,(r_H)} \qquad (3)$$

where m_i is the molar mass and v_i is the volume of a sphere with hydrodynamic radius r_H.

Figure 3 illustrates the concept of the apparent density in different cases, as it occurs in the wine colloids or wine macromolecules. Figure 3a depicts a case in which the apparent density is equal to the material density, with no solvent inside the sphere. Figure 3b refers to the wine colloids expecting density lower than the material density due to the presence of the solvent within the particle. Figure 3c refers to the wine macromolecular fraction with much lower apparent density with a random coil, branched, hyper branched or even gel-like structure of the interior of the particle (molecule).

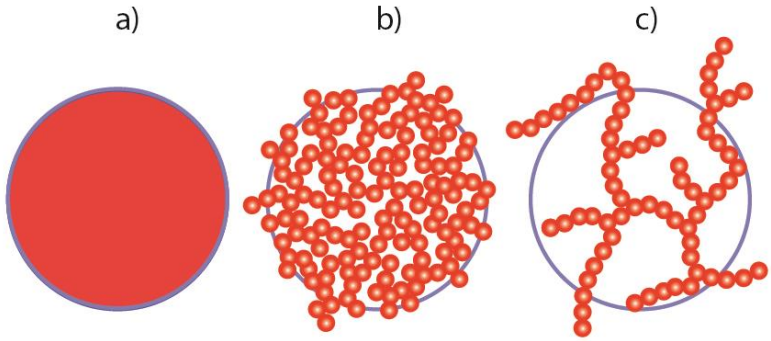

Figure 3. a) Apparent density when it is equal to the material of the sphere $\hat{\rho} = \rho_{material}$, b) Apparent density of the wine colloids $\hat{\rho}_{WC} < \rho_{material}$, and c) Apparent density of the wine macromolecules $\hat{\rho}_{WM} \ll \rho_{material}$.

In order to compare the apparent densities found in wines, table 4 shows some examples of apparent density values in different foods obtained from distinct characterizations, using AF4.

Table 4. Apparent densities based on the r_{rms} obtained by AF4 in different proteins and starch in foods. Modified after Chen et al [64].

Sample	$\hat{\rho}$ kg/m³ Range	Sample	$\hat{\rho}$ kg/m³
Casein Micelles	300-400	Potato Starch	4.2
Gum arabic*	310	Maize Starch	2.8 - 42
Mesquite Gum*	230	Cassava Starch	5.1

* Calculations based on the r_H.

Concentration

The concentration determination of the material present in the fractions is made by using the dRI peak area and *dn/dc* of the respective populations *j* in the AF4 fractograms. The absolute concentration is calculated with the following equation.

$$C_j = \frac{A_j \cdot F_{out}}{V_{inj} \cdot (\frac{dn}{dc})_j} \qquad (4)$$

Where A_J is the dRI area of the population *j*, F_{out} is the detector flow equal to the exit flow of the channel, V_{inj} is the injection volume and the $(\frac{dn}{dc})_j$ value is the characteristic value for each population *j*. The specific $(\frac{dn}{dc})_j$ needs to be obtained or calculated in advance.

Specific absorptivity

The specific absorptivity refers to the molar extinction coefficient (ε) per unit path length, expressed in cm, and per unit of mass concentration, mg, which is a measure of how strongly the chemical species present in the population or peak *j* absorbs UV light at 280 nm.

The determination of the (ε) is made by using a deduced equation from the AF4 fractograms considering the UV intensity of the peak *j* compared with the intensity of the dRI signal of the same peak *j* and using as a comparison reference the same UV/dRI ratios of a BSA standard. The BSA standard is the same as previously used during calibration of the UV signal and channel thickness.

$$\varepsilon_{(j)} = \frac{I_{UV,j}}{I_{UV,\ BSA}} \cdot \varepsilon_{BSA} \cdot \frac{\frac{I_{BSA}}{(\frac{dn}{dc})_{BSA}}}{\frac{I_{dRI,j}}{(\frac{dn}{dc})_j}} \qquad (5)$$

where $I_{UV,j}$ is the UV intensity of the peak *j*, $I_{UV,BSA}$ is the UV intensity of the BSA, ε_{BSA} is 0.66 [mL/mg · 1/cm], $I_{dRI,j}$ is the dRI intensity of the same peak *j*, $I_{dRI,BSA}$ is the dRI intensity of the BSA peak, and the $(\frac{dn}{dc})$ is the corresponding value according to the BSA standard and peak *j*.

dn/dc determination and dialysis experiment

The *dn/dc* is the refractive index increment with increasing concentration of a solution. The property has a temperature, wavelength and solvent dependence.

The *dn/dc* value is a required parameter for the Mw estimation and the concentration of the populations using the MALS and dRI detector, respectively. Subsequently, the dn/dc value affects the quantifications of the apparent density ($\hat{\rho}$), specific absorptivity (ε) properties.

Depending on the sample to be analyzed, the *dn/dc* value can be found listed for some common pure materials or can be determined experimentally by using pure reference materials. For instance, proteins can be estimated based on the amino acid composition [81] or by using online determinations if the protein concentrations are accurately measured [82]–[84]. However, the wine colloids are compounded materials without proper reference material, thus, a practical approach had to be developed in order to obtain a relevant value.

Therefore, the determination of the refractive index, *n*, of the total solids of the retentate from the dialysis experiments allows the determination for the $(dn/dc)_{TS}$ of the total macromolecular and colloidal solids.

It is assumed that *dn/dc*, the total solids of the colloidal and macromolecular fraction, is a mass weighted average of the *dn/dc* of the species in the solution:

$$\sum_{i=1}^{j} \left(\frac{dn}{dc}\right)_i \cdot c_i \,/ \sum_{i=1}^{j} c_i = \left(\frac{dn}{dc}\right)_{TS} \quad (6)$$

Assuming only two species, wine colloids and wine macromolecules, it can be rewritten as:

$$(dn/dc)_{WC} \cdot c_{WC} + (dn/dc)_{WM} \cdot c_{WM} = (dn/dc)_{TS} \cdot c_{TS} \quad (7)$$

Using the mass balance:

$$c_{WC} + c_{WM} = c_{TS} \quad (8)$$

Obtaining the following result:

$$(dn/dc)_{WC} \cdot c_{WC} + (dn/dc)_{WM} \cdot c_{WM} = (dn/dc)_{TS} \cdot (c_{WC} + c_{WM}) \quad (9)$$

Then using the relations to the dRI areas of the peaks:

$$c_{WC} = \frac{A_{WC}}{(dn/dc)_{WC}} \text{ and } c_{WM} = \frac{A_{WM}}{(dn/dc)_{WM}} \quad (10)$$

That gives:

$$A_{WC} + A_{WM} = (dn/dc)_{TS} \cdot \left(\frac{A_{WC}}{(dn/dc)_{WC}} + \frac{A_{WM}}{(dn/dc)_{WM}}\right) \quad (11)$$

AWC and AWM are obtained from the AF4; we assume that $(dn/dc)_{WM}$ is known and we measure $(dn/dc)_{TS}$ using a dialysis technique. Thus, we may determine $(dn/dc)_{WC}$ as:

$$(dn/dc)_{WC} = \frac{A_{WC}\,(dn/dc)_{TS} \cdot (dn/dc)_{WM}}{(A_{WC}+A_{WM}) \cdot (dn/dc)_{WM} - A_{WM}\,(dn/dc)_{TS}} \quad (12)$$

However, this solution is unstable as $(A_{WC} + A_{WM}) \cdot (dn/dc)_{WM}$ may be smaller than $A_{WM}\,(dn/dc)_{TS}$. This could be because the experimentally observed $(dn/dc)_{TS}$ is too high, possibly due to phase separation after the re-dissolution or incomplete dissolution. It could also be because the assumed $(dn/dc)_{WM}$ is too low, as the macromolecular fraction contains a fraction of protein.

To make the function less sensitive to possible errors, we applied a quadratic weighting factor of the $\left(\frac{dn}{dc}\right)_i$ in equation (6) reducing the sensitivity to experimental errors in the determination of $\left(\frac{dn}{dc}\right)_{TS}$ and the assumption errors of $\left(\frac{dn}{dc}\right)_{WM}$. Hence, the equation (6) becomes:

$$\frac{\sum_{i=1}^{j}\left(\frac{dn}{dc}\right)_i \cdot \left(\frac{dn}{dc}\right)_i^2 \cdot c_i}{\sum_{i=1}^{j}\left(\frac{dn}{dc}\right)_i^2 c_i} = \left(\frac{dn}{dc}\right)_{TS} \qquad (13)$$

The solution can then be obtained as:

$$\left(\frac{dn}{dc}\right)_{WC} = \frac{\left(\frac{dn}{dc}\right)_{TS}}{2} + \sqrt{\frac{\left(\frac{dn}{dc}\right)_{TS}^2}{4} - \frac{A_{WM}}{A_{WC}}\left(\frac{dn}{dc}\right)_{WM}^2 + \frac{A_{WM}}{A_{WC}}\left(\frac{dn}{dc}\right)_{TS}\left(\frac{dn}{dc}\right)_{WM}} \qquad (14)$$

This solution is stable as long as $\frac{\left(\frac{dn}{dc}\right)_{TS}^2}{4} + \frac{A_{WM}}{A_{WC}}\left(\frac{dn}{dc}\right)_{TS}\left(\frac{dn}{dc}\right)_{WM}$ is larger than $\frac{A_{WM}}{A_{WC}}\left(\frac{dn}{dc}\right)_{WM}^2$. This condition is satisfied as $\left(\frac{dn}{dc}\right)_{TS}$ in all cases is larger than $\left(\frac{dn}{dc}\right)_{WM}$.

The refractive index, n_{TS}, of the total solids can be determined through a dialysis experiment under similar conditions carried out in the AF4 experiment (model wine carrier with 13% ethanol, 20 mM tartaric acid and pH 3.6). These results allow the determination of the refractive index increase $(dn/dc)_{TS}$ of the total solids, using the formula (14) as explained above.

During the dialysis experiment, samples showed differences in the solubility of polyphenolic compounds since some samples release more color than others through dialysis tubes (see Paper II, table 7 for more details). Figure 4 shows an example of the case.

Figure 4. Release of phenolic/anthocyanin compounds in the dialysis experiments.

Thus, the dialysis experiment purified and concentrated the soluble compounds over a period of four days, for which the same carrier liquid from the AF4 experiments was used. The dialysis tubes used were with 3.5 g/mol MWCO (Spectra/Por of Roth, Karlsruhe, Germany). Furthermore, for two more days, the same experiment was carried out using deionized water instead of the carrier liquid AF4. The fluid was changed twice a day. Soon after, the samples were frozen at -20 °C in a layer 1-2 cm thick and lyophilized (Epsilon 2-6D LSC plus, Osterode, Germany) for three days (see Figure 5).

Figure 5. Freeze dried solids from the retante of the dialysis experiment.

Thereafter, the refractive index of the total solids from the retentate of the dialysis and lyophilization was measured in the AF4 carrier liquid using a digital refractometer (Hanna Instrument, HI 96801, Woonsocket RI, USA).

Recovery calculation

The values of the Recovery calculation can be evaluated using different methods.

1. By comparison of the UV area in the fractograms with and without crossflow. However, the results values can be affected by the solubility of the UV active material with lower molar mass.

2. By considering the total area analyzed, relating the dRI area fraction of each population with the total dRI area.

3. By calculating the relation between the total concentrations obtained with the AF4-dRI with the quantitative material from the dialysis experiment.

Additional Offline Assays Performed

Protein determination

Nitrogen is the most distinguished element present in proteins. Thus, the nitrogen content was analyzed by nitrogen combustion with the Dumas method 32, using a nitrogen analyzer (Flash E.A., 1112 Series, Thermo Electron Corp., Waltham, MA, USA). A correction factor of N × 6.25 was used for the protein quantification. Aspartic acid was used as a reference. Approximately 40 mg of freeze-dried sample coming from the dialysis experiment was weighed into a tin capsule and introduced to a combustion reaction in automated equipment. Nitrogen oxides formed during the oxidation were reduced to N_2 and determined by gas chromatography.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

In this thesis, RP-HPLC is used to identify the presence of phenolic compounds (phenolic acids, flavonoids, etc.) which constitute parts of the building blocks of wine polymeric material.

Only the colloidal fraction separated from the AF4 has been prepared for HPLC analysis. Prior to the HPLC injection, the sample is concentrated and refluxed at 90° C in acidic media for 1 hr to hydrolyze the glycosidic bonds from the tannin complexes.

The HPLC system used (Agilent Technologies 1260 Infinity II, Palo Alto, CA, U.S.A.) is equipped with a quaternary pump with an auto injector and degasser (G1311C), a column oven set at 25 °C, and a diode array detector (DAD, G1315D). The elution of phenolic and anthocyanin compounds was monitored at wavelengths of 280 and 530 nm, respectively. The column was a 3.0 mm × 100 mm × 2.7 μm Halo C18 reversed-phase column (Hichrom, Wilmington, DE, U.S.A.). The separation method was a modification from a method described previously in literature [85]. The flow rate was 0.6 mL/min, and the injection volume was 20 μL. The mobile phase consisted of two eluents: (A) 0.1% formic acid/water and (B) methanol. The gradient was achieved as follows: 25% B at 0 min, 90% B after 10 min, 25% B after 16 min, and 95% B constant for 4 min to reach 20 min. The identification of the peaks in the resulting HPLC chromatogram is made by comparisons to the retention time of external standards and their corresponding UV spectra.

Furthermore, RP-HPLC was also used for sugar determinations (saccharose, glucose, and fructose) in the total wine. The wine samples were centrifuged at

16000× g for 5 min, filtered using 0.20 µm sterile filters, and acidified (5 mM H_2SO_4). The HPLC system used was a Shimadzu Corp. Prominence (Kyoto, Japan), equipped with a system controller CBM-20A, a solvent delivery unit LC-30AD, a refractive index detector RID-20A, a column oven CTO-10 ASVP, an online degassing unit DGU-20A, and an autosampler SIL-30AC. The column was an Aminex HPX-87H column (Bio-Rad laboratories, Hercules, CA, USA). The separation method consisted of an eluent of 5 mM H_2SO_4 at 0.6 mL/min at 65 °C [86].

High-Performance Anion-Exchange Chromatography (HPAEC)

HPAEC was used for monosaccharide identification in the colloidal and macromolecular fractions of the wine samples from the AF4. Similarly, the sample was collected from the AF4 separation and was concentrated and hydrolyzed according to the standardized method previously reported [87]. The HPAEC system was an ICS-3000 chromatography system (Dionex Corp., Sunnyvale, CA, U.S.A.). The separation method used water as an eluent at 1 mL/min.

Total Phenols (TPH), Total Flavonoids (TF) and Total Antioxidant Capacity (TAC)

The Folin–Ciocalteu assay for determining total phenols (TPH) is one of the oldest methods designed for this purpose [88]. In this assay, oxidation of phenols by a phosphotungstate molybdate reagent yields a colored product with absorbance near 750 nm. The results are expressed in gallic acid equivalents (see papers I and II for details).

The TF content was determined using a reagent containing aluminum chloride and sodium nitrite, giving rise to a pink-colored flavonoid–aluminum complex in the alkaline medium (Zhishen et al., 1999). A solution corresponding to 30 µL of sodium nitrite (10%), 60 µL of aluminum chloride hexahydrate (20%), 200 µL of NaOH (1 M) and 400 µL of water was added to 100 µL sample. The absorbance readings at 510 nm were started 5 min after the addition of the sample, and were performed every 20 s for 1 min. A reagent blank containing water instead of sample was used. The final absorbance of each sample was compared with a standard curve made from catechin (69–689 µmol/L). The data were expressed as mmol catechin equivalents (CE) per gram of litter of wine.

The total antioxidant capacity (TAC) was evaluated using the ABTS and FRAP methods, which are based on Electron Transfer Reactions.

The colorless ABTS (7 mmol/L) was oxidized to the green $ABTS^{\bullet+}$ radical cation by the addition of potassium persulphate (2.42 mmol/L) and kept for 12–16 h at room temperature in the dark. The reagent was stable for 2–3 days when stored in

this way. On the day of analysis, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. After the addition of 1.0 mL of $ABTS^{\bullet+}$ solution to 100 µL of sample, the mixture was stirred for 30 s and the absorbance at 734 nm and 25 °C was recorded for 6 min. The decrease in absorbance caused by the addition of sample was compared with that of a standard curve made by use of Trolox (20–200 µmol/L).

The FRAP test is a typical single electron transfer (SET) based method, measuring the reduction of the complex of ferric ions (Fe^{3+})-ligand to the intensely blue ferrous complex (Fe^{2+}) by means of antioxidants in acid environments. Antioxidant activity is determined by colorimetry as an increase in absorbance at 593 nm. The results are expressed in mmol Trolox equivalents per liter of wine (mmol/L) (see Paper I for details).

4. Results

Results Section: Paper I

The characterization of phenolic compounds, antioxidant activity and the main saccharide compounds present in 35 South American red wines was carried out to provide data on their particular characteristic composition and antioxidant capacity, in addition to evaluating the relationship that high altitude may have over these compounds, precisely over the polyphenolic compounds (Paper I). In addition, the results may provide interest in the characterization of colloidal and macromolecular compounds, evaluating the influence that the phenolic content may have over the particle formation in high altitude wines rich in polyphenols, for which alternative techniques (AF4-UV-MALS-dRI) will be used and evaluated using bottled wines and/or the juice wine during the manufacturing process (Papers II, III, IV).

Wine samples

In this study, 35 different commercially available red wines from Argentina, Bolivia, Chile and Uruguay were selected (see table 1 of Paper I for the selected list of South American wines according to their variety, origin, vintage, and altitude).

Total Antioxidant Capacity (TAC), Total Phenols (TPH) and Total Flavonoids (TF) determination

The TAC determination was measured using the conventional ABTS and FRAP methods. Through the ABTS method, higher values were found in the Bolivian red wines, followed by Chilean, Uruguayan and Argentinean red wines. The range of results are in accordance with data previously reported [89], [90].

The FRAP analysis results showed a somewhat similar trend found by the ABTS, possibly due to the same chemical principles that direct the analysis (see full results in Paper I).

The total phenols (TPH) and total flavonoids (TF) analysis determined using Folin–Ciocalteu reagent are expressed in Gallic Acid equivalents (mmol or mg GAE/L) for the TF, and in mmol/L catechin equivalents per liter of wine (mmol CE/L), respectively.

The total average results showed values that varied from 9 to 20 mmol/L for TPH, and from 2 to 5 mmol/L for TF. These results showed a somewhat similar range of results. However, wine samples from countries in an area with higher altitudes above sea level showed higher content of all these variables and properties.

Resveratrol

For the identification of trans-resveratrol, the reversed phase HPLC technique was used. The retention time was 11.5 minutes (Figure 6), and by comparison with the standard (20 ppm), absorption bands between 210 and 250 nm and between 250 and 360 nm in the UV spectra.

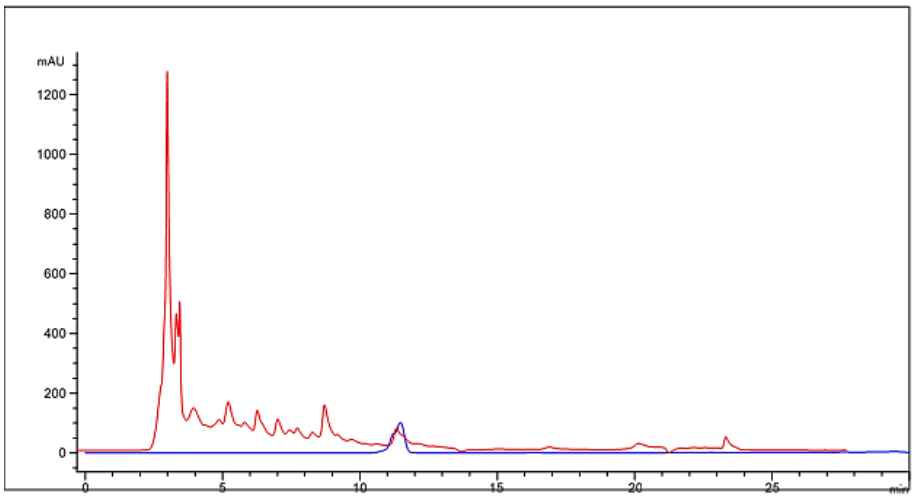

Figure 6. HPLC chromatogram for the identification of trans-resveratrol comparing the trans-resveratrol standard 20 ppm (blue line) with red wine sample BW11 (red line). From Paper II.

The general results showed the trans-resveratrol content ranged 0.1 to 5.0 mg/L in Argentinian red wines, 0.4 to 7.9 mg/L in Bolivian, 0.1 to 2.7 mg/L in Chilean, and 0.1 to 1.7 mg/L in Uruguayan red wines. The highest value was found in the Bolivian Cabernet Sauvignon wines by Campos del Solana 2012 with 7.9 mg/L. The average mean concentration was 2.0 mg/L. However, for many wine samples, the resveratrol content was below the limit of detection (see the result details in Paper I, table 2).

Determination of saccharides (saccharose, fructose, and glucose)

The saccharide composition was determined using HPLC. Figure 7 shows the chromatogram of the sample AW1 and the standard of 0.5 g/L. The highest values were found in Argentinian non-varietal wine (AW1) with 12 g/L for fructose and 8.6 g/L for glucose. The highest value for saccharose content was found in the Argentinian Cabernet Sauvignon (AW2) wine sample. In contrast, the lowest value for fructose was found in the AW6 sample, with 0.24 g/L; for glucose in CW1, with 1.4 g/L; and for saccharose in BW16, with 0.38 g/L.

It is possible to notice that the saccharose content is rather high for some wines; up to about 10 g/L. These high levels of saccharose indicate a possible late dosage of saccharose to the wine, while the high values of fructose noticed may be due to an early stopping of the fermentation. (Results of the determination of sugars for all samples are listed in Paper I, table 3.)

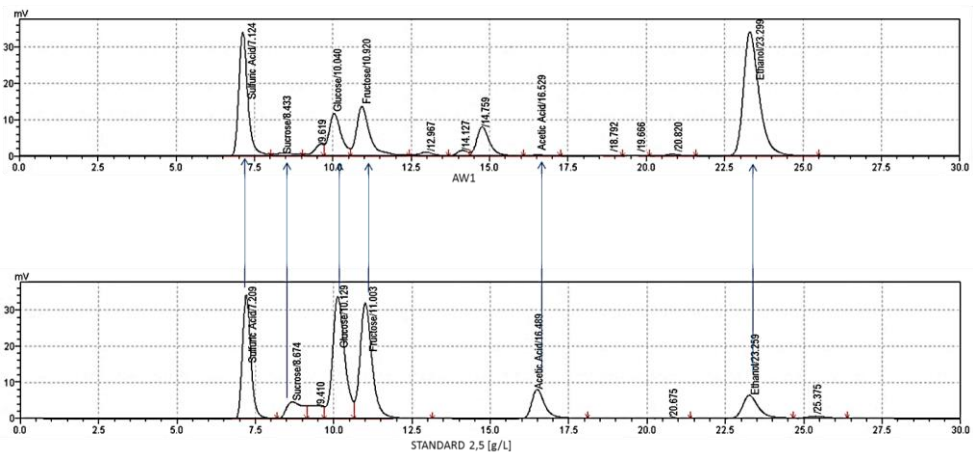

Figure 7. HPLC chromatogram for the all the saccharides elution profile. From Paper II.

In order to correlate all the data found, a Principal Component Analysis (PCA) was performed. Figure 8 shows different clusters with the principal variables and parameters. In the lower right quadrant, a cluster shaped by ABTS, FRAP, altitude, resveratrol and total flavonoids correlates positively. On the other hand, the opposite direction correlates total phenols, saccharose and fructose. It is interesting to notice that the total phenols (TPH) appear opposite distant to the total flavonoids (TF), possibly the total flavonoids (catechins, etc.) come from the grapes and seeds in which their structures show antioxidant activities, while for the total phenols the phenolic compounds may be originates from the oak (phenolic acids), which tends to precipitate along with the tannins and proteins during the aging process.

The results of the chemical characterization of the selected South American wines showed that altitude is an important factor that can affect some properties of the wine. Thus, a direct relationship between the high altitude of the vineyards and the high content of resveratrol, TAC and TF was observed. These results agree with other studies that related UV radiation with the increase of phenolic compounds that show antioxidant activity [91]. In addition, it was noted that the aging (vintage) does not have a direct impact on the phenolic content (TPH). However, the comparison of the phenolic content between different wine samples depends on different factors such as the variety of the grape, the winemaking techniques, type of soils, environmental conditions, UV radiation and type of aging.

All these results have yielded valuable information on the characteristic composition of South American red wines, as well as describing how some properties, such as phenolic content, can be affected by different geographical factors, such as the altitude above sea level of the vineyards.

On the other hand, these results are of potential interest for future studies that might want to consider samples with high phenolic content and the relationship to colloidal particle formation, in addition to its possible relationship with various wine properties.

Furthermore, the results might be interesting for studies that involve analytical applications regarding the characterization of these colloidal compounds. These phenolic aggregates play an important role, both in the structure and in physicochemical properties, such as stability (polymerization and precipitation), thus altering changes in macromolecular properties, with possible consequences on the sensory properties and quality of the wine.

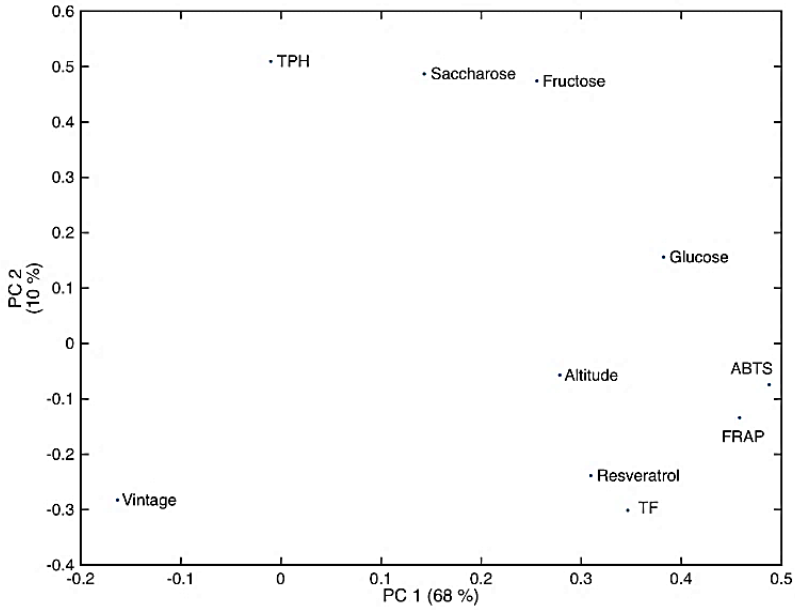

Figure 8. PCA plot representing the variables in study, ABTS, FRAP, Altitude, Resveratrol, Total Flavonoids, Total Phenols, Glucose, Saccharose, Fructose and Vintage. From Paper II.

Developing the separation and characterization methodology for the wine particle matter using the AF4 coupled with online detectors (Paper II)

The separation and characterization of the colloidal and macromolecular matter that constitutes the complex aggregates in wines is an important key factor, allowing the determination of different macromolecular properties related to different physicochemical properties in bottled wines.

The AF4 technique has been used as a method for fractionation and characterization of colloidal particles and macromolecules [57]. Recently, the AF4 technique has also been used for food macromolecules and food beverages [58], [59]. Moreover, it has been suggested as a promising technique in the evaluation of the separation and size of oxidized tannins in a wine-like solvent carrier model [60] using the AF4 technique, coupled with a multidetector system (online detectors UV-MALS-dRI), it is intended to establish a method for separation and for macromolecular characterization (M_W, r_H, $\hat{\rho}$, Concentration (c), Specific Absorptivity (ε)) of the particle properties of the fractionated populations.

In addition, it is necessary to develop a rapid separation and characterization methodology that can be applied at different stages of the vinification process and to a large number of samples, thus providing interesting new data and insights into the changes that occur in properties at the macromolecular level (Papers III and IV).

Samples

For this purpose, and by following the results in Paper I considering a possible high particle formation in wines with high phenolic content, six different high altitude Argentinian red wines samples were chosen. Specifically, samples from vineyards located around 2,000 meters above sea level (m.a.s.l.), with the analysis performed during 2018. Table 5 shows the main characteristics of the collected samples.

Table 5. List of grape variety, origin, vintage, and altitude of wines in this study. (From Paper II, Table I).

No.	Code	Sample	Grape Variety	Region	Origin	Altitude[a]	Vintage
1	CAB	Domingo Molina	Cabernet Sauvignon	Salta	Argentina	2200	2013
2	MAL-1	Quebrada de las Flechas	Malbec	Salta	Argentina	1900	2015
3	MAL-2	El Tapao del Cese	Malbec	Salta	Argentina	1920	2016
4	MALCAB-1	San Pedro de Yacochua	80% Malbec, 20% Cabernet	Salta	Argentina	2035	2013
5	MALCAB-2	Tapadito	Malbec 70%, Cabernet 30%	Salta	Argentina	1920	2012
6	TANN	Coquena	Tannat	Salta	Argentina	2042	2015

[a] The altitude is given in meters above sea level (m.a.s.l.).

AF4 separation

After the AF4-UV-MALS-dRI fractionation, it is possible to notice different types of particles and macromolecular matter. The fractograms in figure 9, according to the different detectors (signals), depict the presence of two principal populations despite the fact that, depending on the wine sample, these two populations can vary in intensities and shape. However, there is a clear trend in that the first population shows active material that is UV absorbing, while the second population has no UV absorbing material.

For instance, the fractogram corresponding to figure 9a (CAB sample) is characterized by high UV-MALS and dRI signals, indicating a higher content of the UV shorter material. The fractograms corresponding to figures 9b (MAL-2), show intermediate UV intensities in the shorter fraction, which may suggest less concentration of UV absorbing material, while figure 9c (MALCAB-2) shows very low UV intensity, indicating a very low number of particles that absorb UV light. However, different concentrations of both populations are estimated with the dRI signal. In order to describe more accurately the nature of these populations, a macromolecular characterization of the properties by the UV-MALS-dRI signals, such as the Molar mass (M_W), Hydrodynamic radii (r_H), apparent density ($\hat{\rho}$), concentration and specific absorptivity ($\mathcal{E}$) is provided in table 6. The values of these properties were calculated according to the equations (1 to 5) previously described in the Method section.

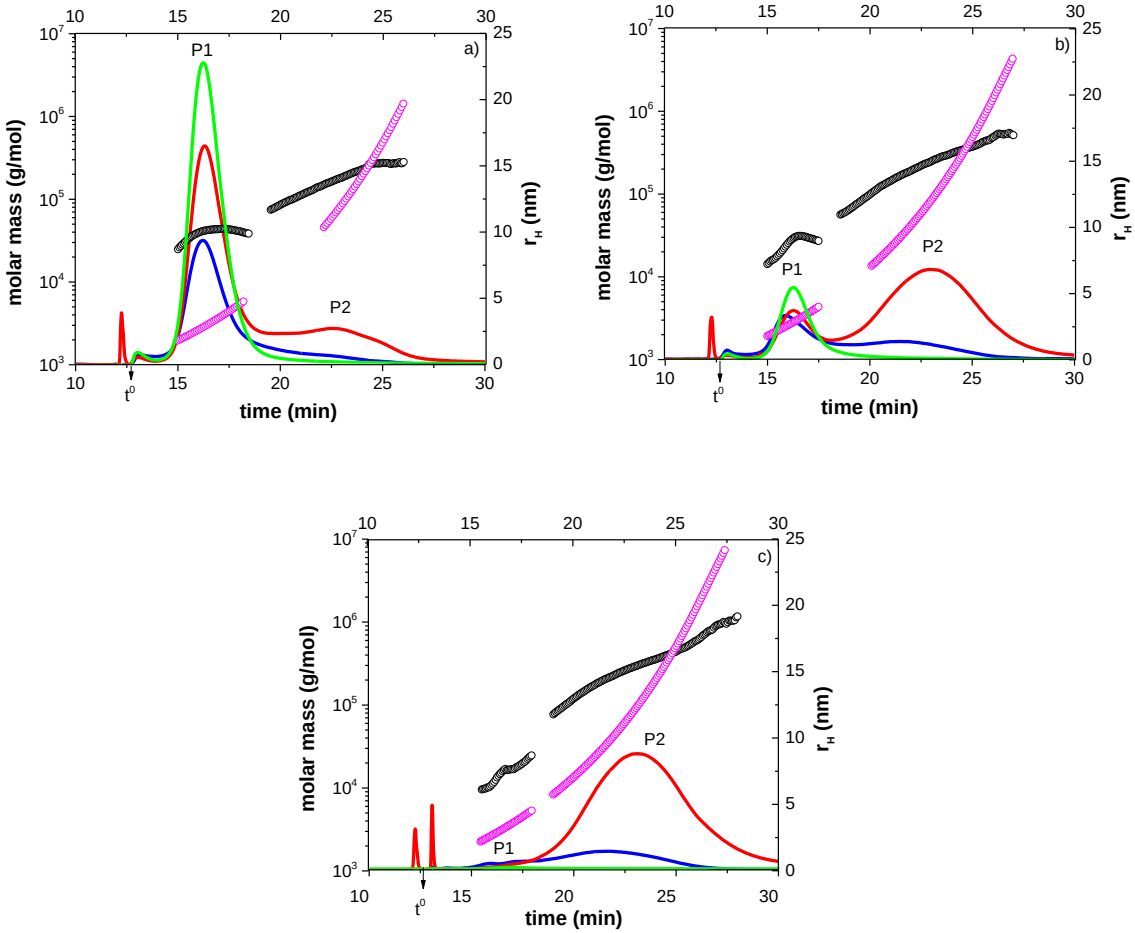

Figure 9. Fractograms from AsFlFFF showing the molar mass (Mw) determined by MALS-dRI detection (o), hydrodynamic radius (o), a) CAB b) MAL-2 and c) MALCAB-2. In red line response form the Multi Angle Light Scatering (MALS), in green line 280 nm UV detector and in blue line refractive index (dRI) detector. From Paper II.

Table 6. Average values for the macromolecular and specific characterization obtained from the AF4-UV-MALS-dRI. (From paper II, tables 3 and 5).

Code	Hydrodynamic Radii[a] r_H (nm)		Molar Mass[b] M_W (g/mol) · 10^3		Apparent Density[c] $\hat{\rho}$ (kg/m^3)		Specific Absorptivity[d] ε (mL/mg · 1/cm)	
Population	**P1**	**P2**	**P1**	**P2**	**P1**	**P2**	**P1**	**P2**
CAB	2.9	11.2	47.1 (0.7)	157 (0.4)	885	34.0	7.15	0.69
MAL-1	2.7	13.1	22.2 (1.9)	277 (0.4)	469	89.6	1.90	0.41
MAL-2	2.8	11.6	25.1 (1.2)	200 (0.4)	539	62.6	5.66	0.50
MALCAB-1	2.8	11.5	44 (1.1)	252 (0.4)	1246	103	7.41	0.40
MALCAB-2	3.5	13	24.5 (2.2)	303 (0.4)	397	64.3	0.78	0.05
TANN	2.9	10.4	26.3 (1.1)	205 (0.4)	551	77.8	6.75	0.47

[a] Hydrodynamic radii, r_H, is estimated at the peak mode from the MALS fractogram.
[b] M_W is the weight-average molar mass from the MALS distribution, and the fitting error is in parentheses.
[c] $\hat{\rho}$ is the apparent density. Calculations are based on r_H and M_W.
[d] is the specific absorptivity based on eq 5.

The results in table 6 in general show that the population 1 is shorter, with low molar mass but higher apparent density and specific absorptivity, suggesting a smaller and denser particle with high absorptivity structure. Meanwhile, the population 2 is approximately 4 times larger with 7 times greater molar mass, is 10 times less dense, and with fairly low specific absorptivity. These characteristic results from the AF4-UV-MALS-dRI show that each population differs somewhat in nature.

Additionally, gravimetric experiments were performed using dialysis to aid the parallel quantification of the amount of the macromolecular and colloidal material present in the different wine samples. Consequently, the specific refractive index increment, $(dn/dc)_{TS}$, for the total solids from the retentate of the dialysis experiment was calculated. As a result, the particular refractive index of the colloidal fraction, $(dn/dc)_{WC}$, value was obtained. Furthermore, the total and individual concentrations of the populations determined with the dRI signal normalized to the *dn/dc* are determined. Tables 7 and 8 summarize these results that complement the wine properties of the soluble and retained fractions. In addition to the total protein values, the total phenols in the retentate (colloidal fraction) and in the soluble fraction (wine) refer in part to the concentration reported in each of the fractions. Table 8 estimates the relationship between both soluble and retained fractions.

Table 7. Values of the refract index increment for the total solids $(dn/dc)_{TS}$ and wine colloids $(dn/dc)_{WC}$ and concentration.
(From paper II, table 5).

			Absolute Concentration[c]		Total Concentration[d]
CODE	$(dn/dc)_{TS}$[a] mL/g	$(dn/dc)_{WC}$[b] mL/g	WC mL/mg	WM mL/mg	WC+WM mL/mg
CAB	0.204	0.212	0.88	0.26	1.14
MAL-1	0.158	0.187	0.18	0.53	0.71
MAL-2	0.200	0.246	0.23	0.53	0.76
MALCAB-1	0.181	0.219	0.40	0.92	1.32
MALCAB-2	0.163	0.251	0.04	0.46	0.50
TANN	0.203	0.245	0.44	0.91	1.34

[a] dn/dc of the total solids present in wine determined by a digital refractometer.
[b] dn/dc refers to the wine colloid refractive index calculated from eq. 14
[c] Absolute concentration of the material in the populations according to eq. 4
[d] Total concentration is the sum of peak 1 and peak 2 in milligrams per milliliter.

Table 8. Total Phenols and Absorbance from the Soluble and Retentate Fractions from the Dialysis Experiment.
(From paper II, table 7).

Code	TPH Wine[a] mg/mL	TPH retained[b] mg/mL	Dialysate fraction[c] %	Retained Fraction[d] %	Total Abs[e] wine	Total Abs Released[f]	Soluble Fraction[g] %	Retained Fraction[h] %	Protein Content mg/mL
CAB	2.44	0.83	66.0	34.0	39.60	27.74	70.0	30.0	0.107
MAL-1	1.92	0.25	87.2	12.8	28.60	24.16	84.5	15.5	0.194
MAL-2	2.72	0.64	76.6	23.4	36.80	24.93	67.7	32.3	0.083
MALCAB-1	2.28	0.91	60.2	39.8	42.80	24.76	57.8	42.2	0.111
MALCAB-2	1.74	0.12	93.0	7.0	34.40	23.97	69.7	30.3	0.085
TANN	2.82	1.19	57.8	42.2	48.10	30.87	64.2	35.8	0.099

[a] Expressed as milligrams of Gallic acid equivalents (GAE) per milliliter of original wine.
[b] Refers to the portion of the total phenols that was not dialyzable and remains present inside the membranes.
[c] Dialysate fraction refers to the percentage of total phenols that was released during the dialysis experiment.
[d] Retentate fraction refers to the percentage of phenols that were retained after dialysis.
[e] Total absorbance of the wine samples at 280 nm.
[f] Total absorbance of the dialysate accumulated after 4 days of dialysis.
[g] Fraction of the total absorbance present in the dialysate in percentage.
[h] Retained fraction of total absorbance in percentage.

The results in table 8 show that the different samples present different ratios of the phenolic fraction that is retained or soluble. The soluble fraction ranged from 58% TANN to 93% for MALCAB-2, which suggests that the MALCAB-2 sample is extremely soluble. This can be supported by the AF4 fractograms 2c results, where the UV/dRI signal was depreciable.

Qualitative identification of the phenolic compounds using RP-HPLC and sugar monosaccharides using HP-AEC

Before the phenolic determination by RP-HPLC, the population-1 of the CAB sample was previously concentrated. After the analysis, the resulting chromatogram showed the presence of anthocyanins and phenolic compounds detected at 530 and 280 nm, respectively. By comparison of the retention time and the corresponding UV Spectra to external standards, the following compounds were identified: Delphinidin, Cyanidin and Malvidin as main anthocyanins, *p*-coumaric acid, and ellagic acid (See Paper II and figure 2).

The monosaccharide composition of the CAB samples was determined for both populations (wine colloids and wine macromolecules). After the concentration of the sample and by comparison with external standards, six peaks were identified in both populations in different ratios: arabinose, rhamnose, galactose, glucose, xylose and mannose. Table 9 shows the relative proportions and the total monosaccharide content in mg/mL.

Higher concentration of the total monosaccharides is found in the second population, WM, with 0.26 mg/mL, dominated by galactose and arabinose, followed by mannose. In contrast, the total concertation for the first population, WC, is 5 times lower with 0.05 mg/mL, with prevalence of glucose.

Table. 9 Relative concentration of the monosaccharides after hydrolysis.
(From Paper II, table 8).

CAB population	Arabinose (%)	Rhamnose (%)	Galactose (%)	Glucose (%)	Xylose (%)	Mannose (%)	Glucoronic Acid (%)	Total Peak[a] Content (%)	Total[b] (mg/mL)
WC	11	18	21	36	< 1	14	ND	5.0	0.05
WM	31	0.8	42	2	ND	20	ND	100.0	0.26

[a] Percentage of the polysaccharide in the colloidal and macromolecular fraction by comparison to the absolute concentration of the wine colloids and wine macromolecules, respectively (See table 4).
[b] Total value found in the fraction by HPAEC in milligrams per millilitre.

The results, using HP-HPLC and HP-AEC, confirm that the wine colloidal material is formed by phenolic compounds, while the wine macromolecular fractions are mainly composed of polysaccharides; this agrees with the concept already indicated by using the UV signal and the specific absorptivity (ε).

Another approach when using the AF4 methodology is to correlate how the specific absorptivity ε, determined by the UV/dRI signals, show a direct relationship with the retained total phenols obtained in the dialysis experiment (see figure 10a). Furthermore, the total concentration (understood as the sum of WC and WM populations), calculated with the dRI area of the respective populations, is related to the retained total solids of the dialysis experiments (see figure 10b). However, the retained solids obtained by the dialysis experiment are higher in comparison with the data obtained as total solids in the colloidal and macromolecular fraction by the dRI signal using the AF4. The greater amount of the solids found using the dialysis experiments may be due to several factors that still needed to be addressed and evaluated.

Thereby, it can be concluded that the results obtained with AF4-UV-MALS-dRI, after allowing an optimal separation, permit full characterization of the properties of these colloidal and macromolecular fractions. In addition, it can be noted that the nature of these fractions varies, which may be due to the particular type of grape, and to the different processes used during the winemaking.

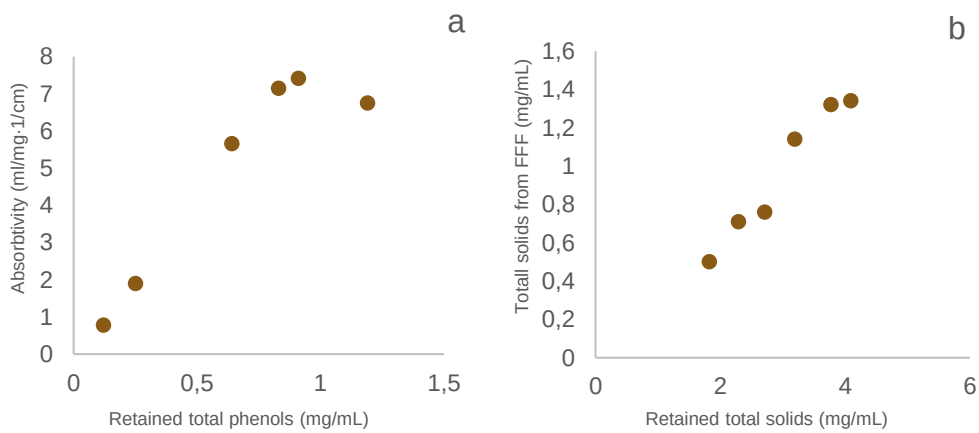

Figure 10. a) Absorptivity of the wine colloid fraction from the AF4 as a function of the retained total phenols from the dialyses experiment. b) Total colloidal and macromolecular solids in the FFF experiment as a function of the total solids in the dialyses experiment. From paper II.

Application of the developed methodology on the clarification process of white wines (Paper III)

The novel approach to colloidal and macromolecular characterization using the AF4-UV-MALS-dRI technique developed in Paper II is applied to white wine processing. The specific aim is to evaluate the clarification operation used to stabilize the wine.

This study aims to evaluate how the AF4-UV-MALS-dRI technique can be used to calculate the removal capacity and determine differences in the application of the use of different clarifiers with different composition (mineral, synthetic and vegetable) on the colloidal and macromolecular compounds of a raw white wine. Additionally, it will evaluate how the clarification process using these different clarifiers can affect some particular properties of the characterized fractions.

In figure 11, an AF4 fractogram of the raw white wine (before the clarification process) is shown. The dRI signal (blue line), shows the presence of three populations; the second and the third populations appear to be higher in their intensities, suggesting higher concentration in these populations. The UV absorbance (green line) at 280 nm shows two well-defined populations for 1 and 2, while population 3 appears as the tail of population 2. MALS signal (red line) shows the third peak or population greater than the previous two, indicating smaller particles in the beginning of the elution and much larger particles as elution time increases.

In the previous study (Paper II), the AF4 analysis characterized two principal populations: the first population was identified as wine colloids (WC) and the second as wine macromolecules (WM). In the present fractograms (raw white wine), populations 1 and 2 are classified as wine colloids based on the UV signal, and population 3 as wine macromolecules. The black dots (o) refer to the molar mass in g/mol, which increases along with the elution time.

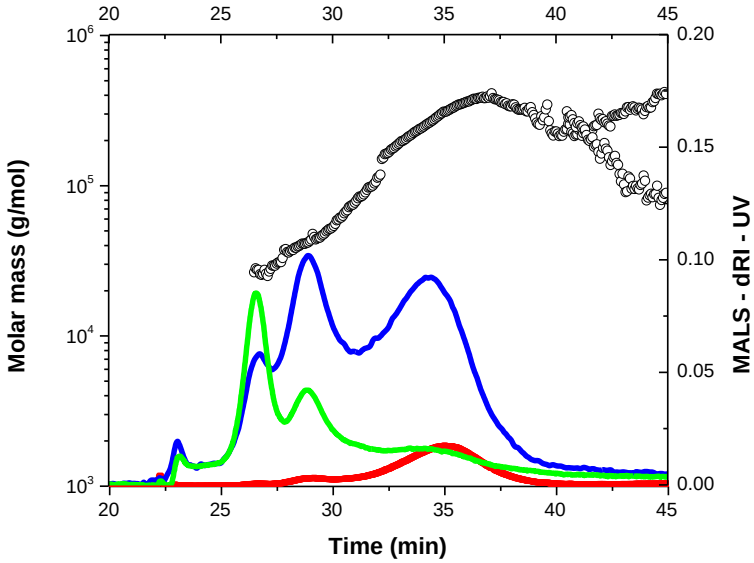

Figure 11. AF4-MALS-dRI-UV fractogram of the elution profile for the raw white wine showing three different populations from the dRI signal. Note that the scale time starts around 20 minutes due to Focus and focus + injection steps were approximately 10 min due to the high volume injected, 400 µL. From paper III.

The following figures 12 a-c show the resulting FFF-fractograms after the clarification process occurred. Precisely, figure 12a shows the fractogram corresponding to the fining process using activated bentonite in high dose (OF15). The UV signal is almost completely removed, showing that the UV absorbing material of the wine colloids of populations 1 and 2 is eliminated after the fining process. In addition, there is a reduction of the dRI signal of population 1, showing a strong removal capacity of the activated bentonite in high dose, especially for the wine colloids.

Figure 12b shows the fining treatment (PP10) of the synthetic polymer (Polyvinylpolypyrrolidone). The fractogram of this treatment shows a decrease in the dRI signal of the three populations when compared to the raw white wine. In addition, the MALS signal looks higher, which may suggest an increment in size of larger particles.

Figure 12c shows the fining treatment using a potato protein (VC5). The fractogram shows a decrease in the UV and dRI signals for the three populations; however, the shape of the peaks indicates that the reduction is significant, although not as drastic as in the case of the activated bentonites.

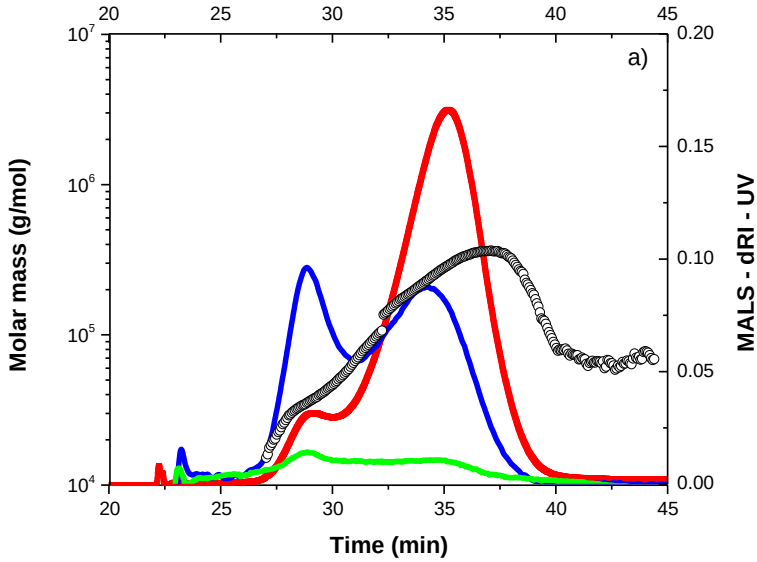

Figure 12a. AF4 fractograms showing the effect of the activated bentonite (OF15) treatment. From Paper III.

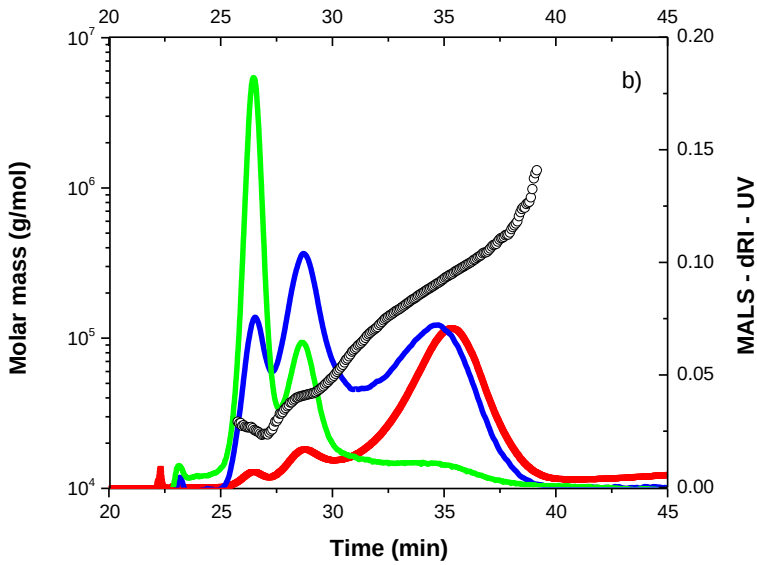

Figure 12b. AF4 fractograms showing the effect of the Synthetic polymer, Polivinilmidazol (PP10). From Paper III.

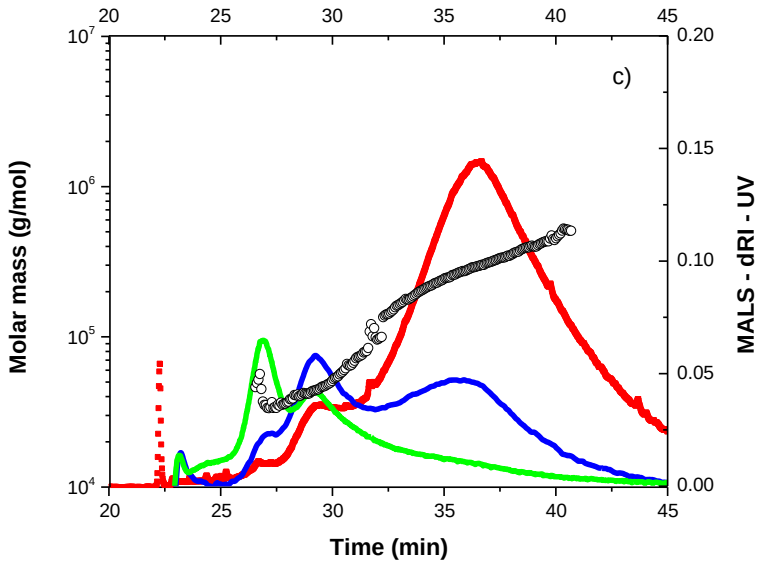

Figure 12c. AF4 fractogram of the fining treatment using the Potato vegetable protein (VC-5). From Paper III.

Thereby, after clarification in the fractograms, it is possible to notice differences in the signal patterns of the elution profile from the different detectors (UV-MALS-dRI). The different treatments have shown different trends according to the nature of the clarifier and the dose used.

These results show differences in the relative intensities, but the retention times of the populations were very similar. The results show the applicability and usefulness of the AF4 method coupled with a multiple detectors technique to characterize the effectiveness of clarifiers in the removal of the undesired colloidal components (see table 10). However, to make a more accurate assessment of the changes occurring in the macromolecular characterization, additional analyses are shown for possible correlations, such as determination of the protein content, total phenols, stability and turbidity (see table 11).

Table 10. Concentration of the material of the fractionated populations and the percentage of the removal efficiency.
(From Paper III, table 2).

Sample	Total Concentration (mg/mL)[a]				Fraction removed (%)[b]			
	Population 1	Population 2	Population 3	Total	Population 1	Population 2	Population 3	Total[c]
Raw Wine	0.018	0.062	0.094	0.174	0.00	0.00	0.00	0.00
Mineral - OF15	0.002	0.052	0.072	0.126	89.3	15.9	23.9	28.0
Synthetic - PP10	0.022	0.058	0.068	0.148	-18.8	5.4	27.8	14.9
Vegetable - VC5	0.005	0.031	0.065	0.102	72.7	49.3	30.8	41.8

[a] Concentration of the material in the populations according to eq.4 assuming $(dn/dc)_{p1}$ and $(dn/dc)_{p2}$ are equal to $(dn/dc)_{WC}$ as determined from eq 14.
[b] The removed fraction is calculated from the ratio of the clarifier concentration with the concentration of the raw wine.
[c] The total percentage is calculated from the total concentration.

Table 11. Specific Absorptivity, total protein content, total phenols, the refract index increment of the wine colloids $(dn/dc)_{WC}$, stability and turbidity.
(From Paper III, tables 4 and 5).

Sample	Specific Absorptivity[a] (mL/mg · 1/cm)			Protein[b] mg/mL wine	TPH[c] mg/L wine	$(dn/dc)_{wc}$[d] mL/g	Stability NTU	Turbidity NTU
	Population 1	Population 2	Population 3					
Raw Wine	0.546	0.142	0.040	0.28	325.0	0.166	19.3	17.4
Mineral - OF15	0.058	0.049	0.042	0.11	300.0	0.167	21.83	0.92
Synthetic - PP10	0.667	0.171	0.036	0.25	272.2	0.163	10.7	11.04
Vegetable - VC5	0.960	0.189	0.072	0.18	316.7	0.171	21.53	1.82

[a] Absorptivity at 280 nm. The concentration normalization is according to the eq. 5.
[b] Total Protein content in mg/mL.
[c] Total Phenols are expressed in mg/L of gallic acid equivalents.
[d]The dn/dc determination of the wine colloids is calculated in carrier liquid (13 % v/v ethanol) according to the eq. 14.

To describe the impact that the characteristics of the clarification (mineral, synthetic and vegetable) has on the different parameters determined for the molecular and macromolecular properties, a Principal Component Analysis (PCA) was performed (figure 15).

In figure 13, the PC1 explains 34% of the variance, and shows how the different groups of clarifiers have arranged according to their nature; the synthetic clarifiers appear in the left side, as well as the mineral clarifiers, with exception of the activated bentonite in high dose (OF15), while the vegetable protein clarifiers appear in the right side.

PC2 explains 21% of the variance, showing how the distinct doses used in the different treatments affect the individual properties.

The properties of the original white wine ("RAW") is in the upper left corner of the diagram. The distinct clarifiers (marked with the sample names) appear grouped around the center of the figure, with the trajectories from the raw wine to the clarified wine shown with arrows. The sample categories are shown with colors; red for the synthetic clarifiers, purple for the mineral clarifiers and green for the vegetable clarifiers. This grouping of the clarifiers after the clarification process makes it possible to differentiate the impact of each group of clarifiers, and even the doses used on the properties of the wine.

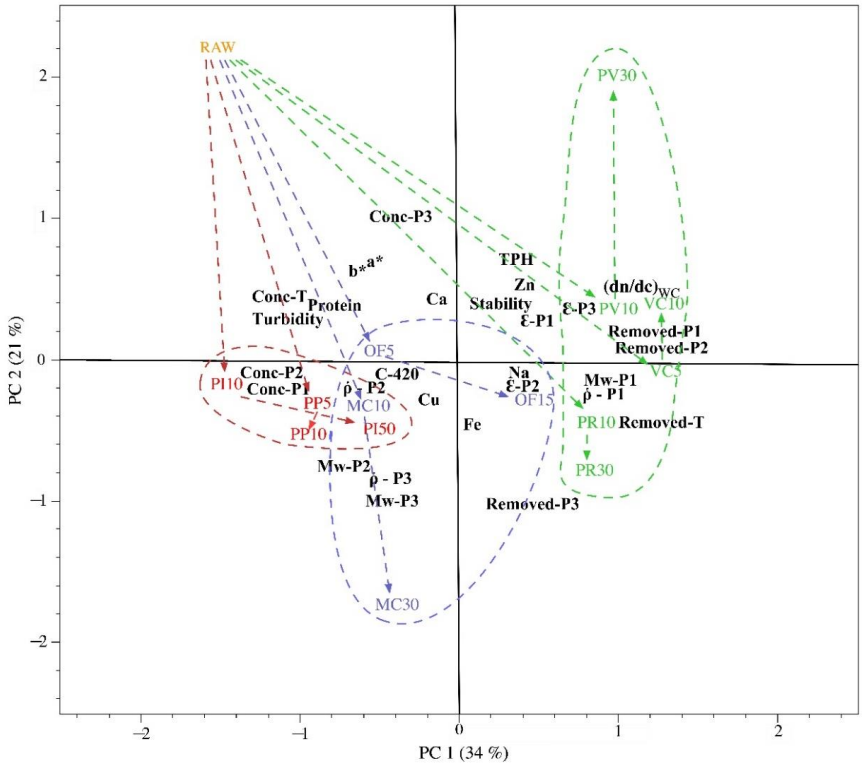

Figure 13. PCA bi-plot representig all clarified samples (colored) and most measured parameters (black) in the study. The arrows shows the development of the clarification process. Green arrows and samples are the vegetable protein clarifiers, the purple arrows and samples are the minearal clarifiers and the red arrows and samples are the synthetic polymer clarifiers. The name of samples and parameters follows Table 1-5, in addition, T refers to total concentration in all three FFF fractions, Rem. refers to removed. RAW (orange) refer to the raw white wine or control. From Paper III.

The mineral clarifiers showed the highest capacity of removal when a high dose is used, for instance the activated bentonite OF15 removed more of the proteins of wine colloids of population 1. In addition, the MC30 removed most of the wine macromolecules of population 3.

The synthetic polymer clarifiers appear along with a cluster of the concentrations of populations 1 and 2, which means that the concentrations of these populations are preserved after clarification. However, it is possible to note that the synthetic polymer clarifiers primarily remove polyphenols and little of the wine colloids and macromolecules, but changes in absorptivity and density are minor. It is also notable that the synthetic polymer clarifiers primarily remove the wine macromolecular population.

The vegetable protein clarifiers considerably reduce the undesirable material of lower molecular weight (population 1 wine colloids). However, these removals do not necessarily correlate positively with all the parameter values measured. Thus, it is interesting to note that during the removal of colloids from the wine of population 1, mainly the protein content has been removed and, on the contrary, the polyphenol content remains considerable (table 11).

The use of the AF4-UV-MALS-dRI technique demonstrated its suitability for evaluating the removal capacity of different clarifiers during the clarification process of white wines. The results show that vegetable clarifiers are more effective in removing colloids from wine from populations 1 and 2, while mineral clarifiers are useful in removing the macromolecular fraction of population 3. It also seems that the use of high doses of clarifiers modifies highlighting different properties of the wine. Therefore, it is possible to monitor the changes through characterization of its macromolecular, molecular and specific properties and improve the clarification process.

The AF4 methodology applied on a full vinification process (Paper IV)

In order to evaluate the capacity of the AF4 methodology when used in oenological applications, we initiated a project where the vinification process was followed. The particle formation and loss of the colloidal fractions, and the changes of present macromolecular compounds were followed through five stages (end of alcoholic fermentation, end of malolactic fermentation, clarification, bottling and during storage that can be interpreted as aging) of the red wine vinification. The principal aim of the study is to evaluate changes in the macromolecular and colloidal composition, as well as in different macromolecular properties (growth or decrease), and to identify potential patterns that occur during the manufacturing process.

For this purpose, six Spanish red wines samples were prepared using different grape varieties, and some were harvested in different regions at different times. The analysis of the wine samples was performed during 2021. Table 12 shows the description of the principal characteristics.

Table 12. Variety, origin, harvest date, and different stages used for the vinification. (From Paper IV, Table 1).

Variety	Origin	Harvest	Stage 1 EAF[a]	Stage 2 EMLF[b]	Stage 3 Clarification	Stage 4 Cold stabilization and Bottling	Stage 5 Storage & Aging
Tempranillo	D.O. Costers del Segre (Lleida)	29 October 2019	5 Nov 2019	12 Nov 2019	22 Nov 2019	25 February 2020	8 April 2021
Merlot	D.O. Montsant (Tarragona)	13 September 2019	5 Nov 2019	25 Nov 2019	29 Jan 2020	23 January 2020	8 April 2021
Cariñena	D.O. Montsant (Tarragona)	7 October 2019	5 Nov 2019	13 Nov 2019	29 Jan 2020	28 January 2020	8 April 2021
Garnacha-1	D.O. Montsant (Tarragona)	28 September 2019	5 Nov 2019	Nm	29 Jan 2020	1 April 2020	8 April 2021
Garnacha-2	D.O. Montsant (Tarragona)	7 October 2019	5 Nov 2019	11 Nov 2019	29 Jan 2020	1 April 2020	8 April 2021

[a] EALF refers to the end of alcoholic fermentation.
[b] EMLF refers to the end of malolactic fermentation.
Nm, not measured.

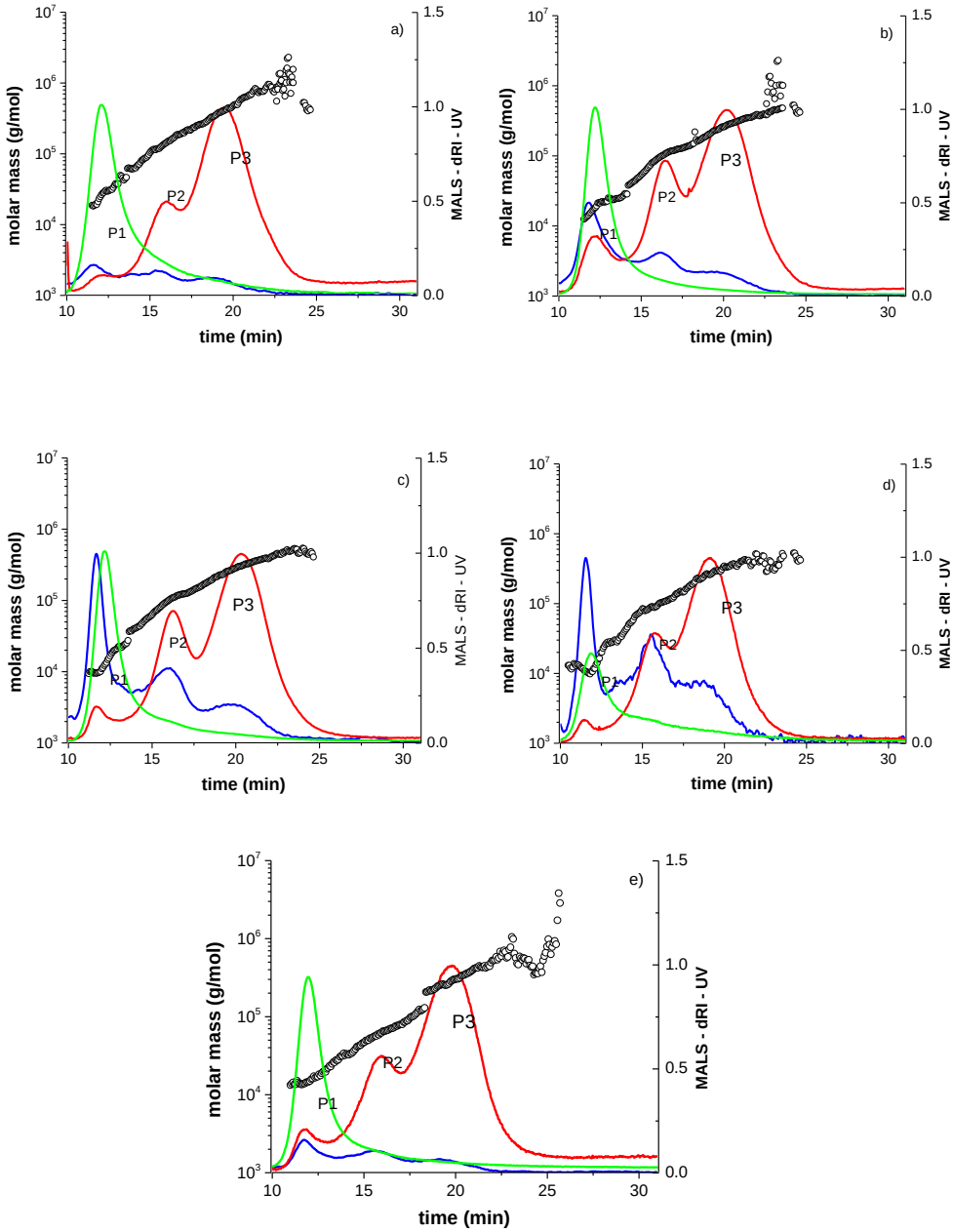

Figure 14. Fractograms for the sample Garnacha-2 showing different stages of the vinification. a) Alcoholic fermentation, b) Malolatic fermentation, c) clarification process, d) after the bottling and e) during storge. In green UV signal at 280 nm, in blue the dRI signal, and in red the MALS signal. Some intensities of the different signals in the fractograms were automatically normalized in relation to the highest peak of the whole elution profile including the void time and the peaks after the end of the cross flow. From Paper IV, supplementary material.

After the AF4 separation, the resulting fractograms for the separation of the Garnacha-2 sample, along the five stages of the vinification, are shown in figures 14a to 14e. In general, is possible to notice three populations with the MALS-dRI signals, and the characteristics of each population is defined, together with the specific and macromolecular characterization in the following sections.

Figure 14a shows three different populations in the dRI and MALS signals. The dRI intensity is comparably low, suggesting low particle concentration during the first fermentation stage. The UV signal clearly shows a small peak for the population 1 of the wine colloids, indicating the presence of UV absorbing material in the colloidal fraction. Figure 14b corresponds to the fractogram after the second, malolactic, fermentation; it is possible to notice an increase of the UV and dRI signal, which indicates that during this process major quantities of particles were released, suggesting a possible major particle formation in both colloidal and macromolecular fractions. This is reasonable since, due to the fermentation process, the extraction of condensed tannins, anthocyanins and polysaccharides is augmented. Thereby, the increase of the UV and dRI signal in the wine colloids of population 1 is augmented. The fractogram in figure 14c corresponds to the clarification process, where the intensity signal of the UV-MALS and dRI signal have decreased in general, and particularly for the wine colloids. During the clarification process, the remotion of the larger particles is expected, but also of undesirable material, such as tannins and proteins that occurred commonly in populations 1 and 2. However, this may depend on the particular grape variety (wine sample) and to the clarification process profile aimed, since it should be remembered that in the clarification process of white wines (Paper III), after clarification, the MALS signal increased. The fractogram in figure14d depicts the bottling stage; it is clear that the dRI signal has increased, indicating that the concentration of the particles is still growing during the bottling stage. This observation must be correlated with the determination of the specific absorptivity in order to fully understand the nature of these growing particles.

The general overview of different fractograms obtained by the AF4-UV-MALS-dRI shows a potential trend based on the unit operations used during winemaking; hence these trends indicates that structural changes in the colloidal and macromolecular fractions occurred with respect to each particular stage of the vinification.

Consecutively, data provided with the retention time and MALS signal can be obtained regarding the size of these separated fractions, such as the molar mass (M_W) and the hydrodynamic radii (r_H) (see table 13).

The tendency shows that the Mw values tend to decrease as the winemaking progresses, with some exceptions for populations 2 and 3 at stage 3 with $65 \cdot 10^3$ and $340 \cdot 10^3$ g/mol, respectively. Though at the last stage 5, it is clear that the

values for the three populations have shown escalating values, with a prominent value for population 3 of $408 \cdot 10^3$ g/mol.

It is noticeable that in the first stage of vinification, higher values of Mw are found, which could be due to the fact that diverse heterogeneous particles were present in the initial stage but were removed in the following stages.

These average values somehow agree with the values previously found in Papers II and III both for the wine colloids and wine macromolecules.

Table 13. Changes in size of the Garnacha-2 sample during five stages of the winemaking. (From Paper IV, Table 2).

Garnacha-2										
	***Mw* (· 10^3 g/mol)[a]**					**r_H (nm)[b]**				
Population	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**
WC-P1	26.5	16.9	12.1	14.5	20.3	3.8	3.0	2.7	3.8	3.7
WC-P2	77.9	63.6	65.0	57.8	70.8	7.9	6.2	6.7	8.2	9.3
WM-P3	369.8	307.0	340.0	257.6	408.2	15.5	13.0	14.7	16.3	18.9

[a] Mw, Average Molecular Mass.
WC-P1 and WC-P2, refers to wine colloids of the population 1 and 2, respectively.
WM-P3 refers to wine macromolecules of the population 3.
[b] Z-Average Hydrodinamic Radii.

Regarding the values of the r_H, the trend to some extent follows the Mw tendency decreasing their values as the winemaking progresses, and in the later stages tends to increase again. In addition, an evident gap in values between stages 1 and 2 is noticeable. The general average results throughout vinification for populations 1, 2 and 3 ranged from 3.4, 7.7 and 15.7 nm, respectively.

The results of the apparent density are shown in table 14. Certainly the values decreased during the winemaking process until the lowest values are reached at the stage 5 (storage and aging). Interestingly, the higher values for the three populations were found at stage 2 (end of malolactic fermentation) followed by stage 3 (clarification), which perfectly agrees with concetration measurments. This higher value of apparent density and concentration found at stage 2 can be well understood as the release of the material, which constitutes the coloidal and macromolecular fraction, during the second fermentation. Concetration agrees with previous data reported [56].

On the other hand, the values of the specific absorptivity (ε) have shown to be interesting (see table 15). The highest values are found at stage 1 and the lowest at stage 4; later at stage 5, the values substantially increase again. Interestingly, the lowest values found at stage 4 imply that neither of the three populations showed particles that absorb UV light. Generally, population 1 must show higher values due to the presence of phenolic and proteinaceous compounds that compose the

wine colloids. Peculiarly, population 2 has shown a somewhat high level of absorptivity at stages 1, 2 and 5, and low values at stages 3 and 4. Thereby, it is interesting to evaluate how considering and combining the different properties together may assist in defining the nature of the populations.

Table 14. Changes in the Apparent densities and concentration for the Garnacha-2 variety along the winemaking. (From Paper IV, Table 4).

Garnacha-2										
	$\hat{\rho}$ (kg/m3)[a]					*c* (mg/mL)[b]				
Population	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**
P1	226	299	243	163	145	0.05	0.24	0.19	0.15	0.09
P2	71	106	91	46	40	0.06	0.20	0.21	0.12	0.18
P3	40	59	46	30	24	0.09	0.17	0.19	0.16	0.12
Total						0.20	0.60	0.59	0.43	0.4

[a] $\hat{\rho}$, Apparent density was calculated using the r_H and M_W.

[b] c, is the concetration and it is calculated using Eq.4.

As an overall view of the presented results (fractograms and macromolecular characterization presented in tables 5-7), the tendency has shown potential trends related to the unit operations used in conjunction with the vinification, which shows that fundamental changes occurred in the colloidal and macromolecular fractions. This suggests some tendency of a dynamic process, where some of the particles are formed, then vanished/removed, and formed again.

Thereby, in order to reveal potential patterns between the properties and processes, a principal component analysis (PCA) was carried out (see figure 15). Figure 15 shows the biplot drawing where PC1 explains 32% of the variance and PC2 explains 26% of the variance, which in combination describes the cyclical evolution of the winemaking process and properties.

As an overall view of the PCA biplot, we can notice that, along with the PC1, the separation of the stages is given as the winemaking process progresses. Thus, S1 and S5 appear on the left side of the plot. Moreover, stages 1 and 5 correlate positively with properties such as the specific absorptivity (ε), molar mass (M_W) and hydrodynamic radii (r_H). On the right side of the PC1, the stages S2 and S4 are correlated with properties such as the concentration and apparent density ($\hat{\rho}$). The PC2 indicates the changes in the magnitudes of the properties as the process occurs.

Table 15. Specific Absorptivity determination (ε) for the Garnacha-2 sample variety throughout the winemaking.

ε (mg/ mL · cm)[a]					
Population	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**
P1	6.66	5.15	1.17	0.06	3.21
P2	2.72	2.07	0.99	0.04	1.49

P3	0.84	0.57	0.25	0.02	0.32

[a] ε, is the Specific Absorptivity calculated using eq.5

On the whole, the different stages describe a cyclical pattern from S1 to S5. As an example, let's look at the Garnacha-2 sample; it seems that it is characterized by a very high absorptivity and shows a trajectory very similar to the Tempranillo variety. While the other samples (Cariñena, Merlot and Garnacha-1) followed different patterns but with similarities between them. Predominantly, Garnacha-2 correlates positively with the absorptivity at stage 1, with the concentration and apparent density at stages 2 and 3; at stage 4 it appears close to the origo, and at stage 5 correlates positively with the M_W and r_H.

Altogether, the results concerning the macromolecular and specific properties, along with the operations process exerted, indicate that the wine colloids of population 1 change completely in their particle nature from the beginning to the end of the process (S1 to S5). This suggests that a transformation of the wine colloids occurs gradually, where the particles fade and tend to reform again, a fact that cannot be noticed clearly with the wine macromolecules.

This study introduces the AF4 technique coupled with online detection (UV-MALS-dRI) to the area of oenology to demonstrate the applicability of the technique and methodology in helping to characterize and monitor changes that occur at colloidal and macromolecular levels at different stages of the winemaking process.

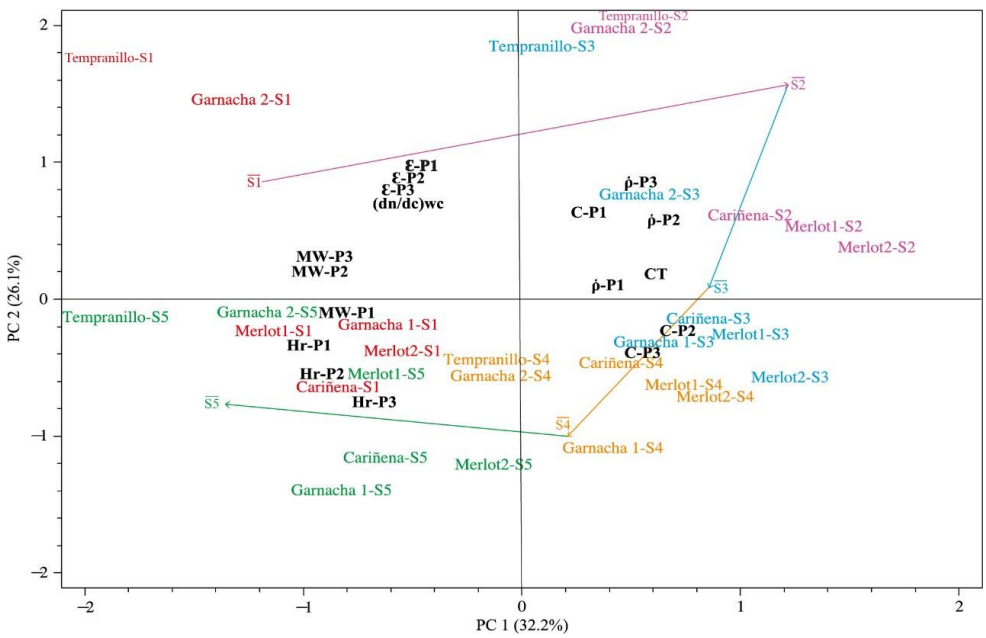

Figure 17. PCA biplot showing the evolution of the wine samples of the five stages (S1, S2, S3, S4 and S5, depicted in red, violet, light blue, yellow and green, respectively) and the macromolecular and specific properties in study (in black) throughout the vinification process. From Paper IV.

5. Discussions

Developing a methodology for separation and characterization

During the development of the separation methodology, different parameters were initially optimized, such as injection volumes, avoiding the possibility of overloading and/or aggregations in the resulting signals of the fractograms. In addition, depending on the injected volume, the relaxation time step in the separation channel (focus, and focus + injection) were optimized. Subsequently, to obtain a better resolution of the separated populations, different values of the elution flows and the crossflow were optimized, including the half-life of the mode. Finally, two membranes, such as regenerated cellulose (RC) as well as polysulfone (PS), were tested, including 5 kDa and 10 kDa cut-offs.

After separation, two different populations are characterized: the wine colloids that are characterized by being more compact and denser, that is, smaller and with lower molar masses but with high UV absorptivity, and the wine macromolecules that, conversely, are less dense and compact but larger in size and in molar mass without UV absorption (see table 16 for the range of values characterized).

In addition, the challenges during the development of the methodology in the present thesis were related to the quantification of the material present that constitutes the wine particle matter. This drawback is due to the wine samples being made up of different colloidal and macromolecular fractions, which show different chemical compositions and therefore properties (M_W, r_H, $\hat{\rho}$, ε, c and dn/dc).

Besides that, the dn/dc property can generally be quantified with well-known material standards, thus, the methodology used had to develop some tricky alternatives to overcome these challenges.

Thus, the determination of the refractive index increment (dn/dc) of each particular fraction allows for quantification which allows to obtain the values of properties such as M_W, $\hat{\rho}$, ε, and the concentration (c) of the fractions, describing the nature of the complex materials present in the fractions.

The wine colloids are complex structures, containing carbohydrates ($dn/dc \sim 0.14$, proteins $dn/dc \sim 0.16$ and polyphenols $dn/dc \sim 0.25$). Thus, to estimate the dn/dc, an experimental value is needed. The dn/dc of the total solids $(dn/dc)_{TS}$ of all

macromolecular and particle material is determined, and then the contribution of the macromolecules is differentiated from the contribution of the colloids.

Hence, through a separate (offline) dialysis experiment, the value of $(dn/dc)_{TS}$ was determined. Although quantification has its limitations, it enables the properties of the different fractions to be described.

Thus, the results using the AF4-UV-MALS-dRI after the separation showed characteristic properties of the different fractions. The first fraction is referred to as the wine colloids, which are shorter, denser, smaller and show UV absorbance. The compounds that make up this colloidal fraction were chemically and qualitatively determined; mainly the presence of polyphenols and proteins with a low portion of polysaccharides were found. The last fraction is referred to as wine macromolecules, and is characterized by being larger, with higher molar mass, less dense and without the presence of UV active absorbing compounds. The chemical and qualitative identification showed that these compounds are mainly composed of polysaccharides. Table 16 shows a comparison of the macromolecular characterization of the different studies. From Papers II to IV we can see that values are well comparable between red wines, although in Paper II, two populations were obtained in contrast to Paper IV, where three populations were characterized. Moreover, by comparison with other authors, the molar masses agree both in the white wines [51] and red wines [92], while the r_H is much lower when compared with other studies [56], since the radius reported referred to the radius of gyration, which is comparably high.

Table 16. Results comparing ranges of macromolecular properties in different sources [51], [55], [92].

Study	Sample	Molar Mass[a] (Mw) (g/mol) · 10^3	Hydrodynamic Radius r_H (nm)	Apparent density $\hat{\rho}$ (kg/m^3)	Total Concentration[b] (mg/L)	Absorptivity (ε) (mL/mg · 1/cm)
Paper II	Argentinian wines	22.2 - 303	2.9 - 11.6	1246 - 34	0.5 - 1.34	7.15 - 0.05
Paper III[c]	White wines	27.7 - 57.9 -278	3.1 - 6.0 - 16	362 – 131 - 38	0.174	0.54 - 0.142 - 0.04
Paper IV[c]	Spanish wines	8.3 - 70 - 565	2.8 - 8 - 20	1135 - 75 - 20	0.23 - 1.01	9.72 - 1.0 - 0.1
Pascotto K.[c]	Red wines	8 - 62 - 278				
Coehlo C.	White wines	10 - > 250				
Marassi V.	Red wines		30 - 50[d]			

[a] Average Molar mass.
[b] Total concertation of the peaks shows the rages found refers to the wine colloids (in the left) and to the wine macromolecules (in the right).
[c] The calculated values include 3 populations where population 1 and 2 are considered as colloids and populations 3 as macromolecules.
[d] The values reported refer to the Radius of gyration.

Application of the methodology and consequences

The methodology was initially evaluated by comparing the properties of a set of bottled Argentinian red wines (originating from the same North Argentinian region). Particular differences and similarities in their molecular and macromolecular composition could be observed in this material.

After that, the intention was to evaluate the methodology not only in sampling and comparing wine samples, but also using red and white wine samples during the wine manufacturing process, to evaluate the impact of various processing operations.

In the study on clarification of white wines, using clarifiers of different origin (minerals, synthetic and vegetable), the results showed changes when comparing the samples before and after the clarification process. Each clarifying agent, according to its nature, showed advantages and disadvantages, both in the capacity to eliminate undesirable material and in the fact of preserving and moderating qualities and characteristics of the wine, affecting its composition, quality, and final flavor.

However, it is necessary here to note that after AF4 fractionation, three populations were observed (as shown in the property results of table 16, ref 51). These results contrast with the two populations obtained previously during the development of the methodology in the study of Argentine red wines (Paper II). For this reason, in the study of the clarification of white wines (Paper III), the methodology was slightly modified. For this new intermediate population, new population 2, the apparent density results were neither too high nor too low, but the specific absorptivity results were comparatively high, indicating that UV absorbing species were present. In addition, the molar mass of this intermediate population was compared with another offline experiment, gel electrophoresis, and it was determined that the Mw found agrees with Mw values of typical proteins in white wines. Thereby, this intermediate population is treated and considered as a second population of wine colloids. Consequently, for both populations, the same value of $(dn/dc)_{WC}$ corresponds.

Similarly, in the Spanish red wines undergoing vinification (Paper IV), three populations were observed. Thus, the procedure to determine the nature of this new intermediate population (population 2) followed the same procedure, treating population 2 as a part of a class of wine colloids. However, it can be considered that the apparent density values were low (lower than those reported in population 2 for white wines), but instead, and interestingly, the specific absorptivity values (ε) depending on the sample and the vinification stage were either high or low. Consequently, new questions arose when assessing the nature of the second population.

On the other hand, if the intermediate population somehow, and by depending on the sample, shows intermediate properties, the use of the same $(dn/dc)_{WC}$ for the new second populations may create some scatter in the results, as for instance in the concentration and apparent density. However, the magnitude of these density results does not vary much more than 20%.

Another detail to highlight is that large volumes of wine samples are necessary for the determination of the refractive index increment of the total solids $(dn/dc)_{TS}$. Consequently, only the *dn/dc* of the total solids could be calculated for one stage of the vinification process. This value was used in the other stages and therefore the calculations of the properties were estimated according to this particular *dn/dc*. However, it was possible to notice that throughout the vinification process, the intermediate population 2 maintained intermediate values and did not show great change in properties.

Remarkably, the most attractive results were obtained with respect to the colloidal fraction of population 1, where it can be seen how some properties of these particles change drastically in the process, specifically the specific absorptivity (ε), and because the concentration does not vary in the same proportion, it indicates that the nature of the population is totally different according to the process carried out during vinification. That is, the particles are formed initially, then they fade away and then reform again, but somehow maintain a similar concentration during the process. This demonstrates that a fairly dynamic process is taking place in the formation of new colloidal particles.

Therefore, considering these results as a whole, it is possible to note that the methodology developed using the AF4 methodology coupled to multiple detectors is capable of separating and characterizing the particulate matter in wine, and that this methodology can be applied for different purposes, including the wine manufacturing process. It is important to state that the advantage is that it allows analysis of several amounts of samples in a quick and efficient manner. In addition, the interpretations provided may infer specific properties of the separated fractions which have to do with important oenological parameters, supporting the choice of decisions in winemaking.

Comparison with other similar studies

Currently, from different groups and researchers, there are three studies dealing with the separation and characterization of wine particle material using the AF4, but the methodological approaches differ: for instance, in relation to comparing the white wines. Coelho et al., 2017 [51], showed the presence of 1 and 2 populations with the UV signal, according to the sample. Moreover, the resulting fractogram was divided in six fractions, and by using the AF4-UV and fluorescence detector the major fraction was collected for analysis, resulting in the

determination of proteins with a M_W between 30-60 · 10^3 g/mol. This agrees with the white wine colloids in population 2 in our study about clarification in white wines. In addition, an MS elucidation analysis was performed to verify the results, concluding the presence of enzymes in association with proteins, which may be related to aroma formation during the winemaking process. The work concludes that if AF4 could be combined with other techniques and detectors, more precise characterizations could be achieved.

Another similar study by Marassi et al., 2021 [56], on two different red wines, separated three main populations using the MALS-dRI signals. The populations were divided in 12 fractions for further offline chemical analysis and quantification. The results showed that different proportions of phenols, proteins and polysaccharides were present in each of the 12 fractions, which were characterized according to their radius of gyration, concluding that different portions of polyphenols, protein and carbohydrate can be found in each fraction, according to the grape variety and the production processes carried out.

Finally, the study by Pascotto et al., 2020 [92], focuses on developing the analytical conditions of the separation process (injection volume, cross flow force and other steps that occur within the separation channel) and the characterization of the molar mass and size distribution according to the multiple detectors. In addition, a comparison with tannin and polysaccharide fractions extracted from apple was performed. The results of this work assumed *dn/dc* values taken from the literature, both for the wine colloids and for the macromolecules, and due to the dRI signal showed three populations; the *dn/dc* value for the intermediate population was calculated according to the average value of population 1 and 3 (see table 17, for comparison of the *dn/dc* values and other properties reported using AF4 and wines). However, four fractions were considered for analysis, according to the UV-MALS-dRI signals. The first three were characterized according to their molar mass, polydispersity, and the coelution phenomenon was reported. The first fraction was assigned as tannins eluting together with polysaccharides, and the following two fractions was composed of polysaccharides. The last fraction was not identified. Later, the same group in a different study reported on the relationship between red wine colloidal fraction and astringency, concluding that astringency increases as the molecular mass of the wine colloidal fraction increases, and the perceived astringency can be counteracted by the polysaccharidic material [25].

Regarding comparison between properties such as the $(dn/dc)_{WC}$, Pascotto et al., 2020 [92], used values taken from literature close to the values determined with the methodology proposed in our study. In addition, very close values were also reported for the protein content of white wines by Coehlo et al., 2017 [51], and for total phenols as well as polysaccharides in the macromolecular fractions by Marassi et al., 2021 [56].

Table 17. Comparisons of wine properties obtained in different studies in the colloidal and macromolecular fractions as well as in the total wine.

Study	Sample	dn/dc_{TS} mL/g	dn/dc_{WC} mL/g	dn/dc_{WM} mL/g	Protein Wine/ Fractions mg/mL	TPH Wine/ Fractions mg/mL	Poly-saccharides fractions mg/mL
Paper II	Argentinian wines	0.158-0.204	0.187 - 0.251	0.14	0.08 - 0.2 (Wine)	1.74 - 2.82 (Wine)	0.05 - 0.26
Paper III	White wines	0.146 - 0.155	0.166	0.14	0.28 (Wine)	0.325 (Wine)	
Paper IV	Spanish wines	0.16-0.18	0.16-0.185	0.14			
Pascotto K. [92]	Red wines		0.247-0.165	0.146			
Coehlo C. [51]	White wines				0.2 - 0.25 (fractions)		0.14 - 0.17
Marassi V. [56]	Red wines					1.2-1.9 (wine) 0.14 - 0.6 (fractions)	0.3 - 0.4

Therefore, when comparing the results obtained from the methodology developed in this thesis with the results from other studies using AF4 for the separation and characterization of particulate matter in wines, it is possible to observe comparable results, such as the number of separated populations, as well as similarities in the magnitudes of some properties, such as molar mass, concentration and even *dn/dc*. However, it is necessary to emphasize that the proposed methodology aims to reduce the use of additional offline analyses, and mainly use online detectors for all macromolecular characterization and quantification. Thus, the parameters (*Mw*, *rH*, $\hat{\rho}$, ε and *c*) calculated using only the UV-MALS-dRI detectors are proposed as fundamental properties that help define the intrinsic nature of the populations, being both the apparent density ($\hat{\rho}$) and the specific absorptivity (ε) fundamental variables for the colloidal and macromolecular characterization.

6. Conclusions and Future Outlook

In this thesis, the AF4-UV-MALS-dRI technique has been used for the separation, characterization and application of the particle matter that constitutes wines.

The developed methodology using AF4 coupled with multiple detectors uses a wine carrier model solvent allowed for wine particle matter separation. In addition, the combination of detectors UV-MALS-dRI permitted an accurate specific and macromolecular characterization, such as the molar mass distribution (M_W), hydrodynamic radius (r_H), apparent density ($\hat{\rho}$), concentration and specific absorptivity (ε) for the separated fractions. The characterization results after the separation showed clear differences in their properties in a wide range of values.

In addition, the resulting AF4 fractograms showed two or three main populations according to the wine sample, and by the nature of their characteristics properties they were attributed as part of the colloidal or macromolecular fractions. However, some samples showed an intermediate population with intermediate values of their properties, therefore making it difficult to consider them strictly as wine colloids or wine macromolecules, regardless of them passing through a dynamic process of formation with changes to the extent of their properties during the wine manufacturing process.

It is worthy to remark that by using a combination of the detectors, the developed methodology allowed for the determination of principal properties such as specific absorptivity and apparent density that together play an important role in online characterization of the properties that defines the nature of the separated fractions. In addition, the methodology took less time and effort compared to the offline analysis. This advantage allows for a precise characterization of a large number of samples during any stage of the vinification process. However, for a correct quantification of properties as concentration, it is necessary to determine the refractive index increment *(dn/dc)* of the population using additional offline analysis, such as a dialysis experiment using similar conditions used during the AF4 separation.

Therefore, the proposed methodology using the AF4-UV-MALS-dRI for the separation and characterization of the wine particle matter may need the support of instrumental online detection. That is, alternatives detectors or techniques that can be coupled to the AF4-UV-MALS-dRI sequence, that can enable more accurate

data to be obtained during characterization, and to quantify determination of the particle properties of the colloidal and macromolecular material.

To address these drawbacks may help to better understand the relationship between the dynamics of the macromolecular and physicochemical properties, and the sensory properties, and possibly to interpret in a more detailed manner the changes of these properties in relation to their interactions, the binding mechanism of these colloidal/macromolecular complexes, as well as possibly including the structure-activity relationships.

Therefore, future AF4 trends or approaches in relation to the wine field could be used to track and evaluate these wine polymers from grape to glass and, therefore, produce and characterize new previously uncharacterized compounds from these colloidal and macromolecular networks. In addition, including the use of complementary spectroscopic analytical techniques and methods could also be an alternate support for areas such as biotechnology and biological systems that have begun to play an important role in the understanding of these processes.

Undoubtedly, this approach can lead to a multifactorial problem for each stage of winemaking. For example, it is possible to use the AF4 to evaluate, monitor and characterize the colloidal and macromolecular fraction in a single step using different types and methods of fermentation procedures. Evaluating different types of yeasts and bacteria and/or fermentation aids (e.g., enzyme preparations), or bacterial fermentations (e.g., malolactic fermentations), the type of yeast strains used (e.g., strains with high release of mannoproteins), etc., will lead to a final composition of the polymer formed and its final impact on the sensory properties of the wine.

Another possibility could be to use AF4-UV-MALS-dRI methodology focused on the red wine fining process using a different approach, that is, using new types of fining agents to evaluate and characterize the effect of atypical thermal instabilities.

It may also be of interest to focus on the aging process by evaluating improved storage properties (e.g., visual appearance, haze, and reduced precipitation) using longer aging times.

The intention is to look for more applications to evaluate the suitability of the AF4 technique in real conditions and how it could possibly be a complementary analytical tool in the winemaking process, for instance in following the changes associated with the cell wall polysaccharides composition of the ripening grape berries. Moreover, it could also be possible to follow the changes in the polymer composition of the berries due to the addition of associated pathogenesis proteins.

It can be assumed that the characterization and monitoring of colloidal and macromolecular properties of the polymeric networks at different stages of the winemaking process may allow us to control and modify some properties, with

possible consequences for the visual properties, stability, and the final quality of wine.

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