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Wine oxidation and aging: a study on the key role of polyphenols and acetaldehyde

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SUMMARY

The present dissertation reports the results obtained from the activities conducted within the framework of the PhD focused on the project "Wine oxidation and aging: a study on the key role of polyphenols and acetaldehyde".

Aging is essential for improving wine quality, especially for red wine. It is no coincidence that high quality wines are traditionally produced by barrel aging, long and expensive methods that significantly affect wineries' cost factors and production capacities. Young, rough, and tart wine after fermentation with a cloudy, dim, and unstable structure requires adequate time for the reactions of oxidation-reduction, esterification, condensation, polymerization to impart a completely different sensory character. During this period, to guarantee chemical and biochemical changes within the wine and avoid alteration phenomena, it is fundamental to manage properly the main actor involved in wine aging process: the oxygen. The traditional aging method, called natural aging, is oak barrel aging. It involves storing wine in oak barrels for several months and has proven to be an effective method for producing many types of quality wines. The influence of this method on wine quality lies in the special permeability of wood to oxygen. Oak barrels have good permeability. Traces of oxygen can enter the vascular bundle of the wood and the cracks between the boards slowly and steadily, causing slow and continuous oxidation of the wine and allowing it to improve color, flavor and astringency character. In more recent years, the use of artificial methods to simulate the aging process of wine to shorten the aging time and improve its quality in a short period of time has become an area of research of great interest. Micro-oxygenation represents the most interesting technique of wine oxidation and aging. This method consists of adding a controlled amount of trace oxygen to wine to meet the oxygen demand required by the physical and chemical reactions of the different varieties that occur during the aging process, thus simulating the micro-oxygenated environment of the oak barrel aging process, promoting the maturation of the wine and improving its quality with particular reference to taste and structure to color stability, aromas as well as astringency. Micro-oxygenation-based aging technology overcomes the problems of time-consuming traditional aging, providing a new idea and a new direction for the development of the wine industry. Although the artificial aging technology, in particular the micro-oxygen technology, has made great progress, the dynamics and changes that occur in the polyphenols as well as the implication of neoformation complexes in the definition of the sensory characteristics of the wine they

are clear enough. Sometimes the results of researchers from different teams are inconsistent and the detailed description of some mechanisms underlying the definition of sensory characteristics, such as astringency, is missing. It is known that acetaldehyde and polyphenols represent two components of extraordinary importance in defining the qualitative characteristics of wine. Understanding their role as single molecules, their evolution as well as their possible interaction among themselves and with other wine components would be of undoubted interest. Furthermore, it is clear that these compounds have a key role also in all issues related to health quality of wines and in the strategies useful for sustainable winemaking approaches. The study of relationship between sustainable techniques and polyphenols as well as the healthy effect of red wine inevitably involves the role of oxidation and acetaldehyde. Then it was the participation of acetaldehyde in the interaction between the different polyphenols and in the formation of new complexes molecules that was investigated. Furthermore, the effect of the each single polyphenol considered as a model and of the newly formed complex molecules involving the ethanal bridge was investigated with computational methods.

This dissertation assesses the observations, and the main significant results were reported in five chapters.

Chapter 1, based on the awareness that quality is not only linked to the alcohol composition of the wine, aims to be an overview of phenolic compounds and their recognized role.

More specifically, the chapter distinguishes and preliminarily describes the different classes of phenolic compounds, then reports the main factors that underlie their presence, concentration and action in wine.

Chapter 2 relying on polyphenols, aimed to estimate the position and role of wine in the Mediterranean diet as well as its relationship with health. In detail, the first part aims to provide a bibliometric analysis of the literature on the association between the Mediterranean diet, wine, and health. The results obtained from the data analysis show a strong relationship between the Mediterranean diet and red wine as well as their interconnection with health protection. Of interest in this regard is the link between wine included in the Mediterranean diet and topics such as 'risk reduction' 'cardiac risk' 'hypertension'. A certain focus on the relationship between red wine polyphenols and sirtuins, a specific family of proteins that mediate a wide variety of pathophysiological events, emerged from the analysis. However, it should be emphasized that there are really few studies related to the specific topic under research and bibliographic analysis. Based

on these considerations, in the second half of chapter, the relationship between the phenolic content and the bioactivity of red wines was investigated. the phenolic profile and bioactivity of Italian red wines Gaglioppo, Magliocco, and Nerello Mascalese were characterized. NMR, HPLC/UV-Vis and spectrophotometric characterization showed that Magliocco was the richest wine in monomeric anthocyanins (two-fold), catechins, and low molecular weight phenolics (LMWP). In vitro evidence on the endothelial cell models of insulin resistance and hyperglycemia showed the ability of Magliocco to reduce reactive oxygen species (ROS) and cytokine release and to upregulate SIRT1 and SIRT6. Overall, the results indicated that the quantitative and qualitative phenolic profiles of red wines influence their in vitro beneficial effects on oxidative and proinflammatory milieu in endothelial cells, showing a positive modulation of SIRT1 and SIRT6, both implied in vascular aging.

Chapter 3 focuses on wine oxidation and ageing, considering the concepts of sustainability and winemaking. The first part of the chapter provides an overview of the non-enzymatic oxidation of wine and the use of conventional protective and antioxidant agents during vinification. From this examination, also emerges that numerous factors, such as increasing consumer attention to health concerns and restrictive legislation on preservatives, together with the quest for environmentally friendly production, have driven the interest of the scientific community and manufacturers in alternative methods to traditional protective and antioxidant agents.

Based on these considerations, two case studies are presented in this chapter. The first case study is based on the need for information on the oxidative evolution of red wines by means of accelerated ageing tests. As already exposed in the previous chapters, phenols in wines vary in quality and quantity. It follows that it is of fundamental importance to assess how different types of red wines react to oxidation. This chapter reports the results of the study that evaluate the effect of three different oxygen addition systems on different red wines (Aglianico, Barbera, Magliocco, Gaglioppo and Nerello) with the aim of developing a tool for predicting the evolution of phenolic compounds in a short time. The results evidenced that oxygen saturation cycles and hydrogen peroxide addition tests have the same impacts on most of the wine chromatic properties and phenolic composition of the treated wines. Thus, both can be used as suitable methods to simulate red wine oxidation and to allow a discrimination among wines based on their possible evolution under oxygen exposure. The second case study aimed to investigate a sustainable tool, such as the use of chitosan, on the evolution of acetaldehyde in red wine

before and after exposure to an oxidative stress designed to simulate wine ageing and, on tannins by assessing their reactivity towards salivary proteins, one of the mechanisms underlying the sensation of astringency in red wines.

Chapter 4 turns its attention exclusively to acetaldehyde and the interactions it establishes with the main polyphenols in wine. After an initial description of the reasons for acetaldehyde accumulation in wine and its changes during ageing, the chapter reports the results of the case study:

How acetaldehyde reacts with low molecular weight phenolics in white and red wines. The results highlighted that the chemical behaviour of acetaldehyde turned out to be different in white and red wines. Specifically, a dramatic loss of monomeric anthocyanins and a simultaneous massive formation of polymeric compounds was detected in red wine. The results regarding red wine highlighted that the anthocyanin moiety is not preferentially attacked by the protonated acetaldehyde, which seems to react first and preferentially with flavanols due to their better nucleophilicity when compared to anthocyanins. Subsequently, after the loss of a water molecule, the acetaldehyde-flavanol adduct attacks either another flavanol or an anthocyanin unit. In our studies conducted on real wines (pH =3.69), we only detected polymers containing just one anthocyanin unit, even if molecules of unreacted malvidin-3-O-glucoside were still present in the analyzed samples. Instead, in white wines the formation of vinyl-flavan-3-ol derivatives was mainly detected. Infact, the comparison of the total ion chromatograms (TIC) of the untreated and treated white wine brought to light a significant decrease of the relative abundance of some chromatographic peaks along with a concomitant increase of others. Based on the results, it can be assumed that flavanols in white wine are helpful to quench acetaldehyde by affording more stable compounds. In addition, in red wines the acetaldehyde led to the formation of ethylidene bridged red pigments. These latter positively enhanced the color properties of red wines.

Chapter 5 addresses the description of the relationship between wine ageing and the perception of astringency.

As reported in the previous sections, important relationships between anthocyanins and flavan-3-ols are established leading to the formation of new, more stable complexes involving the ethanal bridge. These newly formed adducts appear to be involved in the modulation of astringency. However, the prediction of astringency of a specific group of compounds must consider the molecular origin of astringency that is not yet fully understood, and the connection or contribution of the various proposed mechanisms in

scientific literature is still unknown. The most accepted mechanism is based on the precipitation of salivary proteins by polyphenols. To date, the relationship with prolin rich protein PRPs, an important group of proteins subdivided into acidic, basic, and glycosylated PRPs, has been most investigated. PRPs associated with astringency are mostly basic and acidic. In addition to PRPs, other relevant salivary protein families that have been linked to astringency include α-amylase. Although α-amylase is the most abundant enzyme in saliva, the interaction of novel formation complexes and single molecules with α-amylase appears to be less investigated than with other proteins. On these bases, the final phase of the doctoral activity was directed toward the study of computational tools capable of investigating the interactions of anthocyanins and their polymeric pigments against salivary alpha-amylase; thus, an in silico molecular docking study was performed that could predict the preferred orientation of a receptor-bound ligand to form a stable complex. This computational investigation provides several insights into the mechanisms of interaction of salivary α-amylase binding with anthocyanins and their polymeric pigments that may be useful in enriching knowledge regarding the mechanisms involved in salivary protein precipitation and thus initiate the development of new wine aging strategies. Docking results indicated that both the two monomers as well as polymeric pigment showed excellent abilities to actively interact with the 1SMD protein by influencing its conformation. However, the binding energy between the three ligands and the enzyme is significantly different exerting different influence on astringency. The results confirm that catechin has a greater effect on astringency than malvidin. In addition, the astringency produced by the new formed pigment is greater than malvidin but lower than catechin. The results showed that docking tools can offer useful results regarding the interactions between polyphenolic compounds and salivary proteins. The kinetics and evolution of polyphenolic compounds during wine aging combined with computational study can offer extraordinary information for the development of appropriate aging strategies. This last activity was conducted in collaboration with the Randall Centre for Cell & Molecular Biophysics, within the Faculty of Life Sciences & Medicine at King's College London.

RIASSUNTO

La presente dissertazione riporta i risultati ottenuti dalle attività condotte nell'ambito del dottorato incentrato sul progetto di ricerca "*Wine oxidation and aging: a study on the key role of polyphenols and acetaldehyde*". L'obiettivo del progetto di dottorato è studiare il ruolo dei polifenoli e dell'acetaldeide, le interazioni che si stabiliscono tra loro e indagare i loro effetti sulla sfera della percezione sensoriale e non solo, al fine di meglio comprendere i fenomeni ossidativi alla base dell'invecchiamento del vino. L'invecchiamento è talvolta essenziale per migliorare la qualità del vino, soprattutto per il vino rosso. Non è un caso che i vini di alta qualità siano tradizionalmente prodotti mediante invecchiamento in botte, metodi lunghi e costosi che incidono significativamente sui fattori di costo e sulle capacità produttive delle cantine. Un vino giovane, ruvido, aspro, torbido, fioco e instabile, necessita di un tempo adeguato affinché si verifichino le reazioni di ossido-riduzione, esterificazione, condensazione, polimerizzazione tali da imprimere un carattere sensoriale completamente diverso. Durante questo periodo, per garantire i cambiamenti chimici e biochimici all'interno del vino ed evitare fenomeni di alterazione, vengono effettuati una serie di trattamenti che prendono il nome di invecchiamento del vino. Il metodo di invecchiamento tradizionale, definito invecchiamento naturale, è realizzato in botti di rovere che in virtù della loro particolare permeabilità all'ossigeno permettono una lenta e continua ossidazione del vino determinandone il miglioramento del colore, del sapore nonché il carattere astringente. Al fine di accorciare i tempi dell'invecchiamento tradizionale, negli ultimi anni lo sviluppo di metodi di invecchiamento alternativi è diventata un'area di ricerca di grande interesse. A tal proposito, la micro-ossigenazione rappresenta la tecnica più interessante di ossidazione e invecchiamento del vino. Questo metodo consiste nell'aggiungere al vino una quantità controllata di ossigeno in tracce per soddisfare le esigenze necessarie allo svolgimento delle reazioni fisiche e chimiche che si verificano durante il processo di invecchiamento. Sebbene la tecnologia dell'invecchiamento artificiale, in particolare quella basata sulla micro-ossigenazione, abbia fatto grandi progressi, le dinamiche e i cambiamenti che si verificano nei polifenoli, così come l'implicazione dei composti di neoformazione nella definizione delle caratteristiche sensoriali del vino, non sono abbastanza chiari. Inoltre, data la complessità dei fenomeni coinvolti, spesso manca una dettagliata descrizione dei meccanismi alla base della definizione delle caratteristiche sensoriali, come ad esempio l'astringenza. Nell'affrontare la tematica della micro-ossigenazione occorre tener conto che l'acetaldeide e i polifenoli rappresentano due

componenti di straordinaria importanza nella definizione delle caratteristiche qualitative del vino. Comprendere il ruolo delle singole molecole, la loro evoluzione nonché la loro possibile interazione e la conseguente influenza sulle caratteristiche qualitative finali sarebbe di indubbio interesse. Inoltre, appare fortemente auspicabile che l'azione di questi composti sia armonizzata con i due principali cardini attorno ai quali ruotano le scelte del consumatore moderno, quali la qualità salutistica e la sostenibilità del vino. Pertanto, il progetto di ricerca di dottorato nella prima fase ha rivolto l'attenzione alla relazione tra i polifenoli del vino e la salute, nonché al rapporto tra i polifenoli e gli approcci enologici sostenibili. Lo studio di queste relazioni e in particolare del rapporto tra tecniche sostenibili e polifenoli coinvolge inevitabilmente il ruolo dell'ossidazione e dell'acetaldeide. È stata quindi studiata la partecipazione dell'acetaldeide nell'interazione tra i diversi polifenoli e nella formazione di nuove strutture molecolari. Inoltre, l'effetto dei singoli polifenoli e dei composti di neo-formazione è stato studiato con metodi computazionali.

I risultati ottenuti dalla realizzazione delle attività previste dal progetto di ricerca sono riportati in cinque capitoli.

Il **capitolo 1,** partendo dalla consapevolezza che la qualità del vino non è legata solo alla sua composizione alcolica, vuole essere una panoramica sui composti fenolici e sul loro ruolo ampiamente riconosciuto dalla letteratura scientifica. Nello specifico, il capitolo distingue e descrive preliminarmente le diverse classi di composti fenolici, riportando, poi, i principali fattori che ne determinano la presenza, la concentrazione e l'azione nel vino.

Il **capitolo 2**, incentrato sui polifenoli, si propone di valutare la posizione e il ruolo del vino nella dieta mediterranea e la sua relazione con la salute. In dettaglio, la prima parte mira a fornire un'analisi bibliometrica della letteratura sull'associazione tra dieta mediterranea, vino e salute. I risultati ottenuti dall'analisi dei dati mostrano una forte relazione tra la dieta mediterranea e il vino rosso, nonché la loro interconnessione con la protezione della salute. Interessante è il legame tra il vino incluso nella dieta mediterranea e i temi quali "riduzione del rischio", "rischio cardiaco" e "ipertensione". Dall'analisi è emersa una certa attenzione alla relazione tra i polifenoli del vino rosso e le sirtuine, una specifica famiglia di proteine che mediano un'ampia varietà di eventi fisiopatologici. Tuttavia, va sottolineato che gli studi relativi all'argomento specifico oggetto della ricerca e dell'analisi bibliografica sono davvero pochi. Sulla base di queste considerazioni, nella seconda parte del capitolo è stata studiata la relazione tra il contenuto fenolico e la

bioattività dei vini rossi. La caratterizzazione NMR, HPLC/UV-Vis e spettrofotometrica ha mostrato che il Magliocco è il vino più ricco di antociani monomerici, catechine e fenoli a basso peso molecolare (LMWP). Prove in vitro su modelli di cellule endoteliali di insulino-resistenza e iperglicemia hanno mostrato la capacità del Magliocco di ridurre le specie reattive dell'ossigeno (ROS) e il rilascio di citochine e di upregolare SIRT1 e SIRT6. Nel complesso, i risultati hanno indicato che i profili fenolici quantitativi e qualitativi dei vini rossi influenzano i loro effetti benefici in vitro sul milieu ossidativo e proinfiammatorio delle cellule endoteliali, mostrando una modulazione positiva di SIRT1 e SIRT6, entrambi implicati nell'invecchiamento vascolare.

Il **capitolo 3** si concentra sull'ossidazione e sull'invecchiamento del vino, considerando i concetti di sostenibilità e vinificazione. La prima parte del capitolo fornisce una panoramica sull'ossidazione non enzimatica del vino e sull'uso di agenti protettivi e antiossidanti convenzionali durante la vinificazione. Da questo esame emerge anche che numerosi fattori, come la crescente attenzione dei consumatori alle preoccupazioni per la salute e la legislazione restrittiva sui conservanti, insieme alla ricerca di una produzione rispettosa dell'ambiente, hanno spinto l'interesse della comunità scientifica e dei produttori verso metodi alternativi ai tradizionali agenti protettivi e antiossidanti.

Sulla base di queste considerazioni, in questo capitolo vengono presentati due casi di studio. Il primo caso di studio si basa sulla necessità di ottenere informazioni sull'evoluzione ossidativa dei vini rossi attraverso test di invecchiamento accelerato. Come già esposto nei capitoli precedenti, i fenoli nei vini variano in qualità e quantità. Ne consegue che è di fondamentale importanza valutare come i diversi tipi di vini rossi reagiscono all'ossidazione. Questo capitolo riporta i risultati dello studio che mirava a valutare l'effetto di tre diversi sistemi di aggiunta di ossigeno su diversi vini rossi (Aglianico, Barbera, Magliocco, Gaglioppo e Nerello) con l'obiettivo di sviluppare uno strumento per prevedere l'evoluzione dei composti fenolici in tempi brevi. I risultati hanno evidenziato che i cicli di saturazione dell'ossigeno e i test di aggiunta di perossido di idrogeno hanno lo stesso impatto sulla maggior parte delle proprietà cromatiche e sulla composizione fenolica dei vini trattati. Pertanto, entrambi possono essere utilizzati come metodi adatti a simulare l'ossidazione dei vini rossi e a consentire una discriminazione tra i vini in base alla loro possibile evoluzione sotto l'esposizione all'ossigeno. Il secondo caso di studio mirava a indagare uno strumento sostenibile, come l'uso del chitosano, sull'evoluzione dell'acetaldeide dei vini rossi prima e dopo l'esposizione a uno stress ossidativo progettato per simulare l'invecchiamento del vino. Sono state inoltre valutate

le variazioni dei tannini valutando la loro reattività nei confronti delle proteine salivari, uno dei meccanismi alla base della sensazione di astringenza nei vini rossi. I risultati ottenuti suggeriscono il possibile l'uso di chitosano per il trattamento di vini ricchi in tannini condensati e in possesso di una eccessiva astringenza poichè agisce positivamente sia sulla produzione controllata di acetaldeide a seguito dello stress ossidativo che sull'evoluzione dei tannini in grado di precipitare le proteine salivari.

Il **capitolo 4** rivolge l'attenzione esclusivamente all'acetaldeide e alle interazioni che essa instaura con i principali polifenoli del vino. Dopo una prima descrizione delle ragioni dell'accumulo di acetaldeide nel vino e dei suoi cambiamenti durante l'invecchiamento, il capitolo riporta i risultati del caso di studio: "How acetaldehyde reacts with low molecular weight phenolics in white and red wines". I risultati hanno evidenziato un diverso comportamento chimico dell'acetaldeide nei vini bianchi rispetto ai rossi. In particolare, nel vino rosso è stata rilevata una drastica perdita di antociani monomerici e una contemporanea massiccia formazione di composti polimerici. I risultati relativi al vino rosso hanno evidenziato che la parte antocianica non viene attaccata in modo preferenziale dall'acetaldeide protonata, che sembra reagire prima e in modo preferenziale con i flavanoli in virtù della loro migliore nucleofilia rispetto alle antocianine. Successivamente, dopo la perdita di una molecola d'acqua, l'addotto acetaldeide-flavanolo attacca un altro flavanolo o un'unità antocianica. Nei nostri studi condotti su vini reali (pH =3,69), sono stati rilevati solo polimeri contenenti una sola unità antocianica, anche se nei campioni analizzati erano ancora presenti molecole di malvidina-3-O-glucoside non reagita. Invece, nei vini bianchi è stata rilevata soprattutto la formazione di derivati del vinil-flavan-3-olo. Infatti, il confronto dei cromatogrammi ionici totali (TIC) dei vini bianchi non trattati e trattati ha messo in luce una significativa diminuzione dell'abbondanza relativa di alcuni picchi cromatografici e un concomitante aumento di altri. Sulla base dei risultati, si può ipotizzare che i flavanoli presenti nel vino bianco contribuiscano a spegnere la reattività dell'acetaldeide dando origine a composti più stabili. Inoltre, nei vini rossi l'acetaldeide porta alla formazione di pigmenti rossi a ponte etilico. Questi ultimi migliorano le proprietà cromatiche dei vini rossi.

Il **capitolo 5** affronta la descrizione della relazione tra l'invecchiamento del vino e la percezione dell'astringenza. Come riportato nelle sezioni precedenti, si stabiliscono importanti relazioni tra antociani e flavan-3-oli che portano alla formazione di nuovi composti più stabili che coinvolgono il ponte etanale. Questi composti di nuova formazione sembrano essere coinvolti nella modulazione dell'astringenza. Tuttavia,

l'origine molecolare dell'astringenza non è ancora del tutto chiarita e il collegamento o il contributo dei vari meccanismi proposti è ancora sconosciuto. Il meccanismo più accettato si basa sulla precipitazione delle proteine salivari da parte dei polifenoli. Ad oggi, è stata maggiormente studiata, la relazione con le proteine ricche in proline (PRP), un importante gruppo di proteine suddivise in acide, basiche e glicosilate. Le PRP associate all'astringenza sono per lo più quelle basiche e acide. Oltre alle PRP, sono state collegate all'astringenza altre famiglie di proteine salivari che includono l'α-amilasi. A tal proposito, è da notare che sebbene l'α-amilasi sia l'enzima più abbondante nella saliva, l'interazione di nuovi composti di formazione e di singole molecole con l'α-amilasi sembra essere meno studiata rispetto ad altre proteine. Su queste basi, la fase finale dell'attività di dottorato è stata indirizzata allo studio di strumenti computazionali in grado di indagare le interazioni delle antocianine e dei loro pigmenti polimerici con l'alfa-amilasi salivare; è stato quindi eseguito uno studio di docking molecolare in silico in grado di prevedere l'orientamento preferito di un ligando legato al recettore per formare un complesso stabile. Questa indagine computazionale fornisce diversi spunti di riflessione sui meccanismi di interazione dell'α-amilasi salivare con le antocianine e i loro pigmenti polimerici, che possono essere utili per arricchire le conoscenze sui meccanismi coinvolti nella precipitazione delle proteine salivari e quindi avviare lo sviluppo di nuove strategie di invecchiamento del vino. I risultati del docking hanno indicato che sia i due monomeri sia il loro prodotto di reazione hanno mostrato eccellenti capacità di interagire attivamente con la proteina 1SMD influenzandone la conformazione. Tuttavia, l'energia di legame tra i tre ligandi e l'enzima è significativamente diversa ed esercita una diversa influenza sull'astringenza. I risultati ottenuti confermano che la catechina ha un effetto maggiore sull'astringenza rispetto alla malvidina. Inoltre, l'astringenza prodotta dal loro prodotto di reazione è maggiore rispetto alla malvidina ma inferiore alla catechina. I risultati hanno dimostrato che gli strumenti di docking possono offrire risultati utili per quanto riguarda le interazioni tra composti polifenolici e proteine salivari. La cinetica e l'evoluzione dei composti polifenolici durante l'invecchiamento del vino, combinata con lo studio computazionale, può offrire informazioni straordinarie per lo sviluppo di strategie di invecchiamento appropriate. Quest'ultima attività è stata condotta in collaborazione con il Randall Centre for Cell & Molecular Biophysics, all'interno della Facoltà di Life Sciences and Medicine del King's College di Londra.

CHAPTER 1

WINE POLIPHENOLS

1.1 FUNDAMENTALS OF POLYPHENOLS

The PhD dissertation is shaped by the awareness that the quality, value, and charm of wine is not only related to composition in alcohol, acids, minerals, proteins, and volatile compounds, but also to phenolic compounds and their evolution during aging.

Phenolic compounds are natural substances that are composed of one or more hydroxyl groups attached to one or more aromatic or benzene rings. Polyphenols can be found in many vegetables and fruits, including grapes, and therefore in must and wine. However, their content in wine is greatly influenced by the type of grape used, the technological practices applied [Garrido and Borges 2013], the type of yeast used in the alcoholic fermentation, the contact with solid parts of the grape during the maceration and the aging processes [Baiano et al. 2014].

Properties of polyphenols have been widely studied and more recently numerous strategies and factors, during winemaking, have been evaluated to manage the phenolic content in wines [Forino et al. 2019; Gambuti et al. 2020; Rinaldi et al. 2021. The design of a certain type of wine starts with the selection of a grape variety and its behavior under the invariable climate conditions in the growing region. In fact, the climate was found to have the most influential effect on grape composition, followed by soil and cultivar. The results showed that water available on the grapevine is a decisive parameter for the phenolic compound concentration. Autochthonous varieties are usually well adapted to the local climatic parameters. To increase the phenolic content in grapes, bio-stimulants are usually added to the grapevine during the grape ripening process. Once grapes have been harvested, the winemaker may act at different stages. During the pre-fermentative stage, maceration and thermovinification are recommended for young red wines, these having a significant effect on phenolic compounds, especially anthocyanins since they are easily extracted in aqueous media.

Using mixed culture non-*Saccharomyces* plus *Saccharomyces* has been observed to affect the phenolic composition of final wines. Lastly, post-fermentative maceration can be suggested for wine aged in barrels since tannins are easily extracted in alcoholic media. The selection of additives, fining agents, filters, stoppers, and storage conditions play an

important role in the phenolic composition of wine, the choice depending on the type of wine desired.

Regarding phenolic extraction methods to be used for the analysis of phenolic compounds in wine, many authors did not use any kind of sample pretreatment to this end, only dilution in some cases. However, when high sensibility and less interference is required, other alternatives are available such as liquid–liquid microextraction, solid-phase microextraction, headspace sportive extraction, ultrasound- assisted extraction, and the microwave-assisted technique.

It is important to remember that a great difference between red and white wine exists because of red wines are exposed to all grape parts during the winemaking process. For this reason, their polyphenol concentration is higher (1–5 g/L) than in white wines (0.2–0.5 g/L). In the latter the contents of polyphenols are essentially originated from the pulp, while in red grape winemaking polyphenols are extracted from pulp, skin and seeds of grapes [Visioli et al. 2020].

Rose wines present an intermediate polyphenol content, with values between those for red and white wines [Baiano et al. 2014; Paixao et al. 2007]. The polyphenols in wine determine many of its sensory properties such as appearance, color, astringency, bitterness, and flavor [Avizcuri et al. 2016; Alcalde-Eon et al. 2014], and its stability through subsequent oxidative processes (browning in white wines and oxidation in red wines). It has been considered that polyphenol compounds have a significant influence on aroma compounds because they are usually associated with volatile compounds through intermolecular interactions with important consequences on the loss of aroma [Dufour et al. 2000]. It is for example the case of malvidin that can be bonded to acetosyringone, syringaldehyde, acetovanillone, vanillin, 3,5-dimethoxyphenol, and 4-ethylguaiaco. Or catechin, caffeic acid, and quercetin, which are commonly bonded to aroma compounds such as isobutyl methoxypyrazine, 3-mercaptohexanol, 3-mercaptohexanol acetate, and ethyl decanoate. However, a deep study about the influence of these unions in the aroma and sensory properties has not been carried out. In addition, they contribute to the health-promoting properties of wine mentioned in the previous section [Manach et al. 2004].

According to their chemical structure, polyphenols present remarkably diverse structures, from simple phenolic acids to high molecular mass polymeric forms such as hydrolysable and condensed tannins, respectively.

Most of them are found in conjugated forms with sugar residues by *β*-glycosidic bonds (*O*-glycosylated) or by direct bonds of the sugar to a carbon atom of the aromatic ring (C-glycosides) [Visioli et al. 2020].

Glucose is the main sugar in fruit skins, which is why many phenolic compounds are bonded to it. However, they can also be found bonded to galactose, rhamnose, xylose, arabinose, as well as glucuronide, galacturonic and other acids [Manach et al. 2004]. Another factor that could modify the phenolic compounds' nature and that should be considered is malolactic fermentation. Malolactic fermentation is catalyzed by bacteria acid lactic that decarboxylates malic acid to lactic acid, which results in deacidification, which affects polyphenolic compounds in a different manner depending on their structure. It is, for example, the case of anthocyanins that turned into their colorless form during this process due to the changes in pH and the increment in acidity. Or some glucosides can suffer hydrolysis reactions for the change in pH due to malolactic fermentation [Ugliano et al. 2003]. Moreover, malolactic fermentation provides microbiological stability and improves the final aroma balance by modifying fruit-derived aromas and producing aroma-active compounds [Wang et al. 2020]. Apart the fact that that the phenolic composition of red and white wine is affected by a great number of viticultural and technological factors, it also important to underline that grape and wine phenolic compounds constitute an extensive family of phenolic compounds, which are grouped into flavonoids and non-flavonoids.

1.1.1 Flavonoids

Flavonoids are made up a C_{15} (C_6-C_3-C_6) type structure (benzene) linked by a 3-carbon chain cyclized through oxygen (Figure 1.1). This carbon skeleton and the multiple radicals bonded to it are responsible for the chemical diversity of this family [Garrido and Borges 2013]. All the flavonoids found in grapes and wine have a hydroxyl group in position 5 and 7 of the A ring [Waterhouse, 2002]. The antioxidant action of flavonoids depends mainly on their ability to reduce free radicals and chelate metals (Cu and Zn), preventing the catalytic reactions of free radicals. This family comprises anthocyanidins, flavanols, flavonols, flavanones, flavones, chalcones and tannins (condensed tannins).

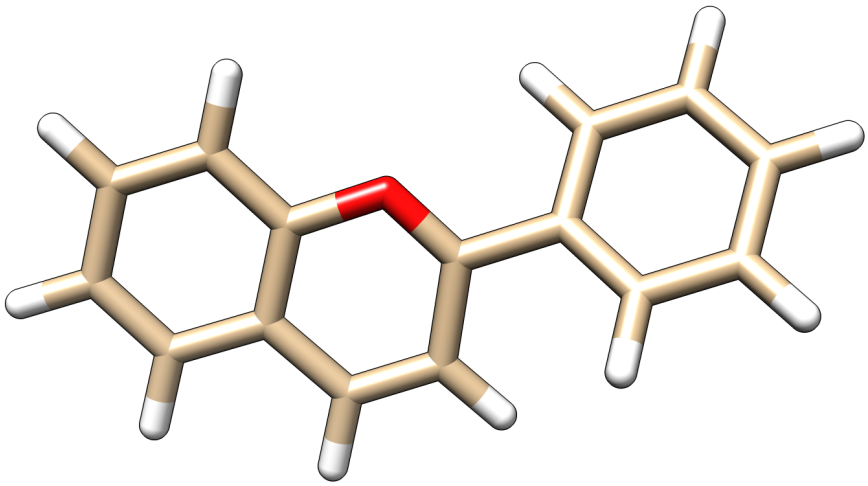

Figure 1.1 *Basic structure of flavonoids*

- *Anthocyanidins* are natural, water-soluble pigments responsible for the red color of grapes and red wines. Anthocyanin pigments are mainly composed of aglycones (anthocyanidins) bonded to sugar (anthocyanins). Five anthocyanidins have been identified in both grape and wine: delphinin, cyanidin, petunidin, peonidin and malvidin. The color of the anthocyanin changes depending on the pH, the concentration of sulfur dioxide, and the copigments present in the wine. At a low pH (less than 4), all greater part of anthocyanidins are in the flavan (red) cation form. When the pH increases, the intensity of color changes from colorless to violet or blue in alkaline or neutral solutions. The anthocyanin concentration can range between 90 and 400 mg/L in red wines [Waterhouse, 2002], sometimes concentrations above 700 mg/L can be found in young red wine, whereas in white wine they are absent [Visioli et al. 2020]. When anthocyanidins interact with other phenolic compounds in wine, a phenomenon known as co-pigmentation occurs, which usually enhance the color [Boulton, 2001].

- *Flavanols* (flavan-3-ols) are found in monomeric form (catechin and epicatechin) and in their polymeric form (proanthocyanidins, also called condensed or non-hydrolysable tannins) [Gambuti et al. 2004]. The following flavan-3-ols are the main ones found in the skin and seed of grapes: (+) catechin, (−) epicatechin, epigallocatechin and epicatechin 3-*O*-gallate [Jeffery et al. 2008]. Flavanols are responsible for the stabilization of the color and for important sensory characteristics (mainly astringency and bitterness) of wines [Hornedo-Ortega et al. 2020]. The concentration of monomeric forma range detected in young white wine is from 15 to 25 mg/L, and from 4 to 120 mg/L in young red wine [Visioli et al. 2020; Piñeiro et al. 2004].

- *Flavonols* [Garrido and Borges 2013; Visioli et al. 2020] are yellow pigments found in the skin of grapes characterized by a double bond between C_2 and C_3 and by the presence of a hydroxyl group in position 1. They are usually present in glycosidic forms, linked to a sugar (glucose or rhamnose), but other molecules such as galactose, arabinose, xylose or glucuronic acid may also be involved. The main flavonols described in grapes and wine are myricetin, quercetin, laricitrin, kaempferol, isorhamnetin and syringetin. Flavonols are present in both white and red wines. In red wines the yellow color is masked by the purplish red of the anthocyanidins. However, they play an important role in the color stabilization of young red wines, through the copigmentation interaction with anthocyanidins [Arcena et al. 2020]. In addition, they have an important role in the sensory perception of astringency and bitterness. In red wine, the maximum content described is 60 mg/L [Zafrilla et al. 2003].

- *Condensed tannins* are the result of the condensation of flavanols (flavan-3-ols). Epicatechin is the most abundant monomer present in condensed tannin in grapes and wine, followed by catechin. B-type proanthocyanidins, and in particular dimers B1, B2 and B4 or trimer as the procyanidin C1, are mainly located in grape skins and seeds [Rasmussen et al. 2005]. These tannins change during the aging of the wine and can form insoluble polymers [Kennedy et al. 2007]. Natural condensed tannins can be found at concentrations levels from 1.2 to 3.3 g/L [Basalekou et al. 2019].

- *Flavanones* have a saturated carbon chain between the C_2 and C_3 atoms, of ten named dihydroflavones by analogy with the flavones [Garrido and Borges 2013]. Naringenin is the main compound in grapes, reaching 25 mg/kg in reds and 7.7 mg/kg in whites [Visioli et al. 2020].

- *Flavones* are characterized by the presence of a double bond between carbons C_2 and C_3 and by the absence of a hydroxyl group in the C_3 position. Isoflavones are isomers of flavones, displaying the aromatic ring B in the C_3 position [Garrido and Borges 2013]. Flavones can be present in wine in levels ranging from 0.2 to 1 mg/L [Agatonovic-Kustrin et al. 2015; Cabrera-Bañegil et al. 2017].

- *Chalcones* are a subclass of flavonoids with two aromatic rings linked by a carbonylic *α, β*-unsaturated system. Chalcone derivatives are important intermediates and are precursors for a vast range of flavonoid derivatives found in grapes or wine [Garrido and Borges 2013].

1.1.2 Non-flavonoids

Non-flavonoids form an extensive family within polyphenols, generally having a simpler structure than that of flavonoids. They are mainly composed of phenolic acids (hydroxybenzoic acids and hydroxycinnamic), and stilbenes [Garrido and Borges 2013; Visioli et al. 2020]. These groups can reach a concentration range from 60 to 566 mg/L in red wine [Castaldo et al. 2019].

- *Hydroxybenzoic acids* have a C_6-C_1 structure derived from benzoic acid. The most abundant are *p*-hydroxybenzoic, gallic, vanillic, gentisic, syringic, salicylic, and protocatechuic acids [Rentzsch et al. 2009]. The total amount of hydroxybenzoic acids in red wine is expected to range from undetectable to 218 mg/L [Castaldo et al. 2019]. Gallic acid is considered the most important phenolic acid in red wine with a concentration of around 70 mg/L, while levels can reach 10 mg/L in white wine. It stands out for being the precursor of all hydrolysable tannins.

- *Hydroxycinnamic acids* have a C_6-C_3 structure, are very abundant, diverse and all come from cinnamic acid. The main examples are caffeic, coumaric, sinapic and ferulic acids, essentially conjugated with tartaric acid esters or diesters [Ferreira-Lima et al. 2018]. Hydroxycinnamic acids are the third most abundant group of polyphenols in grapes and the predominant group in must and white wine. They are easily oxidizable and are associated with wine browning processes. They also are precursors of volatile phenolic compounds [Kallithraka et al. 2009]. The average amount of hydroxycinnamic acids quantified is about 100 and 30 mg/L in red and white wines, respectively although some authors have found higher concentrations: 130 mg/L in white wines and 60 mg/L in red wines [Visioli et al. 2020].

- *Stilbenes* are bioactive compounds consisting of two aromatic rings linked by ethyl positions. The main sources of stilbenes in the human diet are grapes and their derivatives: juice and wine [Guerrero et al. 2009]. The main stilbenes described in *Vitis vinifera* wines are *trans*-piceid and *trans*-resveratrol, with hopeaphenol, ampelosin A, isohopeaphenol, piceatannol, pallidol, ε-viniferin, miyabenol C, r-viniferin, r2-viniferin also being detected [Lambert et al. 2013; Houillé et al. 2015]. They are found naturally in wine, but at low concentrations (0–5 mg/L) [Fernández-Mar et al. 2012]. However, when grapes are exposed to biotic or abiotic stress, the levels of resveratrol (the most studied compound), its glycoside called piceid, and its dimeric and trimeric forms (e.g., pallidol, viniferins) may range from negligible up to more than 100 mg/L [Visioli et al. 2020]. Recently, some stilbenes have been quantified by UPLC-MS/MS, *trans*-piceid

being the most abundant in white wine (average of 155 µg/L), and *cis*- and *trans*-piceids and hopeaphenol in red wine (average of 3.73 and 3.16 mg/L, respectively) (mean 1.55 mg/L) [Guerrero et al. 2020].

- *Tyrosol* is a natural phenolic antioxidant compound found mainly in olive oil, although there are studies that have detected it in white and red wines. Some results showed values up to 45 mg/L in white wine and between 20 and 60 mg/L in red wines [Garrido and Borges 2013].

- *Hydroxytyrosol* (HT) (3,4-dihidroxifeniletanol) is a phenyl ethyl alcohol, mainly responsible for the antioxidant properties of olive oil. In 2011, it was accepted as protective compound against oxidative damage. It is found naturally in red wine at concentrations between 1.98 and 3.89 mg/L [Fernández-Mar et al. 2012; Boselli et al. 2006]. It seems to be synthesized during alcoholic fermentation by yeasts [Rodriguez-Naranjo et al. 2011].

- *Hydrolysable tannins* are high molecular weight molecules, composed mainly of esters of gallic acid (gallotannins) and ellagic (ellagitannins) bonded to glucose or other sugars. They are more susceptible to hydrolysis than condensed tannins induced by pH changes, enzymatic or non-enzymatic processes [Castaldo et al., 2019]. The hydrolysable tannins are not found in *Vitis vinifera*, only in grapes of the muscadine subgenus and in wines aging in barrels and are thus proposed in the literature as a marker of maturity. The final content of hydrolysable tannins can vary widely, from 0.4 to 50 mg/L [Ma et al. 2014; Rentzsch et al. 2009].

All the above polyphenols largely define the quality of wine, due to their contribution to its sensory properties: color, taste, mouthfeel, flavor, astringency, and bitterness [Garrido and Borges 2013; Li and Sun, 2019].

1.2 WINE POLYPHENOLS, THE RESULTS OF SEVERAL FACTORS

Polyphenols have a significant role in affecting wine quality, in particular contributing to sensory properties such as color and astringency [Arnold, Noble & Singleton, 1980].
In addition to technological tools, such as the viticulture practices and the winemaking process [Ricardo-da-Silva et al. 1992; Spranger et al. 2004] or aging and storage conditions of wine [Pérez-Magariño and González-San José, 2004; Sun et al. 2011], the content of polyphenolic compounds in wine can be influenced by grape variety and berry ripening degree [Pérez-Magariño et al. 2004], as well as vine growing methods, related

training systems [Pérez-Lamela et al. 2007; Pérez-Magariño et al., 2004; Peterlunger et al. 2002] and therefore by the *terroir.*

The term "*terroir*" is a French term derived from terre, which was coined to denote the special characteristics of a place, which interact with plant genetics in agricultural products such as wine, coffee, chocolate, tea and cheese [Trubek, 2008]. In particular, French winemakers used the concept of *terroir* extensively by identifying differences in wines from different regions or vineyards. In fact, today the concept of *terroir* is used to describe the unique aspects of a place, which influence and shape the wine produced [Ewing-Mulligan et al., 2011].

Terroir can be defined as an ecosystem in which vines interact with soil and climate. Soil influences grape ripening through soil temperature, water supply, and mineral supply. Soil temperature has a significant effect on vine phenology. Limited water supply to the vines limits shoot and berry growth, which is essential to achieve a grape composition suitable for the production of high-quality red wines. Secondary metabolites, such as polyphenols (anthocyanins, tannins), are mainly influenced by soil and climate conditions, such as altitude, air temperature, UV radiation, soil and vine water status [Van Leeuwen et al. 2018].

1.2.1 Effect of air temperature

High altitude and the related lower air temperature have an influence on the ripening and polyphenolic composition of grapes [Xing et al., 2016]. Coklar [2017] found that under the effect of low temperatures in high elevation vineyards, the total monomeric anthocyanin content in 'Ekşikara' whole berries and skins was significantly higher than that at lower altitude. The author also found that tannin levels in grape seed and skin for the high site were significantly higher than those at the low site. Moreover, higher total phenolic content in whole berries, skins and seeds were obtained for the high site.

The high-altitude regions and their associated climatic conditions seem to be favourable to produce certain types of phenolic compounds, whereas the climatic conditions in low-altitude regions appear to be favourable for the production of other types. Higher levels of anthocyanins monoglucosides and trihydroxylated flavonols in skins of 'Cabernet-Sauvignon', 'Carmenere', 'Syrah' and 'Merlot' were generated in the western regions in China (up to 1214 m a.s.l.) characterised by large day- and night-time temperature differences. However, the grapes from the lower altitude eastern regions near the sea characterized by higher daily minimum temperatures and small day- and night-time temperature differences, had a higher proportion of acylated anthocyanins and

dihydroxylated flavonols [Liang et al. 2014]. Furthermore, Coklar [2017] reported that with decreasing altitude, the relative amount of malvidin-3-O-glucoside in both skin and whole berry of 'Ekşikara' grapes increased, whereas that of petunidin-3-O-glucoside, cyanidin-3-O-glucoside, peonidin-3-O-glucoside and acylated anthocyanins in whole berry decreased. This study, however, did not decouple the effect of temperature from UV radiation.

In another study performed by de Oliveira B. et al. [2019], the difference in altitude had a significant impact on 'Syrah' grapes. The concentrations of total phenols (1,440 mg/ kg fresh fruit), non-flavonoids (200 mg/kg), flavonoids (1,240 mg/kg) and total anthocyanins (890 mg/ kg) at the site at 1100 m a.s.l. were significantly greater than those at the site at 350 m altitude (450, 130, 320 and 350 mg/ kg fresh fruit respectively). This result could be explained by the fact that the high-altitude region is characterized by large day- and night-time differences in temperature and by the fact that the maximum temperatures during the productive cycle stayed below 30 °C, thus favouring the accumulation and preservation of phenolic compounds during the grape ripening period. The grape skins at the high site contained higher levels of total condensed tannins, including monomeric, oligomeric, and polymeric 3-flavanol, whereas the grape seeds contained higher levels of the same compounds at the low site. On the other hand, the concentrations of *trans*-resveratrol in the first and the second year of the study were 5.71 and 8.17 mg/Kg fresh fruit respectively at the low site and 4.11 and 4.72 mg/Kg fresh fruit respectively at the high site. The synthesis of stilbenes is induced by environmental stresses; therefore, the maximum daily temperatures that exceeded 30 °C during berry growth and ripening in the low-altitude area may have stressed the vines, resulting in higher resveratrol levels.

'Cabernet-Sauvignon' wines made from grapes grown at different altitudes (774, 960, 1350 and 1160-1415 m a.s.l.) were studied by Falcão et al. [2007] in Santa Catarina State, Brazil. Altitude did not affect the concentrations of α- and β-ionone and β –damascenone in the produced wines. However, in both winter and summer, the highest levels of 2-methoxy-3-isobutylpyrazine (MIBP) were found in wines of the highest site (1415 m a.s.l.), where the temperature was lower in comparison to the other studied sites. Consequently, these wines were correlated with a "bell pepper" aroma, whereas wines from the lowest altitude (774 m a.s.l.) were correlated with a "red fruits" aroma. Grape MIBP content is closely linked to viticulture parameters, like the growing temperature [Allen et al. 1994]; in a previous study, lower temperatures during the period preceding

véraison had a greater impact on the MIBP content than after the grapes had matured [Lacey et al. 1991].

However, in some winegrowing regions and depending on the geographic location of the vineyards, it is possible for a higher altitude cultivation site to have warmer conditions that affect the sensory attributes of the produced wines; for instance, in a study performed by Alessandrini et al. [2017], the high site (380 m a.s.l.) was warmer than the low one (200 m.a.s.l.) due to its higher heat accumulation degrees favouring the accumulation and preservation of the aroma compounds and enhanced the elegance and the floral aroma of the produced wine. The levels of volatiles were most likely temperature-dependent rather than altitude-dependent. The minimum air temperatures at the low site were approximately 2 ˚C lower than those at the high site and were thus considered to be the main limiting factor for the biosynthesis of the aroma compounds at the lower altitude.

Rotundone is described by Ferreira [2012] as being an important wine aroma-impacting compound, and the anecdotal evidence that rotundone was more common in 'Shiraz' wines from cool climate locations was corroborated in an Australian red wine survey [Black et al. 2015]. Geffroy et al. [2016] evaluated the variability of its concentrations in 'Gamay N' wines in four French wine-growing areas. The results showed that Auvergne - the coolest vineyard over the whole wine-growing season and ripening period - had the highest rotundone concentrations and the most intense peppery notes. Peppery aroma scores and the concentration of rotundone in wine were shown to be significantly correlated. However, the effects of temperature and vine water status were not totally differentiated in this study: the vineyard with the highest rotundone content was also the wettest during the véraison-harvest period.

In cooler vintages and vineyards, higher levels of rotundone are expected to accumulate in 'Vespolina' grapes [Caputi et al. 2011]. The highest levels of rotundone were obtained in 'Shiraz' berries from vines exposed to cooler temperatures, showing a within-vineyard variation and that the topography of the vineyard, particularly the aspect, was the most important factor in the formation of rotundone [Scarlett et al. 2014]. In another study, rotundone was typically present at the top and in shaded areas of bunches of 'Shiraz' grapes, which correlates with lower grape surface temperatures, and its concentration was negatively affected by fruit temperatures above 25 °C [Zhang et al., 2015a]. Furthermore, a study on fifteen vintages of 'Shiraz' wine produced from the same vineyard block at the same winery showed that wines from cooler seasons tend to contain higher levels of

rotundone [Zhang et al. 2015b]. Water abundance was another influential factor in this study, since a higher amount of rotundone was detected in wetter vintages.

1.2.2 Effect of UV radiation

The increase in UV radiation noted at higher altitudes promotes the synthesis of skin anthocyanins; this can explain why wines produced from high-altitude regions have a higher colour intensity [Jin et al. 2017]. At higher altitudes, the increase in UV-B radiation can reach 8 % per decade leading to the enhancement of color, flavonol and tannin synthesis in red grapes on the one hand [Van Leeuwen and Darriet, 2016], and to higher concentrations of 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) - which can give the wine an intense and sometimes unpleasant smell of hydrocarbons.

UV-B up-regulates the biosynthesis of anthocyanin in grapevines, counteracting the negative effects of increasing atmospheric carbon dioxide and increased temperature on anthocyanin content in berries [Gutiérrez-Gamboa et al. 2021]. Exposure to UV-B has been found to slow down berry growth and increase the biosynthesis of anthocyanins and flavonols [Martínez-Lüscher et al. 2016]. It also leads to an increase in levels of both total bound glycosidic secondary metabolites and phenolics [Keller and Torres-Martinez, 2004; Lafontaine et al. 2005], and in the synthesis of anthocyanins in grape skins [Berli et al. 2008; Carbonell-Bejerano et al. 2014].

In Bolivia and Argentina, some wines produced from vineyards located higher than 1500 m a.s.l. were found to have higher total antioxidant capacity and phenolic content (including resveratrol) than wines produced from vineyards located at a lower altitude [Osorio-Macías et al. 2018]. UV-B radiation activates the phenylpropanoid biosynthesis pathway, resulting in phenolic-based resistance against Botrytis cinerea that causes grey mould or *botrytis* bunch rot in grapes [Elmer and Reglinski, 2006; Gutiérrez-Gamboa et al. 2021]. Plant responses to heat stress at high altitudes may be regulated by abscisic acid and salicylic acid [Larkindale and Huang, 2005]. Abscisic acid plays a role in the response of grape leaf tissues to UV-B radiation by augmenting the synthesis of UV-absorbing compounds, antioxidant enzymes and membrane sterols [Gutiérrez-Gamboa et al. 2021]. Salicylic acid might be synthesized by the grapes as a defense response to stress conditions, such as UV radiation that increases with increasing altitude; Jin et al. [2017] found that its amount increased dramatically with altitude in the wines produced from ‘Merlot’ and ‘Cabernet-Sauvignon’.

The phenolic content of ‘Carignan’ grapes grown in two different locations, referred to as early and late ripeness parcels corresponding to a higher (370 m a.s.l.) and lower (305

m a.s.l.) altitude respectively, was studied by Edo-Roca et al. [2013]. The total leaf area (TLA) in the early (warm) parcel (3.4 m2/vine) was smaller than the TLA in the late (moderate) parcel (4.8 m2/vine). As a result, the grapes in the warm parcel were more exposed to solar radiation and synthesised larger amounts of anthocyanins. Furthermore, flavonol compounds can serve as natural sunscreen for grapes; their synthesis is induced by light and their levels are positively related to radiation. Karaoğlan et al. [2015] showed that the highest amount of quercetin-3-O-glycoside (5.16 mg/L) was obtained for the 'Muscat of Bornova' wine from the site at the highest altitude and with the highest solar radiation (587,465 W/m2). Furthermore, the amounts of quercetin-3-O-glycoside found in 2.22 and 3.88 mg/L of lower altitude wines correlated with solar radiation values of 542,875 and 553,782 W/m2, respectively.

Plants accumulate large quantities of various types of compatible solutes and important osmoprotectants (e.g., proteins, proline and carbohydrates) in response to diverse stresses, caused by, for instance, low temperature and UV radiation. These osmoprotectants protect plants from stressing conditions by adjusting cellular osmosis, protecting membrane integrity and contributing to the detoxification of reactive oxygen species and the stabilization of proteins/enzymes activities [Hayat et al. 2012]. Indeed, Berli et al. [2008] showed that the application of a high solar UV-B treatment under field conditions to 'Malbec' grapevine leaves in a high-altitude vineyard (1450 m a.s.l.) in Argentina augmented the levels of photoprotective pigments and proline, thereby increasing the antioxidant capacity of leaves.

1.2.3 The combined effect of soil and vine water status

In many studies, the effect of soil and vine water status cannot be clearly distinguished from the altitude factor. Vines with limited access to water in a vineyard cultivated with 'Carignan' grapes at a higher altitude and characterized by a shallow stony soil produced smaller berries with higher anthocyanin content compared to another vineyard at a lower altitude with a deeper soil [Edo-Rocaet al. 2013].

Soil and water availability affect the branch points of the biosynthetic pathway of flavonoids in grape berries, which are regulated by a number of enzymes [Li et al. 2011]: flavonoid 3'-hydroxylase (F3'H) and flavonoid 3', 5'-hydroxylase (F3'5'H) convert dihydrokamepferol into dihydroquercetin and dihydromyricetin, in addition to their derivatives, respectively. Cyanidin-derived anthocyanins and delphinidin-derived anthocyanins are synthesized from dihydroquercetin in the F3'H branch pathway and dihydromyricetin in the F3'5'H branch pathway respectively in the downstream pathways

of both branches. Li et al. [2011] found that the regional 'Cabernet-Sauvignon' wines from vines at an altitude of 1900-3500 m a.s.l. in China contained the greatest levels of both quercetin derivatives and cyanidin-derived anthocyanins, while the regional wines from vines at an altitude of 214 m a.s.l. contained the second highest level of myricetin derivatives and the highest levels of delphinidin-derived anthocyanins. The authors related this result to different factors, such as soil and water availability, which might have promoted the directional flow of carbon into the myricetin synthetic branch or to the quercetin synthetic branch. The region at the highest altitude is characterized by a brown sandstone soil and a specific water retention curve (the relationship between soil water content and soil water pressure head). This could incite the flow of more carbon to the F3'H branch pathway, leading to higher concentrations of quercetin derivatives and cyanidin-derived anthocyanins in the grapes and in the resulting wines. The authors did not exclude possible interference of other factors, like the cool-warm climate existing in the higher altitudes and the warm climate existing in the lower altitudes.

A study performed on wines from 'Muscat of Bornova' grapes grown in Turkey found that phenolic levels were the highest in wines of the Halilbeyli sub-region (115 m a.s.l.), followed by wines of the Menderes (90 m a.s.l.) and Kemaliye (245 m a.s.l.) sub-regions [Karaoğlan et al. 2015]. In particular, the concentrations of *trans*-caftaric and *trans*-coutaric acids in the produced wines from the Kemaliye sub-region (39 and 5.8 mg/L respectively) were lower than those of the Menderes sub-region (55 and 23.6 mg/L respectively) and the Halilbeyli sub-region (80 and 25.3 mg/L respectively). The lowest values obtained at Kemaliye may be related to the soil composition of this sub-region, which is characterised by having the highest percentage of lime, which affects water retention and drainage.

1.2.4 The effect of altitude other parameters

Many studies have shown that other factors, such as humidity, vintage and grapevine variety, are closely associated with temperature, UV radiation and vine water status. The lower humidity of the high cultivation sites (at approximately 2900 m a.s.l.) in southwest China induced an increase in the production of cyaniding-type anthocyanins and quercetin-type flavonols from the F3'H branch of the flavonoid biosynthetic pathway in Cabernet-Sauvignon, when compared with sites at lower altitudes (2300 and 2150 m a.s.l.) [Xing et al. 2016]. Another study on the effect of altitude on carotenoids of 'Touriga Franca' and 'Touriga Nacional' grape varieties was conducted by Oliveira et al. [2004] in

the Douro Valley. The altitudes ranged from 85 and 145 to 180 m a.s.l. for 'Touriga Franca' and from 90 and 155 to 210 m a.s.l. for 'Touriga Nacional'. High-elevation terraces with higher humidity levels promoted the accumulation of carotenoids, which degrade during the grape ripening period to form odour-active C13-norisoprenoids.

Mateus et al. [2001] showed that at the lower cultivation locations (100-150 m a.s.l.), humidity levels of 95-100 % during the night were not favourable for the biosynthesis of total anthocyanidin monoglucosides in the grape skins of 'Touriga Nacional' and 'Touriga Francesa' compared to the higher sites (250-350 m a.s.l.). Nevertheless, higher concentrations of catechin monomers and low-molecular procyanidin oligomers in the grape skins of both varieties, as well as of total extractable proanthocyanidins in the skins of both cultivars and the seeds of 'Touriga Nacional', and of low-molecular procyanidin oligomers in the 'Touriga Nacional' wine, were obtained at the lower altitudes. None of these studies, however, separated the effect of humidity from temperature.

The latitude effect was more pronounced than the altitude effect for Malbec wines produced by different regions in Argentina. The vineyards at latitudes of 31-33° produced the most desired sensory attributes in wine (e.g., floral, sweetness, cooked fruit, and raisin) in contrast to regions outside these latitudes which exhibited sourness, bitterness, and a strong herbal aroma [Goldner and Zamora, 2007]. The levels and the composition of phenolics in grapes vary because of vine vigour and water inputs. The variability in microclimatic conditions of the grapevines may be caused by vine vigour heterogeneity [Asproudi et al., 2016]. When compared to less vigorous vines, high vigour vines usually have lower fruit exposure and a greater fruit MIBP content [Mendez-Costabel et al. 2014]; as a result of increasing water input from rainfall and irrigation, MIBP synthesis can increase.

Vintage is one of the main factors to influence the concentrations of proanthocyanidins in grapes, in addition to the effect of air temperature, which varies between altitudes. Of the monomeric and the small oligomeric compounds in Syrah seeds, de Oliveira B. et al. [2019] found higher levels of gallocatechin, epigallocatechin and B1, B2, B3 and B4 dimers at a higher altitude (1100 m a.s.l.) in the first year of their study, and high concentrations of catechin and B2 dimers esterified with gallic acid at a lower altitude (350 m a.s.l.) in the second year. Vintage was observed to have a higher effect than altitude for 'Chardonnay' and 'Pinot Noir' cultivated in Minas Gerais, Brazil [Regina et al. 2010]; a greater number of sunny days in the growing season contributed to a greater accumulation of anthocyanins and phenolic compounds in the grapes.

The highest content of total phenols and flavonoids in 'Moscatel' grape stems sampled form three different regions in northern Portugal at 120, 670 and 730 m a.s.l. were found in the lowest altitude region over two consecutive vintages [Gouvinhas et al. 2020]; nevertheless, they increased significantly in the second year of the study, which was characterised by an atypical summer with a 3-day heat wave (temperatures above 40°C) near the beginning of the harvest. Under these stressful conditions, which scalded the grapes, the plants produced secondary metabolites as a defense mechanism. The obtained lower levels of total phenols and flavonoids in the high-altitude regions may be attributed to the absence of significant water or thermal stresses in these regions. However, higher biological capacities were induced by high precipitations and the climate, which has an Atlantic influence in the low-altitude regions.

The number of polyphenols in 'Ancellotta', 'Lambrusco', 'Negroamaro', 'Nero d'Avola', 'Fiano' and 'Garganega' cultivated at 1300 m a.s.l. was greater in the year which had a higher number of rainy days [Malinovski et al. 2016]. This result was correlated with the fact that fungal diseases are common in areas with high rainfall, inducing plants to produce phenolic compounds as a stress response. The intensity of this response was found to vary depending on the grape varieties, showing that different cultivars respond differently even when subject to the same stressful climatic conditions.

The antimicrobial activity in 'Moscatel' grape stems was shown by the multivariate analysis to be lower in the high-altitude regions (730 m a.s.l.) than in the lower altitude regions (120 m a.s.l.) [Gouvinhas et al. 2020]. This antimicrobial activity seemed to be more affected by the genetic characteristics of the grape stem varieties than by the climate conditions and altitude of the growing sites. Jiang et al. [2013] concluded that the magnitude of the effect of environmental factors on the volatile compounds in Cabernet-Sauvignon and Merlot wines produced from four wine-growing regions in China (at 214, 450–600, 1036 and 1100 m a.s.l.) may be related to the cultivar.

Different studies have shown that the effects of temperature and sunlight cannot be isolated from the effect of vine water status. In high-altitude areas in China (2282, 2435 and 2608m a.s.l.), due to a decrease in temperature and an increase in rainfall and sunlight hours, the quercetin, *trans*-resveratrol and tannin content in 'Cabernet-Sauvignon' wines increased with increasing altitude [Jin et al. 2017]; however, no significant effect of altitude on the same parameters was found in 'Merlot' wines. Overall, altitude followed by sunlight hours mostly affected the phenolic characteristics and antioxidant activity of tested red wines. Vine water status is a key determinant of terroir expression, which

depends on climatic conditions, such as rainfall and evapotranspiration [van Leeuwen et al. 2020]. In a study conducted on 'Grenache' berries [Edo-Roca et al. 2013], the effects of vine water status and temperature were not completely differentiated: levels of anthocyanins in grapes declined in the last ripening control in the warmest year of the study, in which dryness and high temperatures occurred during a 3-day heat wave (temperatures reaching an unusual 40 °C) in the period before harvest.

1.2.5 Effect of Viticulture and Viniculture Factors and Wine Elaboration

There are many factors which influence the biodegradation of flavonoids synthesis in vineyard, including nutritional status, microbial interactions, pathogenesis, defoliation, plant growth regulators, and various developmental processes.

The content and composition of polyphenols can be handled by viticultural practices such as canopy management, irrigation, yield regulation and harvest timing. [Downey et al. 2006]. Several Authors [Artem et al. 2016; Van Leeuwen et al 2018] have highlighted the influence of terroir on biosynthesis of phenols and their accumulation in the grape berries. Moreover, during the winemaking process, phenolic content varies with grape crushing, primary fermentation, malolactic fermentation, oak aging, and bottle aging. In fact, generally phenolic concentration shows very low levels at the time of crushing, higher concentrations during the fermentation phase and stable or slightly lower concentrations during the aging period. The age of the oak barrel determines different influences on the quantity of polyphenols [Ginjom et al. 2011; Gambuti et al., 2007].

To increase the content of polyphenols in red wine, appropriate must maceration is necessary. Cell walls and cytoplasmic membranes are resistant to mass transfer, which results in only partial extraction of phenolic compounds from grape skin. Maceration techniques weaken the cell walls in different ways. Traditional maceration involves storage of crushed grapes for several days at low temperature or fermentation with the must. High temperature applied during thermo-maceration induces cell destruction but may also degrade sensitive compounds, such as anthocyanins. Pectinases, hemicellulases or cellulases may be also used for enzymatic hydrolysis of cell walls and for degradation of pectin substances. However, as described by Paranjpe et al [2012], the enzymes may destabilize anthocyanins by affecting their deglycosylation.

In this regard, it must also be considered that maceration techniques must be chosen individually for each kind of wine to achieve optimal properties, as the yield of the target compounds depends on an optimal combination of maceration conditions and grape

cultivar [Carew et al. 2014]. An important aspect of red wine quality and consumer preferences is its color. Various maceration techniques affect the isolation of anthocyanins which are important for obtaining intensely red wine. Several Authors [Barros et al. 2022; Paladines-Quezada et al. 2019] indicated that cold pre-fermentative maceration and autolysis positively influenced the bioactive potential and the color of the red sparkling wines Wojdyło, et al. [2021] highlighted that pre-treatment in the form of microwave maceration may be recommended to produce red wine, due to high efficiency of polyphenolic extraction resulting in better quality of the must. Moreover, Guler et al. [2023] showed that the microwave and microwave and sonication enhanced the polyphenols with strong antioxidant power, such as catechin and *trans*-resveratrol, by comparison with the enzymatic technique.

1.2.6 Wine Storage

Ageing, co-pigmentation and the formation of new pigments.

Ageing of wine, defined as the duration of time after bottling until consumption, is a factor that is crucial for the taste and quality of wines since during the storage time phenolic compounds undergo several chemical transformations. Changes or isomeric transformation or degradation/fragmentation of phenolic compounds cause changes in color and sensory characteristics relative to the original ones. For example, some anthocyanins convert to flavanol–anthocyanin adducts, oligomers, polymeric pigments that develop different colors and tastes [He et al. 2012]. Phenolic compounds undergo condensation and fragmentation with increasing storage time. Older red wines have higher levels of polymeric pigments while younger wines have higher levels of free anthocyanins. However, the formation of polymeric pigments depends on various factors such as pH, phenolic content, oxygen permeability, and microorganisms used during the processing. The effect of aging leads also to the evolution of flavonols and flavones contents in wine with aging time [Feng et al. 2007]. In a study on the effect of aging of red wines prepared from *Vitis vinifera L*. such as Tempranillo, Graciano, and Cabernet Sauvignon it was found that the level of anthocyanins decreased up to 43–66% whereas those of poranthocyanidins were increased. Cassino et al. [2019] analyzed the composition of 10 different red wines that were bottled and stored in a wine storage cellar for 24 months. They found that there was a decrease in the levels of organic acids but an increase in the esters such as ethyl acetate and ethyl lactate. The ageing process is controlled by multiple factors, such as chemical reactions among the ingredients, storage

temperature, barrels, and stoppers. The influence of grape variety, vineyard location, and grape harvest maturity, combined with different oxygen availability treatments, on red wine composition during bottle aging was investigated by numerous authors [Zhang et al. 2020, Koundouras et al. 2006, van Leeuwen et al. 2022.]. Recently Ma et al. [2022] reported that grape variety, vineyard location, and grape maturity had a greater influence on wine composition than bottle-aging conditions. They found that factors such as vineyard location, grape variety, and grape maturity contributed significant influences on the evolution of total and free aldehyde compounds in wine during bottle aging.

Storage temperatures play a significant role in the transformation of phenolic compounds in red wine over time which is directly related to the kinetics of the reactions that occur during the aging process. Cassino et al. [2019] reported that the phenolic contents increased by 40% of their initial levels when some red wine (Barbera, Nebbiolo, Ruchè and Grignolino) were stored for 4 years at 12 °C. In a comparative study to determine the role of different temperatures, de Esteban et al. [2019] observed that red wines preserved at around 15 °C contained higher anthocyanin and tannin levels compared to those preserved at temperatures around 25 °C.

It is well known that the color of red wine is mainly due to presence of anthocyanins. Moreover, the color of anthocyanins in wine is dependent on the pH of the aqueous solution. At a lower pH, the red color of anthocyanin is due to the flavylium cation, as the pH increases the color of the anthocyanins degrades. As a result, the color of the red wines (pH 3.2−4.0) is likely to fade because the colorless hemiketals (>70%) present in such wines are in equilibrium with other forms [Escribano-Bailón et al. 2012]. However, the color is not simply due to the concentration of anthocyanins but is higher than expected. This is possible due to the association of anthocyanin with other compounds (usually non-colored) present in the solution, this phenomenon is known as co-pigmentation. Co-pigmentation stabilizes and enhances the color of red wine [Rustioni et al. 2012]. In nature, the potential color enhancement is fixed for a given pigment-cofactor pair and the observed color in solution depends on the concentration of pigment, the molar ratio of a cofactor to the pigment, pH, the extent of non-aqueous conditions, and the anions in solution. It appears that there should be a minimum concentration of anthocyanin available before significant co-pigmentation is detectable [Rustioni et al. 2012]. The addition of phenolic acids such as caffeic acid and p-coumeric acids during the winemaking process of cabernet Sauvignon and Pinot Noir wines indicated that p-coumaric acid was a stronger co-pigment compound than caffeic acid [Gutiérrez et al.

2005]. Different types of compounds including alkaloids, amino acids, nucleotide, metals, and phenolic compounds act as co-pigments. Flavonoids, particularly flavanols and flavonol, and hydroxycinnamoyl derivatives, have been reported to be some promising co-pigments. Flavonols are one of the major flavonoids but are weaker co-pigments than flavanols owing to their non-planar structures. Epicatechin is a better co-pigment than catechin.

When wine ages new pigments deriving from the reactions of anthocyanins with other wine components determine a stabilization of color by producing new pigments. Some of the these colored adducts occur because of direct reactions between anthocyanins and flavan-3-ols forming the dimeric-type flavanol-(4,8)-anthocyanin (F–A) and anthocyanin-(4,8)-flavanol (A–F) adducts [Escribano-Bailón et al. 2012; Bimpilas et al. 2016].

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CHAPTER 2

MEDITERRANEAN DIET, RED WINE AND POLYPHENOLS

The Mediterranean Diet is characterized by a high intake of phenolic compounds, which are present in the main key foods of this dietary pattern: extra-virgin olive oil (EVOO), nuts, red wine, legumes, vegetables, fruits, and whole-grain cereals. Phenolic compounds, usually called polyphenols, are important candidates responsible for the beneficial effects of the Mediterranean diet. Actually, polyphenols have a wide variety of beneficial properties, including anti-inflammatory and anticancer activity. The mechanisms underlying these properties are not fully understood but are attributed in part to antioxidant activity. In addition, the antitumor effects of polyphenols vary with cancer type, cell lines, and doses. It is noteworthy that some polyphenols, such as tannins, are being investigated to have genotoxic effects at high concentrations. Therefore, the use of these polyphenols in cancer treatment should be cautious. In addition, clinical studies on the antitumor actions of polyphenols are limited. Therefore, in the future, the following are needed: further epidemiological studies using biomarkers of polyphenols to assess the impact of these compounds on cancer risks; further evaluation and comparisons among multiple polyphenols; and further studies on mechanisms of action and bioavailability. Special attention should be paid to safety, so further investigation with effective doses is needed to obtain results regarding both prevention and therapy in humans.

2.1 MEDITERRANEAN DIET, DEFINITION

The Mediterranean diet is usually described as a dietary model that comprises high intakes of extra virgin olive oil, vegetables, including green leafy vegetables, fruits, cereals (unrefined grains), nuts, and legumes with moderate intakes of fish and other animal protein, and low intakes of eggs and sweets [Mennotti et al. 2015]. For the first time, in the 1950s and 1960s, the American physiologist Ancel Keys and his Italian colleague, recognized an inverse association between the Mediterranean diet pattern and several diseases. Through an epidemiological study, known as the "Seven Countries' Study," Keys studied the dietary behaviors, lifestyles and health status of 12,763 subjects aged 40 to 59 years residing in different countries around the world: the United States of America, Finland, the Netherlands, Italy, Greece, the former Yugoslavia and Japan. The results

were surprising because lower rates of mortality and incidence of cardiovascular disease were shown in countries bordering the Mediterranean basin than in the other countries studied. Based on the evidence found, the physiologist emphasizes characters of health interest associated with the particular low saturated fat, high vegetable oil diet observed in Greece and southern Italy.

Over the years, increasing evidence has supported this initial conclusion, thus the Mediterranean diet has been extensively reported to be associated with a favourable health outcome and a better quality of life. In recent decades, it has been promoted worldwide as one of the healthiest dietary patterns and has been reported to have benefits regarding chronic diseases, i.e., cardiovascular illness, breast and colon cancer, cognition and longevity [Estruch et al. 2018; Villarini, et al. 2016; Grosso et al. 2014; McEvoy et al. 2019; Boccardi et al. 2013].

In the past decade, along with numerous studies, an updated meta-analysis of cohort studies associating adherence to the Mediterranean diet with the occurrence of chronic degenerative diseases has been published. The results confirmed a significant reduction in the incidence of mortality and cardio- and cerebrovascular diseases [Sofi et al. 2010].

Regarding the relationship between the Mediterranean diet and cardiovascular disease (CVD), an updated meta-analysis of cohort studies associating adherence to the Mediterranean diet with the occurrence of chronic degenerative diseases has been published in the past decade, along with numerous studies. The results confirmed a significant reduction in the incidence of mortality and cardio- and cerebrovascular diseases. Estruch et al. [2018] demonstrated, in a prospective randomized, controlled clinical trial enrolling patients at high risk of CVD, decreased incidence of cardiovascular events in patients assigned to a Mediterranean diet supplemented with extra virgin olive oil or walnuts compared to those as- marked to a reduced-fat diet. The synergism between cardioprotective nutrients and foods of the Mediterranean Diet appears to trigger the mechanisms responsible for reducing the risk of CVD. These mechanisms include reductions in blood pressure, lipids, endothelial dysfunction, and increased nitric oxide (NO) bioavailability, antioxidant properties, and anti-inflammatory effects [Jacobs et al. 2009].

In a recent review, Finicelli et al. [2022] asserted that the Mediterranean diet and its components appear to have a positive impact on the recovery of endothelial dysfunction in CVD patients due to its regulatory effect on fatty acids. The same authors also highlight the beneficial impact on cardiovascular risk regardless of sex and that the

Mediterranean diet can be considered as a possible nutraceutical supplement to enhance the beneficial effect of medical therapy on cardiovascular risk factors.

Moreover, the relationship between cancer occurrence and diet has been demonstrated by numerous studies [Piccirillo et al. 2022; Merra et al. 2022]. Many features of the Mediterranean diet have been shown to be beneficial in reducing the incidence of neoplastic diseases, hypothesizing that adoption of a Mediterranean-style diet can prevent about 25 percent of colorectal cancer, 15-20 percent of breast cancer, and 10-15 percent of prostate, endometrial, and pancreatic cancers [Sofi et al. 2013; 2014; Djuric et al. 2022].

In addition, the broad-spectrum neuroprotective activity of the Mediterranean diet is widely documented, and its nutritional and caloric balance seems likely to induce modulation of the endocannabinoid system. In recent decades, many studies have shown how the enhancement of endocannabinoid tone may be a promising new therapeutic strategy to counteract the major hallmarks of neurodegeneration. From a phylogenetic point of view, human co-evolution between the endocannabinoid system and dietary habits could play a key role in the pro-homeostatic activity of the Mediterranean lifestyle: this adaptive balance among our ancestors has been compromised by the modern Western diet, resulting in the "clinical endocannabinoid deficiency syndrome" [Scoditti et al. 2002].

The Mediterranean Diet's health power lies on the foods' heterogeneity, allowing the proper intake of key molecules, such as long-chain polyunsaturated fatty acids, antioxidant vitamins, carotenoids, and polyphenols. In detail, special attention has been paid to the protective function expressed by polyphenols with special reference to the antioxidant function in preventing cellular and extracellular damage related to oxidative stress [Annunziata et al. 2018; Kawabata et al. 2019; Fakhri et al. 2022; Rudrapal et al. 2022; Gasmi et al. 2022; Kruk et al. 2022]. Several authors have shown that polyphenols have several functions, including antioxidant, antimicrobial, anti-inflammatory, antiangiogenic, and antitumor [Cory et al. 2018]. In addition, numerous phenolic substances are determinants in the mechanisms of action in reducing inflammatory responses in the human body [Tangne et al., 2013; Hussain et al. 2016; Yahfoufi et al. 2018].

Raw vegetables, together with the processed products derived from them, represent the most important source of polyphenols. They are classified into several groups based on their different chemical structures. Flavonoids, lignans, stilbenes and phenolic acids are

the four main families of polyphenols. So far, a great deal of in vivo and in vitro research has been conducted to evaluate their health consequences. Polyphenols play a crucial function in protecting the organism from external stressors and in eliminating reactive oxygen species (ROS), which are the cause of various diseases.

Therefore, foods naturally rich in polyphenols have the potential to positively affect human health. For example, flavan-3-ols in cocoa have been associated with reduced risk of myocardial infarction, stroke, and diabetes. Polyphenols in the diet also contribute to improved lipid profiles, blood pressure, insulin resistance, and systemic inflammation. Quercetin, a flavonoid, and resveratrol, a stilbene, have been linked to improved cardiovascular health. The potential of dietary polyphenols to elicit therapeutic effects could be attributed, at least in part, to a bidirectional association with the gut microbiome. Indeed, polyphenols are known to influence the composition of the gut microbiome in ways that lead to improved human health. Specifically, the gut microbiome converts polyphenols into bioactive compounds with therapeutic effects [Rana et al. 2022].

2.1.1 Mediterranean Diet-Wine Association

The cultural and nutritional aspects of the Mediterranean civilization also include wine, when used in moderation, as a central element of health and well-being [Sofi et al. 2014]. Indeed, Mediterranean meals provide food microcomponents including polyphenols, vitamins, fibers, polyunsaturated fatty acids, and oligoelements present in fruits, vegetables, olive oil, fish, infusions. In addition, wine, especially the red variety, provide additional unique polyphenols with antioxidant properties, such as resveratrol, procyanidins, and monophenols, including hydroxytyrosol and tyrosol [McEvoy et al. 2019].

Interestingly, red wine is conceived to be one of the three pillars of the Mediterranean diet along with bread and olive oil. In Mediterranean diet the alcohol intake is primarily in the form of red wine, which is consumed in moderate amounts [Diolintzi, et al. 2019]. Since the early St. Leger et al. [1974] and Framingham studies [Hubert et al. 1983], a lot of epidemiological evidence has accumulated, pointing to the existence of inverse relationships between light to moderate alcohol consumption and incidences and mortality of cardiovascular diseases [Corraro et al. 2000; Di Castelnuovo et al. 2006; Larsson et al. 2018], as well as of other chronic disorders like type 2 diabetes or dementia and cognitive decline in old age [Huang et al. 2017]. The relationship has been described

as a U- or J-shaped curve, with a minimum situated at a level of consumption around 10 to 30 g of alcohol/day. These studies are not free from debate, as they have been attributed to suffer from methodological limitations, which may have led to misinterpretations or biased conclusions [Naimi et al. 2019]. Nevertheless, despite possible bias, many authors agree that when confounding factors are specifically adjusted, epidemiological trials still continue to be remarkably consistent regarding the beneficial effects from low to moderate alcohol/wine intake on CVD morbidity and mortality, as well as diabetes, osteoporosis, and neurological disorders [Rehm and Roerecke 2017].

A point of discussion is whether the purported wine benefits are due to ethanol or to other components. It is known that ethanol itself is able to increase HDL-cholesterol, prevent platelet aggregation, and enhance fibrinolysis, which may have positive effects on the cardiovascular system [Covas et al. 2010]. However, when differentiation among drinks is made, it is generally concluded that wine provides superior health benefits to other alcoholic drinks either regarding protection against CVD type 2 diabetes, or dementia. This perception has also been supported by the results obtained in human clinical studies and observations over Mediterranean cohorts [Liberale et al. 2019; Vázquez-Fresno et al. 2016].

The intended superior benefits of wine have been related to its phenolic compounds, which are absent or in very low concentrations in other alcoholic drinks. Wine contains a variable mixture of flavonoid and non-flavonoid compounds, extracted from the grape during winemaking. Phenolic contents in red wine are usually well above 1 g/L, concentrations that are higher than those that can be found in most fruits and vegetables while, in white wine, it does not commonly exceed a few hundred mg/L, due to the fact that it is not normally submitted to maceration with grape solids during winemaking [Waterhouse, 2002]. Actually, red wine is one of the richest dietary sources of procyanidins, a type of compound recognized to possess a range of biological activities, and that is related with the disease preventive properties of plant-based diets [Rasmussen et al., 2005]. Red wine is also rich in anthocyanins (especially young red wine) and flavonols, with acknowledged biological activities, including antioxidant, anti-inflammatory, antiproliferative, or gene modulating abilities, which are also considered to contribute to the health protective effects of fruits and vegetables.

Polyphenols, and especially flavonoids, have been proposed to be the main vasoactive components in red wine. They have been reported to be able to modulate the plasmatic lipid profile to a healthy shape, reducing triglyceride and LDL-cholesterol circulating

levels [Rifler et al. 2012]. They may also improve both systolic and diastolic blood pressure, stimulate endothelial-dependent vasodilation by enhancing nitric oxide (NO) generation, decrease platelet aggregation, and inhibit the activity of inflammatory enzymes and the production of several types of proinflammatory and oxidant mediators. Many recent reports have been published dealing with the putative health effects of polyphenols, either from wine or other plant sources, and their possible mechanisms of action [Fraga et al. 2019].

In addition to polyphenols, other bioactive phenolic and non-phenolic components can also be present in wine that might contribute to the putative health effects and that are usually less considered. Thus, during must fermentation, yeasts catabolize aromatic amino acids such as tyrosine, tryptophan, and phenylalanine to their respective aromatic alcohols, tyrosol, tryptophol, and phenyl ethanol, which also possess bioactive properties and are also associated with some of the beneficial effects of moderate wine consumption [Mas et al. 2014]. Tyrosol has been indicated to be the second most abundant non-hydroxycinnamate phenolic in many wines, with concentrations that may reach up to 95 mg/L. Its antioxidant and anti-inflammatory properties were suggested to contribute to the beneficial effects attributed to a moderate consumption of wine. Among others, tyrosol was found to be able to inhibit the LPS-induced production of pro-inflammatory cytokines tumor necrosis, like factor alpha (TNF-α), and interleukins IL-1β and IL-6 in human peripheral blood mononuclear cells at nanomolar concentrations, either alone or in synergy with caffeic acid. Hydroxytyrosol is also present in wine in levels under 10 mg/L [Bordiga et al. 2016], but it can also be formed in the human organism from hydroxylation of tyrosol. De la Torre et al. [2006] found that the consumption of moderate doses of wine or olive oil by healthy subjects led to a higher increase in urinary concentrations of hydroxytyrosol in the wine group, even though the amount of hydroxytyrosol administered was fivefold greater in the olive oil group (1.7 mg vs. 0.35 mg). This was explained by the biotransformation of tyrosol to hydroxytyrosol; besides, the alcohol could help to increase the bioavailability of the tyrosol present in the wine. The authors indicated that a single glass of wine was at least equivalent to 25 mL (22 g) of virgin olive oil in its capacity to increase hydroxytyrosol concentrations in the body, leading to similar beneficial effects. The same Authors found that there was a direct association between wine consumption and the urinary concentrations of tyrosol and hydroxytyrosol determined in individuals at cardiovascular risk [Pérez-Mañá et al. 2015], suggesting that the endogenous formation of hydroxytyrosol might explain part of the

cardiovascular benefits associated with light-to-moderate wine consumption.

Another bioactive compound that may contribute to the health benefits of wine is melatonin (n-acetyl-5-methoxytryptamine). This is a neurohormone secreted from the pineal gland, with well-characterized antioxidant, anti-inflammatory, and immune-modulating properties. It also contributes to the regulation of the circadian rhythms and has been attributed to tumor inhibitory activities and positive effects on the cardiovascular system, lipid, glucose metabolism, and neuroprotection [Meng et al. 2017]. It is present in grapes and can also be formed in wine from tryptophan metabolism by yeasts [Fernández-Cruz et al. 2019]. Its content in wine is mostly influenced by the fermentation process, where the yeast strain and the fermentation time are the most influential factors [Fernández-Cruz et al. 2019]. It has been shown that blood levels of melatonin and total antioxidant capacity in plasma increased after the dietary intake of food containing it [Aguilera et al. 2016]. Melatonin concentrations ranging from a few μg/L to more than 150 μg/L have been reported in wine, which is higher than those found in most fruits and vegetables. Moreover, most fruits and vegetables are usually situated in the low ng/g level, with only a few products, such as mushrooms, coffee beans, or some berries showing contents in the μg/g range [Meng et al. 2017]. Therefore, wine can be considered a significant source of dietary melatonin, though it is not unlikely that it could be a contributor to the beneficial effects associated with wine consumption.

A moderate consumption of wine, especially red wine, has been proposed to provide some degree of protection against cardiovascular diseases, diabetes mellitus, or cognitive decline, which has been related to its polyphenol content. The available studies in this respect are, however, limited by their observational nature, and there is a lack of randomized clinical trials that may prove a causal relationship. Furthermore, wine contains alcohol, which even at moderate consumption increases the risks of liver disorders and several types of cancers, among other diseases, Although the Mediterranean habit of drinking wine with meals may delay ethanol absorption and favor its more rapid clearance, while it may contribute to a decrease in postprandial oxidative stress produced after a meal. Furthermore, although polyphenols present in wine are also found in fruits and vegetables that lack the risks associated with alcohol, the concomitant presence of ethanol in the food bolus might make wine polyphenols more bioavailable. Some authors have, however, highlighted that a high wine and total alcohol intake, particularly by men, can represent a problematic aspect of the Mediterranean diet that may have not been critically evaluated. Indeed, the potential risks of wine consumption, even at moderate

doses, may have been overlooked or undervalued by many authors, which inadvertently may have disclosed a confusing message, although not only restricted to the context of the Mediterranean diets. Certainly, we can't think to wine or any other alcoholic drink as an element for health promotion, but the risks of alcohol should always be considered in the first place. Releasing any message that might induce people to drink in the hope of gaining health benefits could likely have more harmful than beneficial consequences. All in all, it is not easy to give a simple answer to the question of whether wine should be considered a key food contributing to the beneficial health outcomes of the Mediterranean Diet. Despite the fact that it is excluded from the diet in many Mediterranean areas for religious reasons, we do think that it definitely constitutes a distinguishing feature of many Mediterranean cultures and plays an undeniable part of their historical legacy. In those regions, wine can be a relevant contributor to polyphenol intake and could be considered a side element in the beneficial health effects of the Mediterranean Diet, if it is consumed in the 'traditional' way, that is, light to moderate regular consumption with meals.

2.1.2 Wine and Nutrients for Longevity

Wine, especially red wine, is a complex beverage in continuous evolution, even when it is in the bottle. It is a hydroalcoholic solution (~78% water) that includes many different chemical components, such as minerals, sugars, lipids, soluble proteins, vitamin aldehydes, esters, ketones, phenolics, and organic acids [Fernandes et al. 2017].

Among the many bioactive compounds contained in wine, the polyphenols represent the main class. White wines usually contain less polyphenols than red ones: total polyphenol content has been reported in amounts of about hundreds of mg GAE·L−1 (gallic acid equivalents·L−1) in white wine, whereas in red wine the amounts are about thousands of mg GAE·L−1 [Li and Sun 2019].

Polyphenols contained in grapes, also highlighted recently by Lucarini et al. [2019] predominantly represented by proanthocyanidins (oligomers and polymers) and anthocyanins, with lower amounts of other phenolics. Wine polyphenols include both grape polyphenols and new phenolic products formed from them during the winemaking process. This leads to a great diversity of new polyphenols and makes wine polyphenol composition more complex.

A phenolic compound that has been frequently associated with the putative beneficial effects of wine is the resveratrol (3,4′,5-trihydroxy-trans-stilbene), a naturally occurring polyphenolic stilbene that has been steadily gaining attention in the management of

cancer, owing to its abundance in nature as well as satisfactory tolerance in patients, in addition to marked health benefits following administration. Resveratrol can be found in grape skin and is extracted into wine during winemaking. The average contents of resveratrol in wine does not usually exceed a few mg/L [Waterhouse, 2002]. Since white wine is not usually submitted to maceration with grape solids, it possesses lower resveratrol concentrations than red wine.

Dietary sources of resveratrol are scarce and, in addition to grapes, they include rhubarb, peanuts, or berries, though they are always present in low levels. Actually, grapes and wine are considered the most relevant food sources for humans [Benbouguerra et al. 2021]. Stilbenes are synthetized by plants in response to biotic or abiotic stress, so that exposure to UV radiation can induce the formation of resveratrol in grapes, increasing its concentration by up to tenfold. Post-harvest UV irradiation has been employed as a strategy to increase resveratrol levels to "functionalize" grapes [Triska et al. 2012].

The presence of resveratrol in wine was firstly described in 1992 [Jang et al. 1997], suggesting that it might be an active component in the lowering effects of serum lipids associated with wine consumption. Since then, a high number of studies have been published reporting a diversity of bioactivities and multiple potential health outcomes for stilbene, including antioxidant, anti-inflammatory, anti-obesity, chemopreventive, glucose-modulating, cardiovascular protective, or calorie restriction mimicking effects. The ability of resveratrol to inhibit the enzymatic activity of both forms of cyclooxygenase (COX1 and COX2), suggesting that it may behave as an anti-inflammatory and anticarcinogenic agent [Baur et al. 2006].

Further studies showed that it was able to enhance stress resistance and extend lifespan in various model organisms, including Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster, fish, and mice. Those effects were related to the activation of Sir2 proteins (*sirtuins*), a family of NAD^+-dependent deacetylases and mono-ADP-ribosyltransferases involved in key regulation processes, such as glucose and insulin production, fat metabolism, the regulation of the p53 tumour suppressor, and cell survival [Valenzano et al. 2006].

Later on, several authors have also explored the effects of resveratrol on obesity, brain function, and visual performance. The results obtained in a number of studies in cell, animal, and human trials revealed that resveratrol and related stilbenes were able to inhibit adipocyte differentiation and proliferation, decrease lipogenesis, and promote lipolysis and fatty acid beta-oxidation, pointing out that it may be used as an anti-obesity

agent [Wang et al. 2014]. Regarding brain function, Kennedy et al. [2010] found that the oral administration of a single dose of resveratrol (250 or 500 mg) to healthy adults increased cerebral blood flow during task performance in a dose-dependent way without affecting cognitive function. Furthermore, Evans et al. [2017] reported that daily consumption of 150 mg of resveratrol for 14 weeks enhanced verbal memory and overall cognitive performance in postmenopausal women. Another study in postmenopausal women concluded that supplementation with 75 mg of trans-resveratrol twice a day for a year improved overall cognitive performance and cerebrovascular responsiveness to cognitive stimuli, which was also associated with a reduction of fasting blood glucose [Thaung Zaw et al. 2020]. By contrast, a nutritional intervention with 200 mg/day of resveratrol failed to show significant improvements in verbal memory after 26 weeks in healthy elderly individuals. Moreover, in a meta-analysis on the results obtained from four randomized clinical trials, Farzaei et al. [2018] did not conclude significant effects on memory and cognitive performance assessed by auditory verbal learning test.

Similarly, Marx et al. [2018] concluded that, despite the fact that resveratrol supplementation might improve cognitive performance, the results obtained among clinical trials are limited and inconsistent. As for visual performance, studies carried out in different retinal cell lines found that resveratrol at micromolar concentrations was able to protect them from damage caused by oxidative stress and hyperglycemia-induced low-grade inflammation, suggesting that it might contribute to preventing age-related ocular disorders like cataracts, glaucoma, or macular degeneration. Additionally, oral administration of resveratrol (5 to 200 mg/kg for 5 days) to mice was seen to prevent endotoxin-induced uveitis by inhibiting oxidative damage, leading authors to propose that supplementation with resveratrol is a possible strategy to treat ocular inflammation [Kubota et al. 2009].

However, despite the range of evidence on the potential benefits of resveratrol obtained in model and preclinical studies, attempts have failed to come to clear and consistent outcomes in cohort and clinical trials. It must also be highlighted that the available studies have been performed using relatively high doses of resveratrol, which are unlikely to be provided by the diet when considering the scarcity of food sources and the very low concentrations at which stilbenes are present. It does not seem that Mediterranean diets, either with or without wine, can represent further improvements in this sense. Thus, it should not be expected that resveratrol may have a relevant contribution to the beneficial health effects associated with Mediterranean diets or any other type of diet.

Supplementation or therapeutical approaches might, therefore, be the way to take advantage of its potential benefits. Nonetheless, much work seems still required in this respect.

As recently reviewed by Ren et al. [2021], poor pharmacokinetics and low potency as well as possible toxicity issues, including gastrointestinal disorders, headache, rash, or nephrotoxicity seem the main bottlenecks to overcome for its nutritional or therapeutical application. The development of more potent analogues and/or novel resveratrol formulations to enhance its bioavailability may be promising strategies to take it from bench to people.

2.1.3 Quantitative Research Literature Analysis

Red wine is an intricate beverage in continuous development represented by a hydro alcoholic solution that includes many different chemical components, such as minerals, sugars, lipids, soluble proteins, vitamin aldehydes, esters, ketones, phenolics, and organic acids. Among the many bioactive compounds contained in wine, the polyphenols represent the main class.

This PhD action aims to provide a bibliometric analysis of the literature related to research on the association between the Mediterranean diet, wine and health. The analysis can reveal the subject areas covered by the largest number of publications and research opportunities the components of wine of health interest.

Accordingly, a substantial contribution to the study has been identified. First, the paper focuses on the association between Mediterranean diet and red wine. The results of this study can provide an overview of a bibliometric review of the literature on the importance of red wine in the Mediterranean diet. Second, the research reveals the subject areas of most publications and shows the evolution of publications related to the health value of wine. Therefore, the paper fulfills the following objectives. First, the paper aims to examine the trends and efficiency of publications on red wine as a component of health interest in the Mediterranean diet. This analysis is an approach to sort articles by document and source type, year of publication, subject area, and titles of the most active sources, and to visualize the relationships among them. In addition, the paper addresses cluster analysis based on keyword analysis of the examined publications and words appearing in titles and abstracts.

Bibliometric analysis is a widespread and accurate method for examining and analysing large volumes of scientific data. This technique is intended to comprehend interconnectedness among journal citations and sum up the up-to-date situation in terms

of a current or rising research topic. In the research, the data used in a bibliometric analysis is retrieved from Scopus. Visualization of similarities (VOS) viewer that aims to provide easy formation and visualization of bibliometric maps is gaining popularity in bibliometric research. This method allows us to efficiently collect literature and establish the interrelationships between chosen publications within the options.

Two analyses were conducted using two different sets of keywords as follows:

- a. 525 publications have been retrieved by the database Scopus with the use of the following keywords search title-abs-key (Mediterranean diet and wine) and limit-to publication year, 2013-2023;
- b. 72 publications have been retrieved by the database Scopus with the use of the following keywords search title-abs-key (Mediterranean, Polyphenols, Wine and Disease) and limit to publication year, 2013-2023;

The received results have been downloaded in RIS format to be processed using VOSviewer to visualize and analyze the trends in the bibliometric form. VOSviewer makes the creation of country maps possible based on a network (co-occurrence), builds a keyword map based on shared networks, and creates maps with many items [Rahmawati 2022]. Data mining, mapping, and grouping of articles retrieved from the database can be done using the VOSviewer software [Effendi et al. 2021].

An analysis approach based on co-occurrence mapping was adopted using the scientific software, VOSviewer which creates data mining, mapping and clustering of articles retrieved from the database. The use of VOSviewer software provides graphical analysis of bibliometric data and visualization of search results. All keywords that contributed to the full counting method were considered as an analysis element during co-occurrence mapping. To obtain a more accurate result, the study placed some constraints on the analysis. The number of keywords to be used can be adjusted by eliminating less relevant keywords. A minimum of 5 occurrences of a keyword was applied as a limiting factor. Regarding the analysis based on the search combination Mediterranean diet and wine, 720 keywords out of 533 met several criteria.

Figure 2.1 represents the visualization of the co-occurrences map between the selected keywords (*Mediterranean diet* and *Wine*) processed in VOSviewer software.

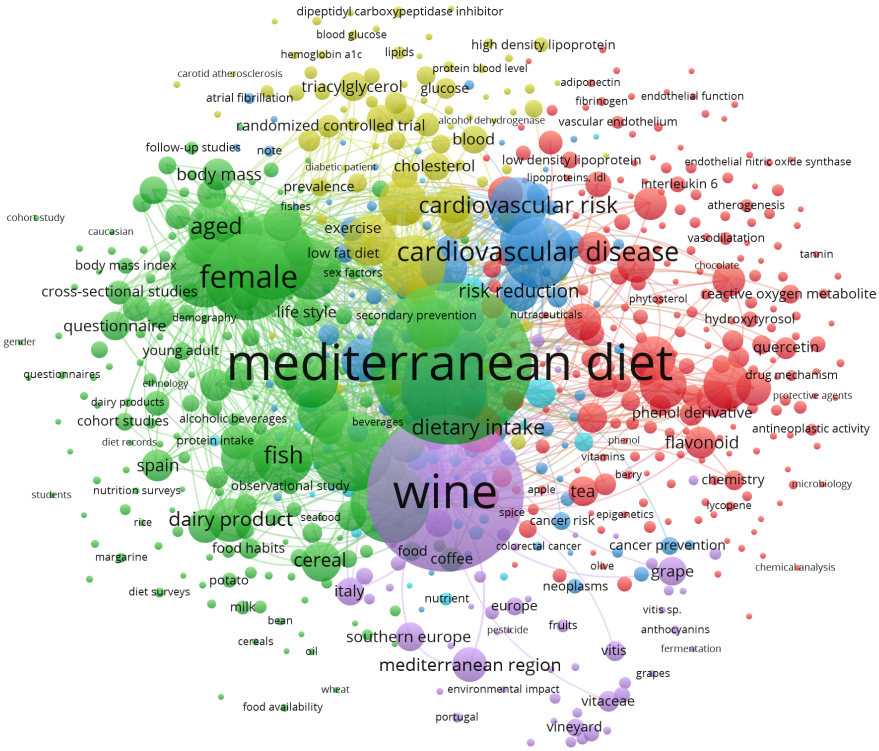

Figure 2.1 *Co-occurrences map and clusters relationship between the selected keywords processed in VOSviewer software.*

The map shows the number of occurrences of the word in the article and the relationship between the keywords. In the network, each term is presented by a circle, while the size of the circle is commensurate with the number of publications in which the term is found. Each color represents a group of terms joined in clusters, and the length of the curved lines specifies the approximate connection of the term's repetition, while the thickness of those indicates the strength of the pairs of topic areas or keywords. Clusters represent the relationship between one topic and another.

The data were analyzed using VOSviewer and the results were divided into 6 clusters (Figure 2.1). Two clusters out of six, red and green are larger than the others. The red area consists of topics related to health-related substances of interest, with special reference to polyphenols, resveratrol, or even quercitin. The green cluster groups the strong interest in the Mediterranean diet as well as studies related to the foods and countries characteristic of the Mediterranean diet. It should be pointed out, however, that wine is not in this cluster but in the purple cluster, fifth in size.

The software analyzes each keyword by calculating links, total strength of links, and co-occurrences with other keywords. The occurrences indicate the number of items in which the keyword is observed. The keywords with the most frequent co-occurrences are "Mediterranean diet"; "wine"; and "cardiovascular disease." A strong link between

vascular risk reduction and wine as well as between "cardiovascular risk reduction" and "Mediterranean diet".

Based on these results, the second analysis took into account in the search for additional keywords, such as polyphenols and disease reduction. The analysis based on the search combination "*Mediterranean*, *Polyphenols*, *Wine* and *Disease*", 181 keywords out of 1387 met several criteria.

The co-occurrences map between the selected keywords (*Mediterranean*, *Polyphenols*, *Wine* and *Disease*) processed in VOSviewer software.

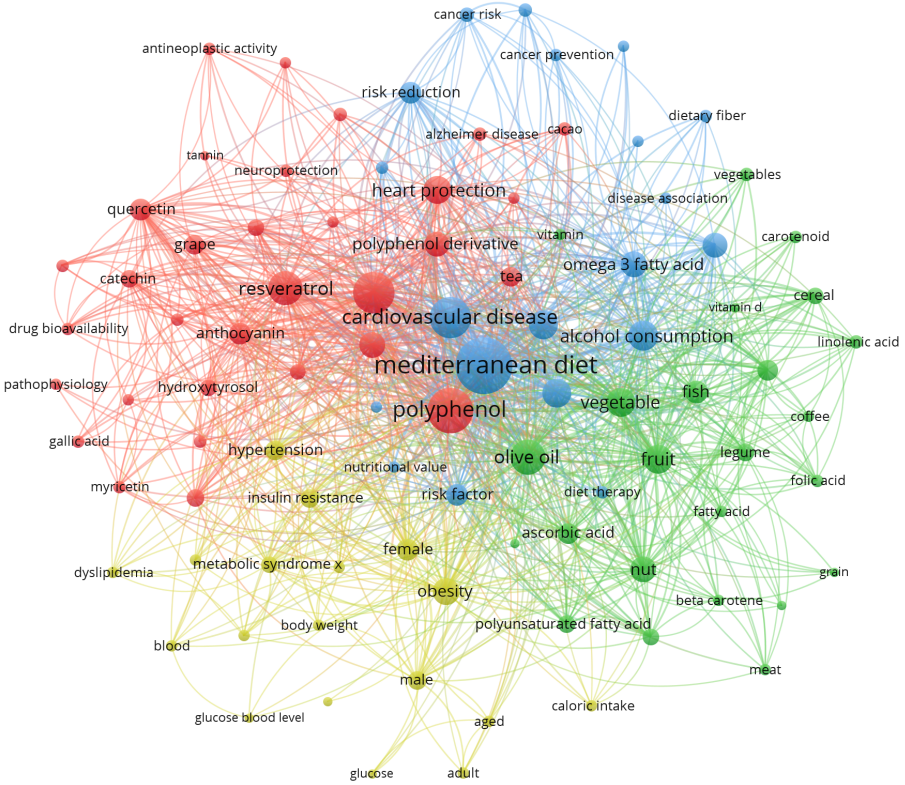

Figure 2.2 *Co-occurrences map and clusters relationship between the keywords Mediterranean, Polyphenols, Wine and Disease processed in VOSviewer software.*

The co-occurrences map highlighted four cluster reported in four different colour (red, green, blue and yellow) The red cluster is larger than the others and consist of 32 items all related to polyphenols.

The blue cluster groups together topics related to the Mediterranean diet and disease reduction as well as omega-3 fatty acids. The green cluster groups together foods and substances (other than polyphenols) related to the Mediterranean diet. Finally, the yellow cluster, less extensive, groups items mainly related to variables such as sex (male or female) age, blood group as well as some diseases such as obesity, hypertension and metabolic syndrome. A strong relationship between the Mediterranean diet and red wine

emerges from the map shown in Figure 2.2. The relationship between Mediterranean diet and red wine and their relationship with the other items is further clarified by the maps focused on the relationships produced by specific words. Of interest are the maps obtained by the focus on Mediterranean diet (Figure 2.3A) or on red wine (Figure 2.3A).

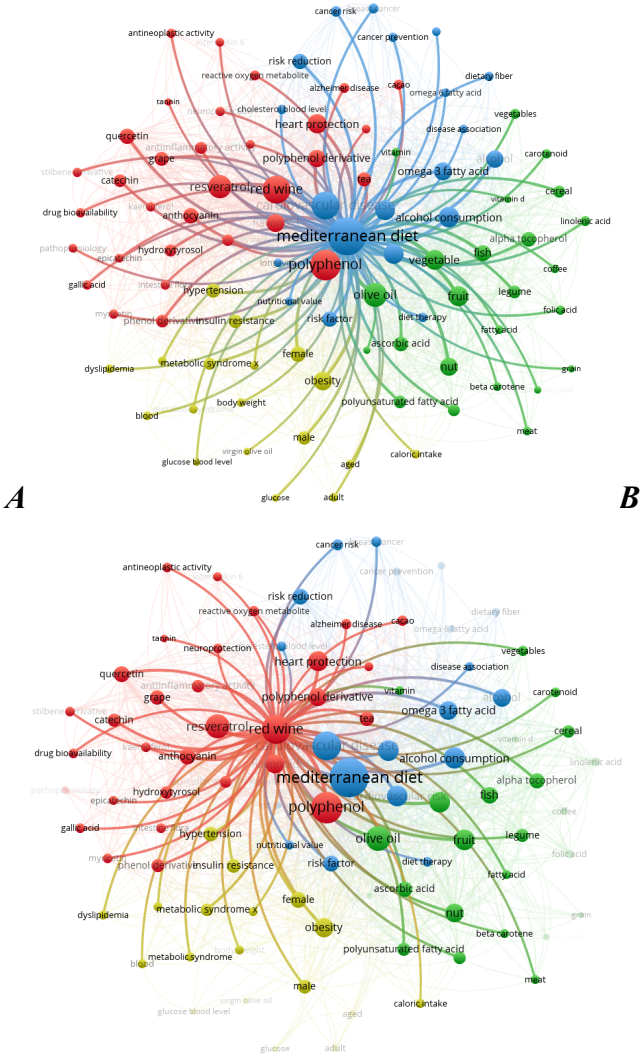

Figure 2.3 *Co-occurrence maps focused on A) Mediterranean diet item and B) red wine*

In both cases (focus on *Mediterranean diet* or *red wine*), the relationship with health items such as "risk reduction" "cardiac risk" "health protection" "hypertension" "obesity" etc. appears to be evident.

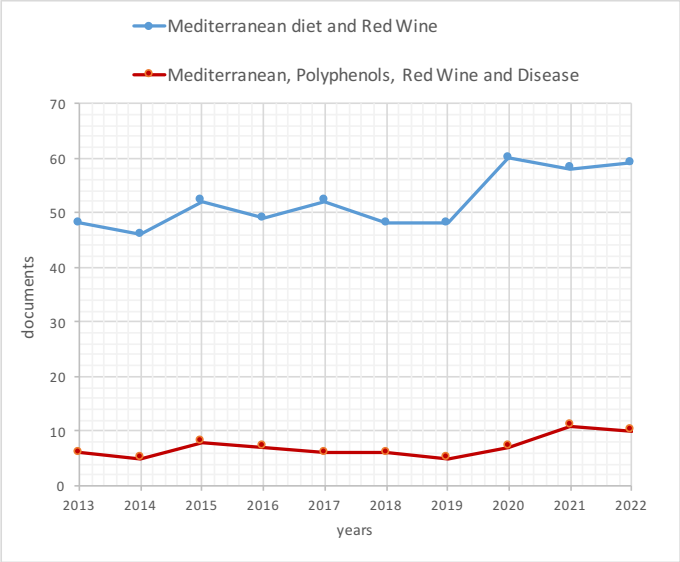

Figure 2.4 *Number of publications per year that match the research criteria*

Analysis of the data, however, shows that the trend in the number of publications per year that meet the research criteria are essentially constant over the years. A slight increase was appreciated in 2020 but this was followed by a further steady trend. The number of papers per year that meet the research criteria Mediterranean, Polyphenols, Wine and Disease can be considered low compared to the copious scientific production related to wine. Therefore, even in light of the current discussion around the wine-health relationship, the study of the effect of wine and its polyphenolic substances on reducing the risk of certain diseases is worthy of investigation.

2.2 THE CASE STUDY: PHENOLIC PROFILES OF RED WINE RELATE TO VASCULAR ENDOTHELIAL BENEFITS

Mammalian SIRTs (SIRT1-7) are a family of NAD^+-dependent deacetylases (class III histone deacetylases) signaling proteins involved in metabolic regulation and biological processes such as cell survival, apoptosis, proliferation, cellular senescence, stress response, genome stability, and metabolism [Hussain et al. 2021]. Red wine polyphenols are particularly attractive for their properties as activators of sirtuins proteins, a family of proteins mediating a large variety of pathophysiological events, including protection of the circulatory system function and prevention of endothelial dysfunction at the early stages of the pathogenesis of type 2 diabetes and atherosclerosis [D'Onofrio et al. 2018; Vitiello et al. 2017; Balestrini et al. 2015]. In this PhD study first a bibliometric analysis of the literature related to research on the association between the red wine and sirtuins

was perfermod and then the corelation between phenolic composition and antioxidant, antinflammatory and *SIRT1 and SIRT6 activation* properties of red wine was evaluated in a specific case study. In detail, the data used in bibliometric analysis is retrieved from Scopus retrieving 27 documents by the database with the use of the following keywords search title-abs-key (Red Wine, Polyphenols, Sirtuin) and limit-to publication year, 2013-2023. The received results have been downloaded in RIS format to be processed using VOSviewer software. A minimum of 5 occurrences of a keyword was applied as a limiting factor. Figure 2.5 represents the visualization of the co-occurrences map between the selected keywords processed in VOSviewer software.

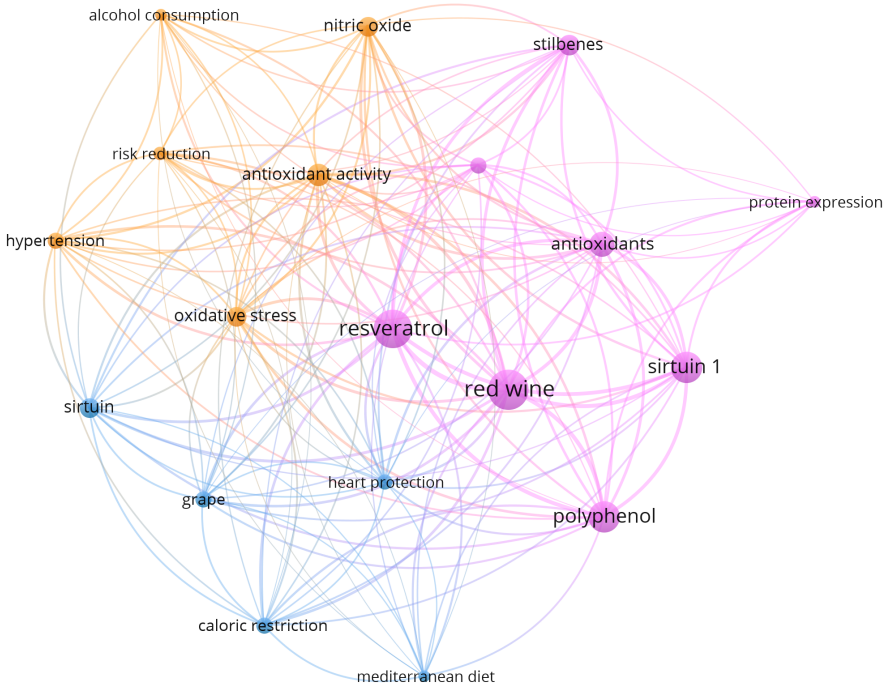

Figure 2.5 *Co-occurrences map and clusters relationship between the selected keywords (Red Wine, Polyphenols, Sirtuin) processed in VOSviewer software.*

The map shows the number of occurrences of the word in the article and the relationship between the keywords highlighting 3 clusters (Figure 2.5).

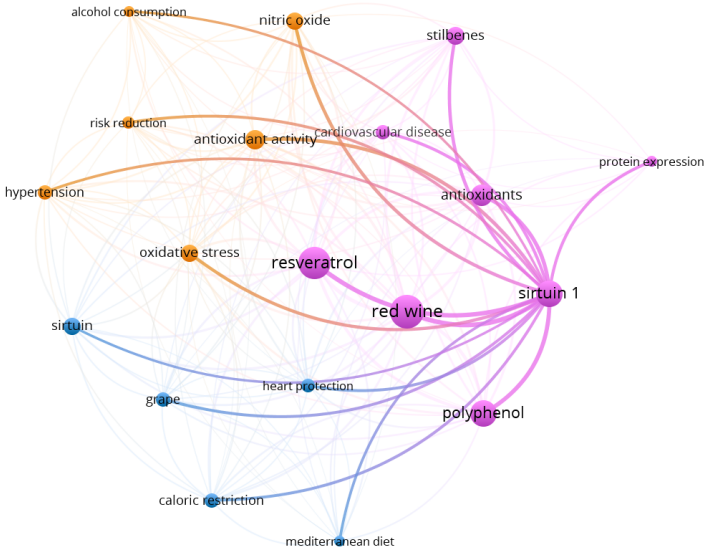

Figure 2.6 *Clusters relationship between Sirtuin and other selected keywords (Red Wine, Polyphenols, Sirtuin) processed in VOSviewer software.*

The purple cluster grouping 8 items from which the polyphenols, resveratrol and sirtuins relationship emerges. However, it should be emphasized that there are really few studies related to the specific topic under research and bibliographic analysis. Natural polyphenols, first identified as sirtuin activators, compose a diversified class of molecules usually divided into two categories: flavonoids and nonflavonoids [Rahnasto-Rilla et al. 2018]. Based on the previous considerations, in this section of PhD activities, the relationship between the phenolic content and the bioactivity of red wines was investigated. To this end, three red wines were selected based on the comparable pedoclimatic conditions under which grapes were grown and the same technological protocol adopted for their production. This was instrumental in ruling out any technological variables, thus allowing a straightforward correlation between the bioactivities of the wines and their specific content of healthy natural metabolites. The Italian red wines chosen were Magliocco, Gaglioppo, and Nerello Mascalese, all produced in the Calabria region (Italy). In particular, two main aspects were covered by this study: (a) chemical characterization and differentiation of the most abundant phenolics by means of an integrated approach of NMR, HPLC, and spectroscopic techniques and (b) the in vitro bioactivity in endothelial models of insulin resistance and hyperglycemia and potency as SIRT1 and SIRT6 activators.

2.2.1 Materials and Methods

2.2.1.1 Experimental Wines

Monovarietal wines were obtained from Nerello Mascalese and Magliocco grape produced in the Calabria region (Italy) by Marrelli Wines (Le Verdi Praterie Società Agricola a.r.l, Crotone, Italy) in a 2019 vintage, while the blend Gaglioppo-Magliocco (50–50%) was produced in the same area but in a 2018 vintage, as reported by Coppola et al. [2021]. As for the standard winemaking process, grapes were destemmed and crushed. They were treated with K2S2O5 (60 mg/Kg of grapes) and 50 mg/kg of ascorbic acid. Fermentation took place at 18–21 ◦C with indigenous yeast, and the cap was immersed two to four times per day. Maceration of the pomace lasted twelve days. Wines were aged in contact with oak wood. The base parameters of the wines were determined by using official methods of analysis at bottling. For the chemical analysis, two bottles for each treatment were analyzed. Alcohol, residual sugar, titratable acidity, volatile acidity, and free and total sulfur dioxide were determined by the official method of analysis (OIV-MA-F1-07, RESOLUTION OIV-OENO 419A/2011, www.oiv.int, accessed on 16 February 2021).

2.2.1.2 Analysis of Wine Monomeric Anthocyanins

The analysis of anthocyanins was carried out by high-performance liquid chromatography analysis HPLC-DAD (RESOLUTION OIV-MA-AS315-11, www.oiv.int, accessed on 16 February 2021) [Coppola et al 2021]. A HPLC SHIMADZU LC10 ADVP apparatus (Shimadzu Italy, Milan), consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, an SPD-M 10 AVP detector, and an injection system full rheodyne model 7725 (Rheodyne, Cotati, CA) equipped with a 50-µL loop, was employed. A Waters Spherisorb column (250 × 4.6 mm, 4 µm particles diameter) with a pre-column was used. Twenty microliters of wine or calibration standard (malvidin-3-O-glucoside chloride, purity >95%, purchased from Sigma-Aldrich, St. Louis, MO, USA) were injected onto the column. Detection was performed by monitoring the absorbance signals at 518 nm. All the samples were filtered through 0.45-micron, durapore membrane filters (Sigma Aldrich, Milan, Italy) into glass vials and immediately injected into the HPLC system. The HPLC solvents were solvent A: water/formic acid/acetonitrile (87:10:3) *v*/*v*; solvent B: water/formic acid/acetonitrile (40:10:50) *v*/*v*. The gradient used was: zero-time conditions 94% A and 6% B, after 15 min the pumps were adjusted to 70% A and 30% A, at 30 min to 50% A and 50% B, at 35 min to 40% A, and 60% B, at 41 min through

the end of the analysis, to 94% A and 6% B. After a 10-min equilibrium period the next sample was injected. The flow rate was 0.80 mL/min. For calibration purposes, the external standard method was used: the calibration curve was plotted for the malvidin-3-O-monoglucoside (Extrasynthese, Lyon, France) based on peak area, and the concentration was expressed as mg/L of malvidin-3-O-glucoside. All the analyses were conducted in duplicate on each experimental replicate.

2.2.1.3 Analysis of Wine Phenolic Compounds by Spectrophotometry

Total anthocyanins, short polymeric pigments (SPP), large polymeric pigments (LPP), and BSA reactive tannins indicating the high molecular weight phenolics HMWP were determined by the Harbertson-Adams's assay, as previously reported [Coppola et al 2021]. Color intensity, hue, and vanillin reactive flavans, indicating low molecular weight phenolics (LMWP), were determined as described by Gambuti et al. [2020]. Additionally, total phenolics were also determined by the Harbertson-Adams assay by using a Shimadzu UV-1800 (Kyoto, Japan) UV spectrophotometer.

2.2.1.4 NMR Analyses of Wines

Aliquots of each wine (100 mL) were concentrated under vacuum in order to remove ethanol and then partitioned twice with ethyl acetate (EtOAc). Twenty milliliters of each EtOAc extract were dried under vacuum, fully solubilized in 0.6 mL of CD_3OD (99.8% purity; ARMAR Isotopes GmbH, Leipzig, Germany) containing 2.0-mM deuterated 3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (d4-TMSP; min 99 atom%D ARMAR Isotopes GmbH, Leipzig, Germany), and submitted to the NMR-based analysis. Each sample was prepared in duplicate.

NMR spectra were recorded on a Bruker Avance Neo 700 MHz (700 and 175 MHz for 1H and 13C NMR, respectively) using a Norell® Select SeriesTM 5-mm NMR tubes. Chemical shifts are referenced to the residual solvent signal (CD_3OD: δ_H 3.31, δ_C 49.0). Standard Bruker pulse sequences were employed for 1H NMR spectra. d4-TMSP was employed as a calibration standard for NMR-based quantification of analytes. 1H NMR spectra were acquired setting the d1 value at 5.0 s in order to allow a complete relaxation of the d4-TMSP standard to equilibrium [Peterson and Waterhous, 2016]. Quantitation of compounds was conducted by selecting representative NMR signals and their areas determined by integration. The molar ratio of each analyte to TMSP was calculated as follows:

M"analyte" /M"TMSP" = N"TMSP" /N"analyte" × A"analyte" /A"TMSP" (1)

where M is the molarity, N the number of nuclei generating the investigated NMR signal, and A the peak area. Peak areas (A) were determined by peak picking in combination with line-fitting deconvolution by Mestrenova 9.0 software.

2.2.1.5 NMR Data of Major Polyphenols Identified in Wines

Catechin. 1H NMR data in CD3OD at 25 ◦C (700 MHz): H2 4.57 (doublet J 7.4 Hz); H3 3.97 (multiplet); H4a 2.50 (doublet of doublets J 16.5, 9.0 Hz); H4b 2.86 (doublet of doublets J 16.5, 1.7 Hz); H6 5.86 (doublet J 2.2 Hz); H8 5.92 (doublet J 2.2 Hz); H2′ 6.84 (doublet J 1.6 Hz); H5′ 6.76 (doublet J 7.1 Hz); H6′ 6.72 (doublet of doublets J 7.1, 1.6 Hz). Catechin was detected in all wines.

Epicatechin. 1H NMR data in CD3OD at 25 ◦C (700 MHz): H3 4.18 (multiplet); H4a 2.74 (doublet of doublets J 16.6, 1.5 Hz); H4b 2.84 (doublet of doublets J 16.6, 2.4 Hz); H6 5.93 (doublet J 2.3 Hz); H8 5.95 (doublet J 2.3 Hz); H2′ 6.97 (doublet J 1.6 Hz); H5′ 6.76 (doublet J 7.1 Hz); H6′ 6.80 (doublet of doublets J 7.1, 1.6 Hz). Epicatechin was detected in all wines.

Ethyl Caffeate. 1H NMR data in CD3OD at 25 ◦C (700 MHz): H2 7.04 (doublet J 1.9 Hz); H5 6.78 (doublet J 8.1 Hz); H6 6.94 (doublet of doublets J 8.1, 1.9 Hz); H7 7.53 (doublet J 15.8 Hz); H8 6.22 (doublet J 15.8 Hz); H21′ 4.20 (quadruplet J 7.1 Hz); H32′ 1.34 (triplet J 7.1 Hz). Ethyl caffeate was detected in Nerello Mascalese and Gaglioppo.

Gallic acid. 1H NMR data in CD3OD at 25 ◦C (700 MHz): H2/H6 7.06 (singlet). Gallic acid was detected in all wines.

2-phenylethanol. 1H NMR data in CD3OD at 25 ◦C (700 MHz): H21 3.74 (triplet J 7.1 Hz); H22 2.81 (triplet J 7.1 Hz); aromatic ring constituted by H2′/6′, H3′/5′, H4′: overlapped resonances centered at 7.17, 7.21 and 7.26. 2-phenylethanol was detected in all wines.

Pyrogallol. 1H NMR data in CD3OD at 25 ◦C (700 MHz): H4/H6 6.32 (doublet J 8.1 Hz); H5 6.49 (triplet J 8.1 Hz). Pyrogallol was detected in all wines.

Tyrosol. 1H NMR data in CD3OD at 25 ◦C (700 MHz): H22/6 7.03 (doublet J 8.4 Hz); H23/5 6.70 (doublet J 8.4 Hz); H27 2.71 (triplet J 7.2 Hz); H28 3.67 (triplet J 7.2 Hz). Tyrosol was detected in all wines.

2.2.1.6 Antioxidant Assays

The total antioxidant capacity was determined using a colorimetric assay (Abcam, Cambridge, UK, ab65329) based on the Cu^{2+} conversion to Cu^{+} by antioxidants and, to the following release of a colorimetric probe, proportional to the total antioxidant power. The assay was performed following the manufacturer's instructions. Briefly, samples were mixed with 100 µL of the Cu^{2+} working solution and then incubated for 90 min at room temperature in the dark. The reaction was detected by measuring, with a microplate reader model 680 Bio-Rad (Bio-Rad, Hercules, CA, USA), the absorbance at 570 nm. Recorded absorbances are interpolated with the standard curve of Trolox, a known antioxidant, and the total antioxidant capacity expressed as nM equivalents. The ability of wines to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by generating a colorimetric reaction was on the basis of the ferric reducing antioxidant power assay (Abcam, Cambridge, UK, ab234626). The reducing power of the samples was calculated by reacting 10 µL of sample with 190 µL of the reaction mixture and monitoring the increase in absorbance at 594 nm for 1 h at 37 °C. The antioxidant potential of samples was determined using a ferrous iron standard curve and results are expressed as Fe^{2+} equivalents (nM).

2.2.1.7 Cell Culture and Treatment

Endothelial cells (EC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA, CCL 209). Cells were maintained in minimum essential medium (MEM, Gibco, Life Technologies, Carlsbad, CA, USA, 11095-080), supplemented with 20% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (0.1 mg/mL) at 37 ◦C in a humidified atmosphere, 95% air, 5% CO2. Exposure to high-glucose (hGlu) (30 mM) and palmitic acid (PA) (0.5 mM) were used as a pro-oxidant and inflammatory stimuli [D'Onofrio et al. 2019; Mo et al. 2019] to mimic oxidative microenvironment. To exclude any interference with ethanol, cell treatments were performed using lyophilized wine samples dissolved in Hanks' balanced salt solution (HBSS)–10 mM of Hepes (0–10 µg/mL). Cells were pretreated with wine samples for 12 h before exposure to hGlu (30 mM) or PA (0.5 mM) added to the same culture medium. The coincubation of wine + hGlu (30 mM) or wine + PA (0.5 mM) was performed for 24 h, 48 h, and 72 h at 37 ◦C in a humidified atmosphere, 95% air, 5% CO2. Control cells

(Ctr) were treated with corresponding volumes of Hanks' balanced salt solution (HBSS)–10 mM of Hepes.

2.2.1.8 Cell Viability and Cytotoxicity

Cell viability was detected using Cell Counting Kit-8 (CCK-8 Donjindo Molecular Technologies, Inc., Rockville, MD, USA) following the manufacturer's instructions, as previously described [D'onofrio et al. 2021]. Briefly, 10 µL of CCK-8 solution was added to each well, and then, the cells were incubated at 37 °C for 4 h. Thereafter, the absorbance was measured at 450 nm using a microplate reader model 680 Bio-Rad (Bio-Rad, Hercules, CA, USA). Cellular membrane integrity was assessed using cytotoxicity LDH Assay Kit-WST (Donjindo Molecular Technologies, Inc., Rockville, MD, USA, CK12). The release of lactate dehydrogenase (LDH) into the medium was measured according to the manufacturer's instructions. Briefly, after treatments, 100 µL of the working solution was added to 50 µL of endothelial cell suspension. The 96-well culture plate was then incubated at room temperature for 30 min protected from light. Sample absorbance was determined at 490 nm on a microplate reader model 680 Bio-Rad (Bio-Rad, Hercules, CA, USA), and the percent of cytotoxicity was calculated by the following equation: (substance—low control)/(high control—low control) × 100. All experiments were performed with $n = 5$ replicates.

2.2.1.9 Cell Lysates and Western Blotting

After the above-mentioned treatments, the cellular total protein content was extracted, as previously described [D'Onofrio et al. 2020], and the total protein concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). According to the target protein band size, protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8–12%) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 10-mM Tris-HCl, pH 8.0, 150-mM NaCl, and 0.05% Tween 20 (TBST) supplemented with 5% nonfat dry milk for 1 h at room temperature. Membranes were incubated overnight at 4 °C with specific primary antibodies anti-SIRT1 (1:1000, Biorbyt, Cambridge, UK, orb306144), anti-SIRT6 (1:1000, Abcam, Cambridge, UK, ab191385), anti-NF-κB p65 (acetyl K310) (1:1000, Abcam, Cambridge, UK, ab218533), anti-NF-κB (1:1000, Abcam, Cambridge, UK, ab16502), anti-p53 (acetyl K382) (1:700, Abcam, Cambridge, UK, ab75754), anti-p53 (1:1000, Biorbyt, Cambridge, UK, orb323871), and

anti-tumor necrosis factor-α (TNF-α) (1:1000, Abcam, Cambridge, UK, ab6671). Anti-α-tubulin (1:5000, Cell Signaling Technology, Danvers, MA, USA, 3873), anti-β-actin (1:5000, Cell Signaling Technology, Danvers, MA, USA, 3700), and anti -GAPDH (1:10.000, Abcam, Cambridge, UK, ab9485) were used as loading controls. After 1 h incubation with HRP-conjugated secondary antibodies (NC GxMu-003-DHRPX and GtxRb-003-DHRPX, ImmunoReagents Inc., Raleigh, NC, USA), the immunocomplexes were examined by the Excellent chemiluminescent sustrate kit (Elabscience Biotechnology Inc., Houston, TX, USA, E-IR-R301) and visualized by using the ChemiDoc Imaging System with Image Lab 6.0.1 software (Bio-Rad Laboratories, Milan, Italy). The analyses of immunoblotting data were performed with ImageJ 1.52n software (National Institutes of Health) by measuring the density of each band and, after background subtraction, comparing it with the loading control signal. Results were reported as arbitrary units (AU) ± SD of at least three independent experiments.

2.2.1.10 Intracellular ROS Detection

Endothelial cells (5×10^3 cells/well) were seeded in a 96-well microplate. Intracellular ROS levels were determined by using the cellular reactive orange fluorescence oxygen species detection assay kit (Abcam, Cambridge, UK, ab186028), as previously described [D'Onofrio et al. 2021]. Briefly, 100 µL of ROS working solution was added to each well, and the colorimetric reaction proceeded for 60 min until the fluorescence intensity was measured at an excitation wavelength of 540 nm and an emission wavelength of 570 nm using a Tecan Infinite 2000 Multiplate reader (Tecan, Männedorf, Swiss).

2.2.1.11 Extracellular ROS Evaluation

To evaluate the extracellular H_2O_2 released from endothelial cells, the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA, A22188) was used as described by D'Onofrio et al. [2021]. Briefly, the suspension of live cells (2×10^4) prepared in a Krebs–Ringer phosphate glucose buffer (145 mM NaCl, 5.7 mM sodium phos- phate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, and 5.5 mM glucose, pH 7.35) was mixed with 100 µL Amplex Red reagent containing 50 µM Amplex Red and 0.1-U HRP/mL. After a 60 min incubation at 37 °C, the fluorescence was measured at excitation wavelength of 530 nm and emission wavelength

of 590 nm, using a Tecan Infinite 2000 Multiplate reader (Tecan, Männedorf, Swiss). Extracellular ROS content was calculated interpolating the sample fluorescence values with an H_2O_2 standard curve (0–2 μM concentration range).

2.2.1.12 Mitochondrial ROS Measurement

To detect the generation of mitochondrial ROS, the Mitosox Red Mitochondrial Superoxide Indicator (Thermo Scientific, Rockford, IL, USA, M36008) was used as indicated by its manufacturer's protocol. EC were seeded in 24-well plate containing microscope glass (12 mm) (Thermo Fisher Scientific, Waltham, MA, USA) and stained for 10 min with 5-μM Mitosox at 37 °C before the paraformaldehyde fixing, as reported in the "Confocal laser scanning microscopy" section. The superoxide levels were assessed by confocal laser microscopy, and the fluorescence intensity, expressed as Arbitrary Fluorescence Units (AFU), was calculated with ImageJ1.52n software (National Institutes of Health, Bethesda, MD, USA). Menadione (50 μM) (Sigma Aldrich, St. Louis, MO, USA, M57405) was the positive control.

2.2.1.13 Assessment of Cytokine Levels

Cytokines (IL-6, IL-8, and TNF-α) and monocyte chemoattractant protein 1 (MCP-1) levels were determined by ELISA assays (human interleukin-6 ELISA, BioVendor Labora- torni medicina a.s., Brno, Czech Republic, RD194015200R; human interleukin-8, BioVendor, Laboratorni medicina a.s., Brno, Czech Republic, RD194558200R; ELISA Cymax TNF-alpha ELISA, AbFrontier, Seoul, Korea, YIF-LF-EK0193; human MCP-1 ELISA, BioVendo r, Laboratorni medicina a.s., Brno, Czech Republic, RAF081R, respectively), according to the manufacturer's instructions. Briefly, 100 μL of EC lysates were incubated in microplate wells precoated with specific anti-cytokine antibodies. After 60 min incubation and wash- ing to remove non-bound cytokines and other components of the sample, biotin-labeled anti-IL-6, -IL-8, -TNF-α, and -MCP-1 antibodies were added and incubated for additional 60 min. After another washing, streptavidin-HRP conjugate is added, following 30-min incubation. The remaining conjugate is allowed to react with the substrate solution, and then, absorbance was measured at 450 nm using a microplate reader model 680 Bio-Rad (Bio-Rad, Hercules, CA, USA). Concentrations of cytokines in samples were resulted by plotting absorbance values against concentrations of each standard curve.

2.2.1.14 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy analysis was performed as previously reported by D'Onofrio et al. [2021]. After treatments and Mitosox Red staining, EC were fixed with 4% (*v*/*v*) paraformaldehyde solution for 20 min and then permeabilized with 0.1% (*v*/*v*) Triton X-100 in PBS for 10 min at room temperature. For SIRT1 (1:500, Abcam, Cambridge, UK, ab32441), SIRT6 (1:500, Abcam, Cambridge, UK, ab191385), p53 (1:500, Biorbyt, Cambridge, UK, orb304644), and NF-κB (1:500, Cell Signaling Technology, Danvers, MA, USA, C22B4) immunofluorescence detection, primary antibodies were incubated overnight at 4 °C, followed by incubation with Alexa Fluor 633 (1:1000, Life Technologies, Carlsbad, CA, USA) for 1 h. For immunofluorescence study, anti-vimentin antibody (1:1000, Sigma Aldrich, St. Louis, MO, USA, V6630) was used to stain cytoskeleton, followed by Alexa Fluor 488 (1:1000, Life Technologies, Carlsbad, CA, USA) secondary antibody incubation. As for Mitosox Red staining, cellular architecture was marked by Phalloidin 488 (1:1000, Abcam, Cambridge, UK, ab176753). The nuclear staining, performed for 7 min with 2.5-µg/mL 4′, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, MO, USA), was included to samples involved in mitochondrial stress investigation. Microscopy analyses were performed with a LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) equipped with a plan apochromat X63 (NA1.4) oil immersion objective, and fluorescence intensity values were evaluated with ImageJ 1.52n software (National Institutes of Health, Bethesda, MD, USA).

2.2.1.15Statistical Analysis

For the biological assays and in vitro experimentation, all reported data referred to at least three independent experiments. Values are presented as the mean ± standard deviation (SD). Results were statistically evaluated using one-way ANOVA, followed by Bonferroni's post-hoc test. $p < 0.05$ was assumed as a statistically significant difference. For correlations between antioxidant properties and phenolic contents, all data were processed in the R environment. Therefore, Spearman's correlation coefficients were computed with the rcorr function to measure the strength and direction of association between the variables. The correlation plot was generated by the corrplot package [Stiglic et al. 2019].

2.2.2 Results

2.2.2.1 Characterization of Wines by NMR/ HPLC-UV/Vis and Spectrophotometric Analysis

The analysis of the base parameters of wines were determined by using official methods of analysis. To identify the major phenolic compounds, both monomeric and polymeric, occurring in the analyzed wines, we resorted to three different analytical approaches: NMR, HPLC, and spectrophotometry. The choice of these three techniques was driven by the different, yet complementary, pieces of information they can provide. First, NMR constitutes a valid untargeted analysis to rapidly obtain a comprehensive qualitative and quantitative picture of the most abundant low molecular weight metabolites present in a given sample. The HPLC-based analysis, conducted according to the OIV official methods, is a robust tool to define the monomeric anthocyanins in red wine; finally, spectrophotometric measurements give crucial information on polymeric pigments that would be quite difficult to be analyzed otherwise. In addition to the compounds recently characterized in these red wines [Coppola et al. 2021], the NMR-based analyses of Gaglioppo, Magliocco, and Nerello Mascalese brought to light different polyphenolic contents from both a qualitative and quantitative standpoint (Table 2.1).

Table 2.1. *Chemical characterization of wines. Polyphenols in Gaglioppo, Magliocco, Magliocco, and Nerello were identified by NMR. Concentration of monomeric anthocyanins were determined by HPLC analysis; total phenolics were determined by spectrophotometric methods. Their quantifications are expressed as mg/L. All data were expressed as the mean ± standard deviation (SD) of three replicates. Statistical analysis was performed through an analysis of variance (ANOVA), followed by the Tukey's multiple comparisons. Statistical significance was attributed to p-values < 0.05.*

	Nerello Mascalese	Magliocco	Gaglioppo–Magliocco
	NMR analysis		
Polyphenols (mg/L)			
Pyrogallol	18.6 ± 0.5	96.1 ± 2.2	105.2 ± 2.5
Gallic Acid	62.1 ± 1.3	19.7 ± 0.7	81.8 ± 1.9
Tyrosol	77.2 ± 3.2	65.6 ± 3.7	78.9 ± 2.2
Ethyl Caffeate	81.2 ± 4.1	N. D.	40.2 ± 0.3
Catechin	32.2 ± 1.7	170.0 ± 2.5	74.1 ± 0.8
Epicatechin	10.2 ± 0.2	72.3 ± 2.6	37.0 ± 1.2
	HPLC analysis		
Monomeric anthocyanins (mg/L)			
Dp3glc	1.73 ± 0.14	9.22 ± 0.94	11.49 ± 0.63
Cy3glc	0.76 ± 0.11	2.49 ± 0.25	5.66 ± 0.61
Pt3glc	2.67 ± 0.56	15.08 ± 3.18	12.22 ± 0.54
Pn3glc	2.78 ± 0.06	11.39 ± 0.62	11.91 ± 1.62
Mv3glc	25.53 ± 0.74	238.39 ± 1.40	52.02 ± 2.13
Mv3acglc	1.02 ± 0.05	9.57 ± 0.32	0.64 ± 0.24
Mv3cmglc	1.76 ± 0.04	24.69 ± 1.29	0.69 ± 0.28
	Spectrophotometric analysis		
Total phenolics (mg/L)	4722 ± 252	4291 ± 122	3083 ± 122

The identity of each metabolite was ascertained either by comparing the detected chemical shifts with those reported in the literature or with those of authentic samples. More specifically, besides anthocyanins, the major low molecular weight polyphenols detected in all the three wines were catechin and epicatechin, belonging to the flavan-3-ol class, gallic acid, pyrogallol, tyrosol, and 2-phenylethanol. Additionally, ethyl caffeate was detected in Gaglioppo and Nerello Mascalese, while, in Magliocco, such a metabolite was either absent or occurring below the detection limit of NMR. All mentioned polyphenols were quantitated by means of Equation (1), apart from 2-phenylethanol because of its high volatility.

Further chemical analyses were conducted to better characterize the phenolic contents of the three wines under investigation. By employing the HPLC-based official OIV method, the contents of the monomeric anthocyanins in each wine were evaluated. Malvidin turned out to be the most abundant anthocyanin in the Magliocco wine, where it accounted for 86% of the total monomeric anthocyanins (Table 2.1), consistent with the previous data obtained on Magliocco and in accordance with what has been reported for most red grape cultivars. Malvidin usually occurs as malvidin-3-O-glucoside (Mv-3-glc), but it can also present either coumaroyl (Mv-3-cum) or acetyl (Mv-3-ace) moieties. In Magliocco, the malvidin composition expressed as mg/L was found to be the following: Mv-3-glc >> Mv-3-cum > Mv-3-ace. Such a composition is similar to that of several Italian grape varieties. Even in Gaglioppo and Nerello, the most abundant anthocyanin was malvidin, with a relative abundance of 76% in Gaglioppo and 56% in Nerello, respectively. Again, the little amount of acylated anthocyanins we found in the Gaglioppo wine might be due to the significant percentage of Magliocco it contained. Finally, the spectrophotometric measures — instrumental in obtaining crucial information on polymeric phenolics — on the three wines under investigation allowed us to obtain further insights into their polyphenolic profiles (Table 2.1). Magliocco showed a total amount of anthocyanins more than twice as abundant as that of either Gaglioppo or Nerello. The amount of total phenolics confirmed that Nerello is the poorest in these important compounds. For the other two autochthonous. wines, the amount of low molecular weight phenolics (LMWP) is almost twice as abundant as that of the high molecular weight phenolics (HMWP) (Table 2.1).

2.2.2.2 Antioxidant Activity and Phenolic Content

The antioxidant capacity and the reducing power of red wines determined by the TAC (Figure 2.7 a, b) and FPAP assays (Figure 2.7 c, d) showed higher values for Magliocco (20,240 ± 2139 and 22,945 ± 1596, respectively) compared to Nerello (10,123 ± 427 and 10,740 ± 197, respectively) ($p < 0.01$) and Gaglioppo (17,014 ± 590 and 18,447 ± 1632, respectively) ($p < 0.05$). No differences in ferrous equivalents (nmol/L) were observed be- tween the Magliocco and Gaglioppo wines. Of note, significant correlations were observed between the antioxidant activity and biomolecules mainly responsible for the antioxidant properties (Figure 2.7e) ($p \leq 0.05$).

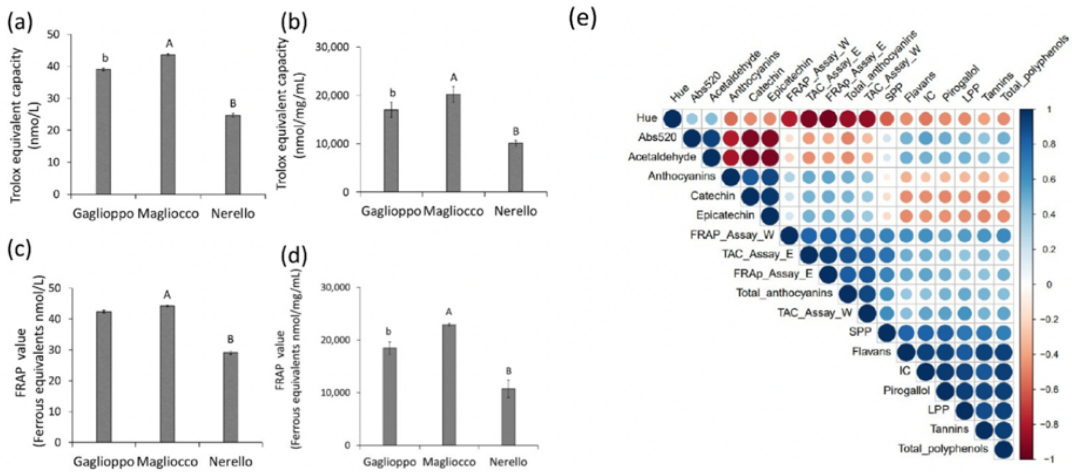

Figure 2.7. Antioxidant potential of red wines and correlation with the polyphenol contents. Total antioxidant activity and ferric reducing power were reported for (**a,c**) whole red wine samples and (**b,d**) lyophilized wine samples of Gaglioppo, Magliocco, and Nerello. Values represent the mean ± SD of n = 3 independent experiments. (**e**) Correlation plot is between the antioxidant activity and the phenolic contents of the wines. Stronger correlations are depicted by larger circles, with shades of blue representing positive correlations and shades of red representing negative correlations. Ab, $p < 0.05$ and AB, $p < 0.01$

2.2.2.3 Cytoprotective Effects

In vitro assays on the endothelial cell models of insulin resistance and hyperglycemia were used to test the cytoprotective effects of the three red wines studied. To this end, Magliocco (Ma), Gaglioppo (Ga), and Nerello (Ne) were firstly tested for their effects on the cell viability by incubating EC with concentrations of wine up to 10 µg/mL for 24 h, 48 h, and 72 h. The results showed that the cell viability was not affected up to 72 h of treatment. When tested in combination with hGlu or PA, coincubation counteracted the negative effects of hGlu and PA on cell viability ($p < 0.01$ vs. control cells). In particular,

Ma+hGlu and Ma+PA showed the highest potency at the concentration of 6 µg/mL after 48 h ($p < 0.01$ vs. hGlu; $p < 0.01$ vs. PA), compared to Ga+hGlu and Ga+PA or Ne+hGlu and Ne+PA. Among the wines tested, Magliocco was found to be more effective in protecting endothelial cells against the cytotoxicity induced by hGlu and PA (Figure 2.8 a, d) ($p < 0.01$ vs. hGlu; $p < 0.01$ vs. PA), compared to Gaglioppo (Figure 2.8 b, e) and Nerello (Figure 2.8 c, f) ($p < 0.05$ vs. hGlu; $p < 0.05$ vs. PA). Based on these results, Magliocco (6 µg/mL), displaying the highest antioxidant and cytoprotective properties, was chosen to investigate the molecular mechanism of endothelial protection.

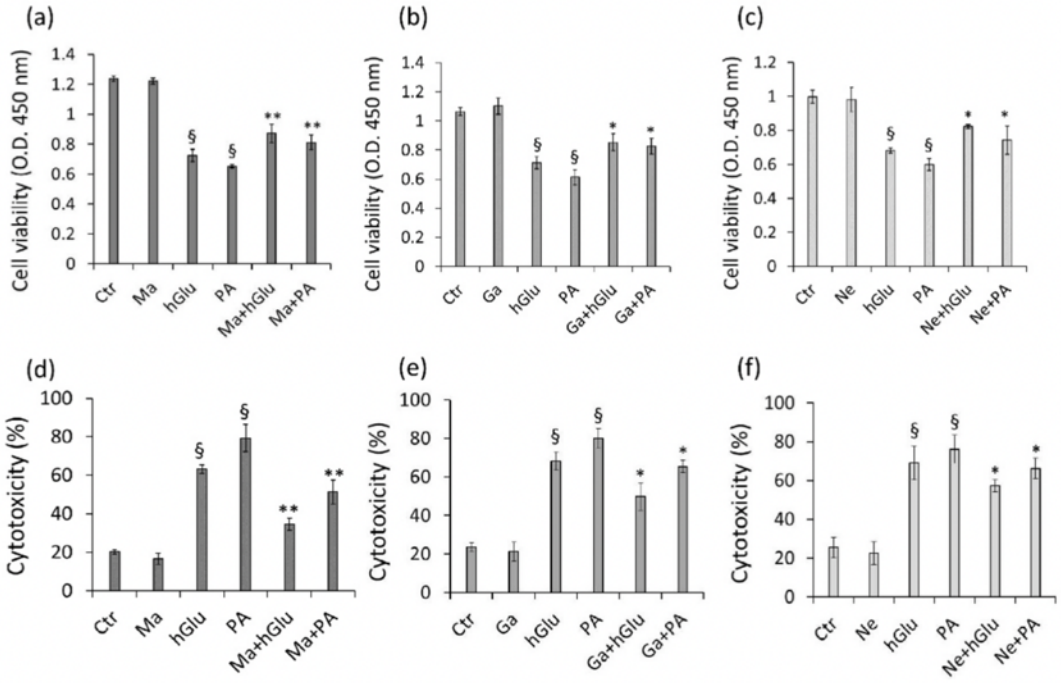

Figure 2.8 Endothelial protective effects of red wines during insulin resistance and hyperglycemia. (**a**–**c**) Cell viability and (**d**–**f**) cytotoxicity were evaluated by pretreating (12 h) EC with Ma, Ga, or Ne (6 µg/mL) before exposure to hGlu (30 mM) or PA (0.5 mM) for 48 h. Control cells were treated with corresponding volumes of Hanks' balanced salt solution (HBSS)–10 mM of Hepes. Cell viability was assessed by Cell Counting Kit-8 (Donjindo Molecular Technologies, Inc., Rockville, MD, USA). Cytotoxicity by LDH Assay Kit-WST (Donjindo Molecular Technologies, Inc., Rockville, MD, USA) and expressed as the mean ± SD of $n = 5$ replicates. § $p < 0.01$ vs. Ctr, * $p < 0.05$ vs. hGlu or PA, and ** $p < 0.01$ vs. hGlu or PA.

2.2.2.4 Anti-Inflammatory and Scavenger Action

The cytoprotective effect of Magliocco was accompanied by its efficacy in reducing IL-6, IL-8, and MCP-1 release induced by hGlu or PA ($p < 0.001$ vs. c Ctr) (Figure 2.9 a–c). Coincubation with Magliocco also reduced TNF-α levels and protein expression ($p < 0.05$ vs. hGlu and $p < 0.05$ vs. PA) (Figure 2.9 d, f). Moreover, the increased production of ROS by PA and hGlu (2.6 and 1.9 times, respectively; $p < 0.01$ vs. Ctr) was inhibited by cotreatment with Magliocco ($p < 0.01$ vs. hGlu and PA) (Figure 2.9 g). Extracellular ROS

measurements with the Amplex Red assay confirmed that the exposure to Magliocco was effective in protecting cells from ROS accumulation ($p < 0.01$ vs. hGlu and PA (Figure 2.9 h). Finally, the mitochondrial redox status evaluated by chemical probe MitoSox (Figure 2.9 i, j) showed that the mitochondrial ROS activated by hGlu and PA ($p < 0.001$ vs. Ctr), was consistently reduced by cotreatment with Magliocco (39.3 ± 7.6 vs. 67.89 ± 2.82 AFU in hGlu; 43.68 ± 3.4 vs. 76.09 ± 10.2 AFU in PA).

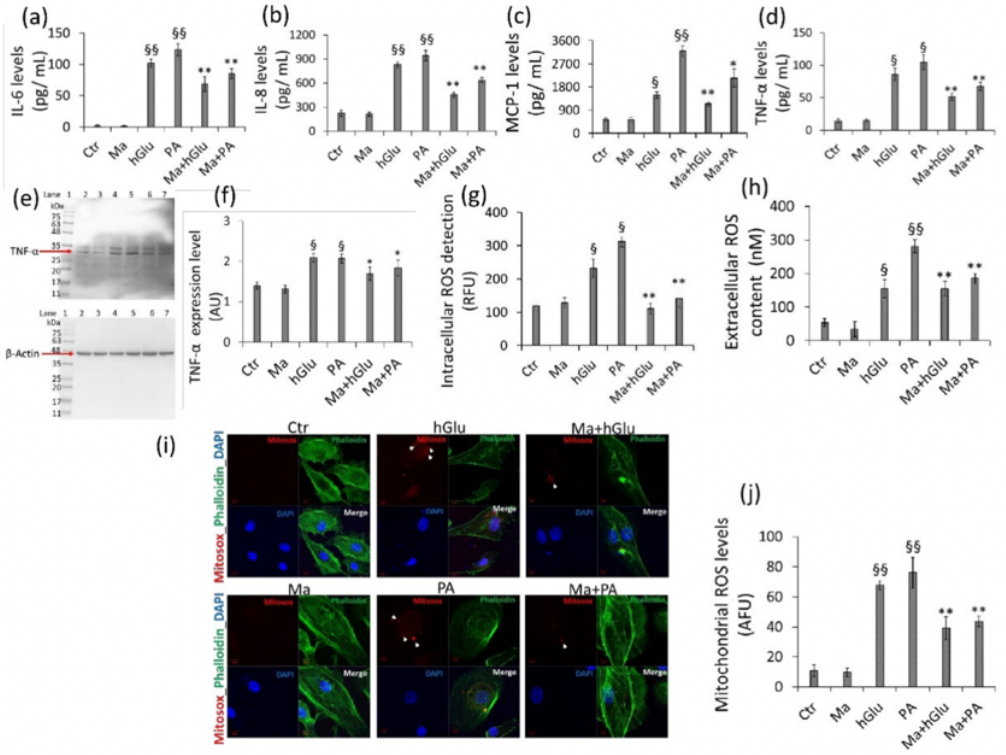

Figure 2.9. Antioxidant and anti-inflammatory activities of Magliocco. Endothelial cells were exposed to Magliocco (Ma), high glucose (hGlu) (30 mM), palmitic acid (PA) (0.5 mM), or combined Ma+hGlu and Ma+PA for 48 h. (**a**–**d**) IL-6, IL-8, MCP-1, and TNF-α-level measurements. (**e**–**f**) TNF-α protein expression. Lane 1 = protein ladder molecular weight markers, lane 2 = Ctr, lane 3 = Ma, lane 4 = hGlu, lane 5 = PA, lane 6 = Ma+hGlu, and lane 7 = Ma+PA. The analysis of densitometric intensity was calculated with ImageJ software and expressed as arbitrary units (AU) ± SD of $n = 4$ replicates. β-Actin was used as internal control. (**g**) Intracellular and (**h**) extracellular ROS content evaluations. (**i**) Representative images of confocal laser scanning analyses of mitochondrial ROS generation (indicated with white arrows) detected by MitoSOX probe and (**j**) mitochondrial superoxide level assessment. The results are expressed as arbitrary fluorescence units (AFU) ± SD of $n = 3$ replicates. Scale bars = 10 µm. The cytoskeleton is marked with Phalloidin 488 (green), while DAPI was used as a nuclei counterstain(blue). § $p<0.01$vs. Ctr, §§ $p<0.001$vs. Ctr, *$p<0.05$vs. hGluorPA,and**$p<0.01$vs. hGluorPA.

2.2.2.5 SIRT1 and SIRT6 Activation

The capacity of Magliocco to modulate SIRT1 and SIRT6 protein expression was further investigated by confocal laser scanner microscopy and Western blot analyses, thus providing cellular localization and protein expression on whole extracts, respectively. The results indicated that Magliocco was opposed to the nuclear downregulation of SIRT1 and SIRT6 induced by hGlu and PA (Figure 2.10) ($p < 0.01$ vs. Ctr). Specifically, compared with hGlu- and PA-treated cells, SIRT1 arbitrary fluorescence units (AFU) were higher in cells cotreated Ma+hGlu (38.08 ± 4.33 vs. 26.03 ± 2.49 AFU in hGlu, $p < 0.01$) and Ma+PA (35.11 ± 3.55 vs. 26.03 ± 2.49 AFU in PA, $p < 0.01$), as confirmed by Western blot analysis (Figure 2.10 a–e). Moreover, as for SIRT1, an increased SIRT6 expression was observed in cells treated with Ma+hGlu (51.61 ± 3.13 vs. 39.97 ± 4.95 AFU in hGlu) and Ma+PA (41.61 ± 3.77 vs. 29.01 ± 3.855 AFU in PA) (Figure 2.10 f–j). Less consistent effects in counteracting SIRT1 and SIRT6 downregulation occurring under hGlu and PA stressful conditions were observed following Gaglioppo and Nerello treatments ($p < 0.05$ vs. hGlu and $p < 0.05$ vs. PA).

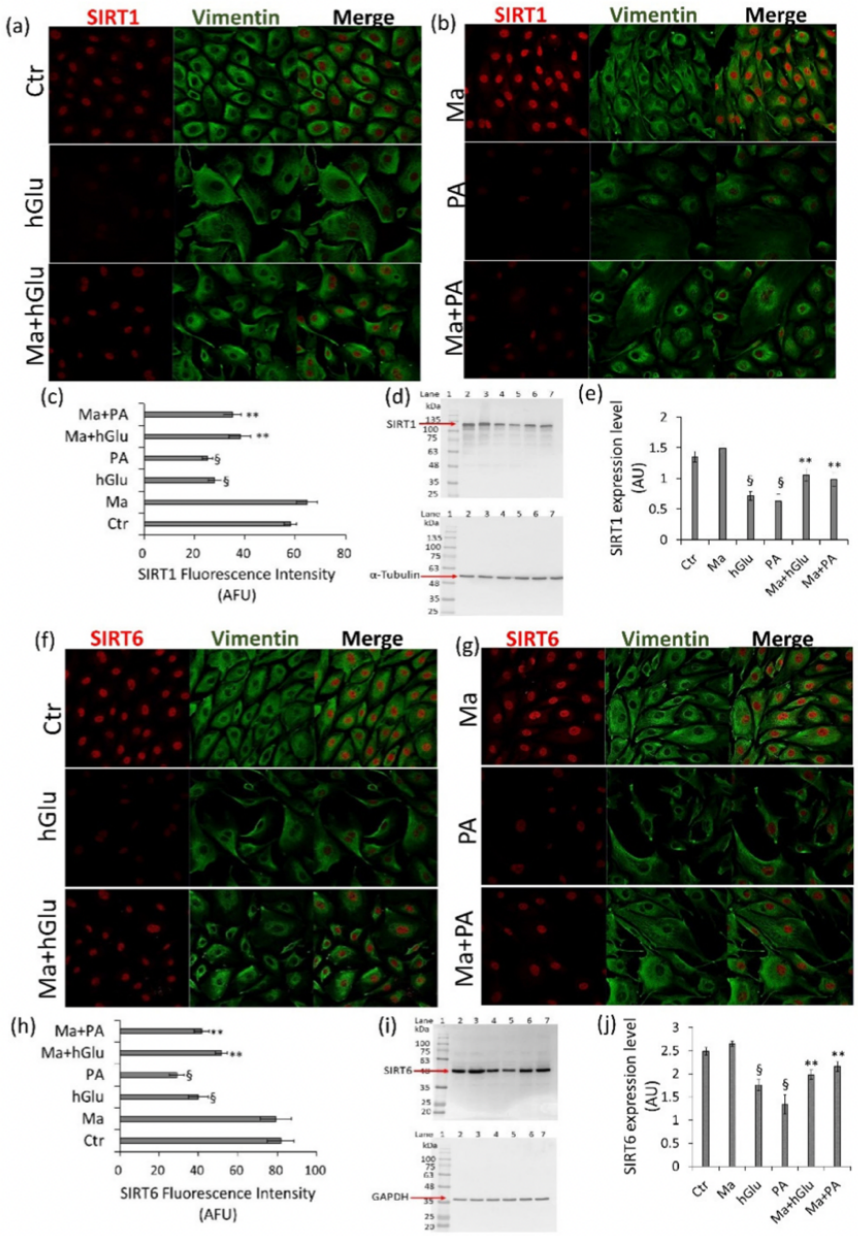

Figure 2.10. *SIRT1 and SIRT6 activation by Magliocco. (a,b) Representative confocal images of SIRT1 (red) and vimentin (green) and (c) fluorescence intensity determination performed by ImageJ software and expressed as arbitrary fluorescence units (AFU) ± SD of n = 3 replicates. (d,e) Western blot analysis of SIRT1 expression levels. (f,g) Representative confocal images of SIRT6 (red) and vimentin (green). (h) Fluorescence intensity analysis. (i,j) Western blot analysis of the SIRT6 expression levels. Lane 1 = protein ladder molecular weight markers, lane 2 = Ctr, lane 3 = Ma, lane 4 = hGlu, lane 5 = PA, lane 6 = Ma+hGlu, and lane 7 = Ma+PA. The analysis of densitometric intensity was calculated with ImageJ software and expressed as arbitrary units (AU) ± SD of n = 4 replicates. α-Tubulin or GAPDH was used as the internal control. § $p < 0.01$ vs. Ctr, ** $p < 0.01$ vs. hGlu and vs. PA.*

The activation of SIRT1 and SIRT6 was also reflected in the modulation of their downstream targets. Indeed, the increased levels of total and acetylated NF-κB protein expression during treatments with hGlu and PA ($p < 0.01$ vs. Ctr) were reduced by

cotreatment with Magliocco (0.6 µg/mL) (0.72 ± 0.062 vs. 1.01 ± 0.115 AU in hGlu; 0.88 ± 0.045 vs. 1.13 ± 0.115 in PA-treated cells) (Figure 2.11 a–g).

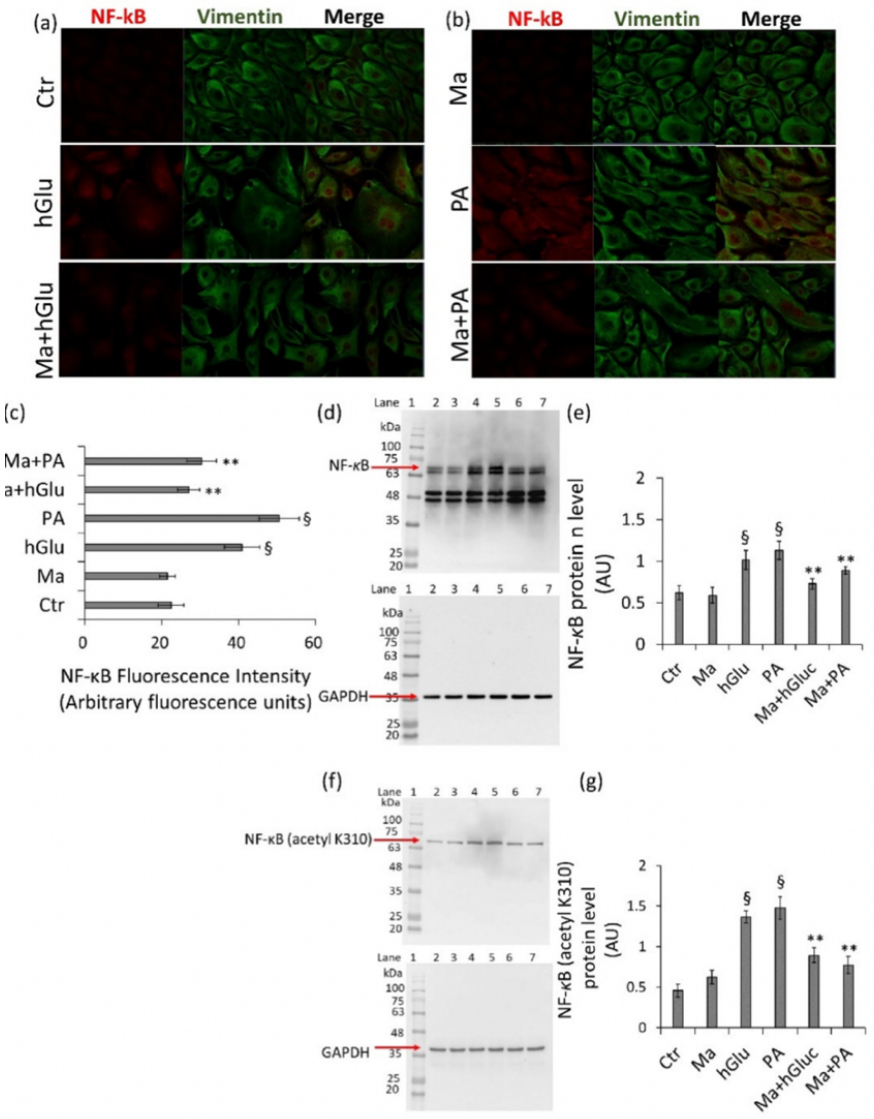

Figure 2.11. Modulation of NF-κB by Magliocco. (a,b) Representative confocal images of NF-κB (red) and vimentin (green) and (c) fluorescence intensity analysis performed by ImageJ software and expressed as arbitrary fluorescence units (AFU) ± SD of n = 4 replicates. (d,e) Western blot analysis of total and (f,g) acetylated (acetyl K310) NF-κB expression levels. Lane 1 = protein ladder molecular weight markers, lane 2 = Ctr, lane 3 = Ma, lane 4 = hGlu, lane 5 = PA, lane 6 = Ma+hGlu, and lane 7 = Ma+PA. The analysis of densitometric intensity was calculated with ImageJ software and expressed as arbitrary units (AU) ± SD of n = 3 replicates. GAPDH was used as the internal control. § $p < 0.01$ vs. Ctr, and ** $p < 0.01$ vs. hGlu or PA.

Finally, treatments with hGlu and PA caused the increase of total and acetylated p53 levels ($p < 0.01$ vs. Ctr), which were restored by Magliocco coincubation ($p < 0.05$ vs. hGlu and PA alone) (Figure 2.12 a–g).

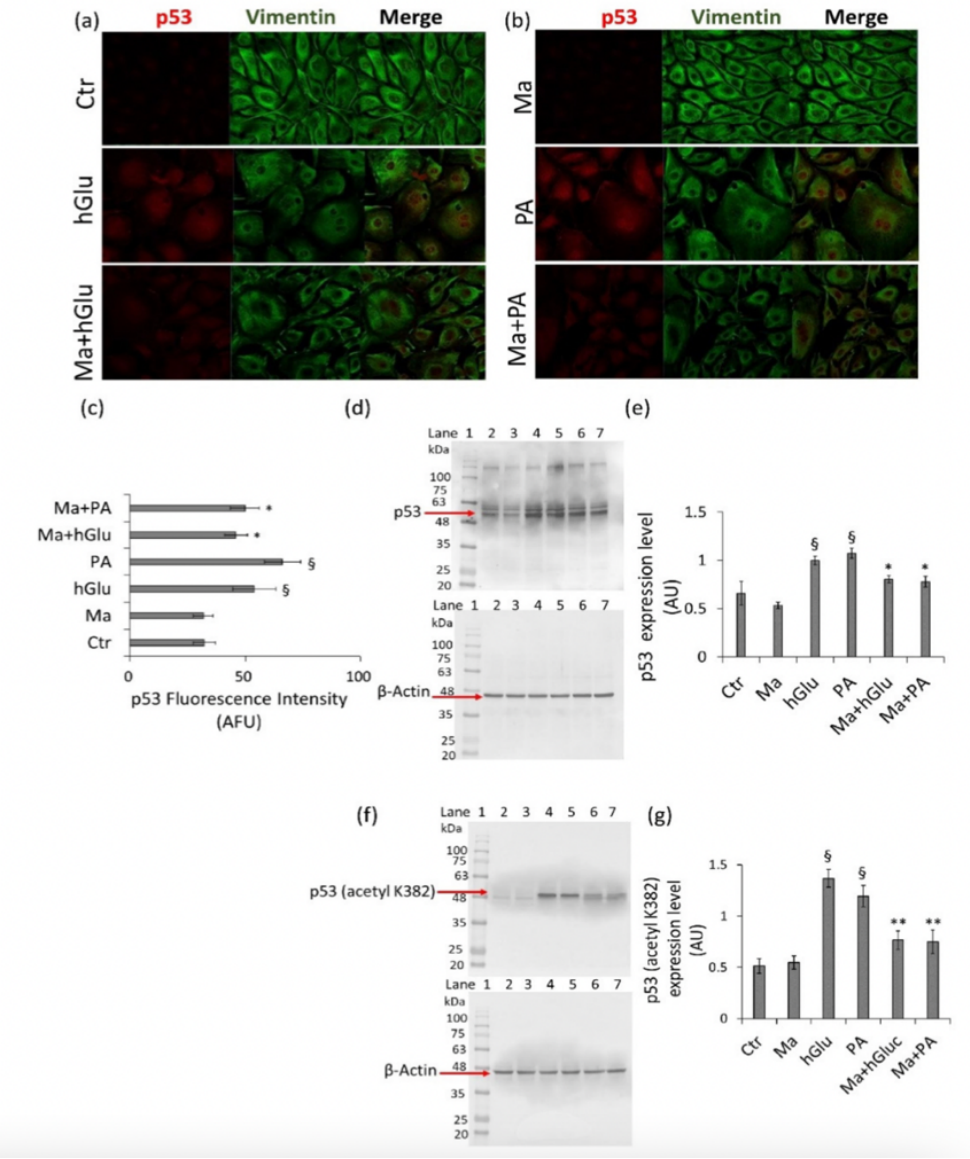

Figure 2.12. *Modulation of p53 by Magliocco. (a,b) Representative confocal images of p53 (red) and vimentin (green). (c) Fluorescence intensity performed by ImageJ software and expressed as arbitrary fluorescence units (AFU) ± SD of n = 3 replicates. (d,e) Western blot analysis of total and (f,g) acetylated (acetyl K382) p53 expression levels. Lane 1 = protein ladder molecular weight markers, lane 2 = Ctr, lane 3 = Ma, lane 4 = hGlu, lane 5 = PA, lane 6 = Ma+hGlu, and lane 7 = Ma+PA. The analysis of densitometric intensity was calculated with ImageJ software and expressed as arbitrary units (AU) ± SD of n = 3 replicates. β-Actin was used as the internal control. § p < 0.01 vs. Ctr, *p<0.05vs. hGluorPA,and**p<0.01vs. hGluorPA.*

2.2.3 Discussion

In the present study, we provide novel evidence on the in vitro bioactivity of red wines in endothelial cell models of insulin resistance and hyperglycemia related to the quantitative and qualitative phenolic profiles. The Italian red wines, Magliocco, Gaglioppo, and Nerello Mascalese, displayed different polyphenol profiles, with Magliocco being the richest in polyphenols and exhibiting the most potent of cytoprotective effect as activator of SIRT1 and SIRT6.

The chemical analysis of red wines was conducted by three different techniques to achieve information on both the major monomeric and polymeric polyphenol contents. Firstly, the untargeted NMR-based analysis was employed to provide a comprehensive picture of the low molecular weight polyphenols, including phenolic acids, flavonoids, and hydroxycinnamic acids. The results revealed that Magliocco displayed the highest concentration of flavan-3-ols and of catechin but presented the lowest content of gallic acid. Pyrogallol was quite abundant in both Gaglioppo and Magliocco, while Nerello Mascalese showed the highest content of ethyl caffeate. Structurally, pyrogallol is related to gallic acid, from which it is industrially produced by thermal decarboxylation under drastic conditions of pressure and temperature. The occurrence of pyrogallol in wines has been suggested to be the result of the aging in certain types of wood and is of great interest, as it has been shown to exert lung cancer cell growth inhibition, antioxidant, antiseptic, and antipsoriatic properties [Sarikaya, 2015]. Additionally, tyrosol and 2-phenylethanol, produced during the fermentation of tyrosine and 2-phenylalanine, respectively, by the Ehrlich pathway mediated by yeasts, display several health enhancing activities, such as antioxidant, anticancer, antimicrobial, and even cardio-preventive actions [Covas et al. 2003]. Regarding flavan-3-ols, the detected amounts of catechin and epicatechin evaluated in the analyzed wines are quite common and have important enological impacts. Indeed, flavan-3-ols influence both the organoleptic qualities, as well as biological properties, of wines. As bitter and astringent compounds, flavan-3-ols have been extensively studied to investigate their role in determining the gustative equilibrium of wines. Additionally, catechin and epicatechin are of great pharmacological interest, owing to their many health-related effects, such as the antioxidative, antihypertensive, anti-inflammatory, antiproliferative, antithrombogenic, and antihyperlipidemic ones [Bernatova et al. 2018]. The results obtained from the HPLC/UV-Vis analysis allowed the identification and quantification of the anthocyanin contents. These latter, which constitute a class of natural polyphenols responsible for both the quality and longevity of

red wines, are native grape pigments responsible for the color of red wine, and their relative amounts are a specific feature of each grape variety and, consequently, of the derived wines. We found that malvidin was the most abundant anthocyanin in Magliocco wine, while a lower percentage of this anthocyanin was detected in Gaglioppo and Nerello. Similar, anthocyanin profiles have been reported for Nerello, while studies conducted on Gaglioppo wine have described a clear dominance of peonidin [Mattivi et al. 2006]. We hypothesized that the discrepancy between our data and what was reported in the literature for Gaglioppo could be related to the fact that the Gaglioppo wine we analyzed was, in fact, a blend of Gaglioppo and Magliocco (1:1 *v*/*v*).

The oligomeric and polymeric wine polyphenols analyzed by spectrophotometric investigation also revealed that Magliocco was the richest one in terms of the anthocyanin amount with respect to the other two wines. The formation of polymeric pigments in red wines is a long and slow process modulated by the oxygen uptake, as well as by the initial phenolic composition [Gambuti et al. 2020]. Our data suggested that Magliocco abundant anthocyanins likely continue to react, thus further stabilizing the wine color. Indeed, a previous study demonstrated that red wine color stabilization depends on reactive monomeric anthocyanins and on their ratio to LMWP [Gambuti et al. 2015; 2019]. Therefore, Nerello is poised to improve its color too, but to a lesser extent than Magliocco, while Gaglioppo appears not to be able to further stabilize its pigments because of its quite low concentration of anthocyanins. The content of LMWT determined in Gaglioppo is similar to that reported by Bosso et al. [2019], and when LMWT are more abundant than HMWT, wines usually taste less astringent and more bitter. However, the astringency intensity in wine depends on a great number of factors [Soares et al. 2017] and is strictly correlated to the binding affinity of tannins, whose chemical structures are crucial for their interaction with saliva. Under an enological point of view, the evidence that there is still a large quantity of reactive compounds present in Magliocco, such as monomeric anthocyanins, catechins, and LMWP, suggests that, in this wine, the phenolic profile is likely to evolve over time, thus changing the wine stability, sensory properties, and health-related effects.

The definition of the chemical compositions of Magliocco, Gaglioppo, and Nerello Mascalese paved the way to investigate the possible correlations with their bioactive properties. The antioxidant capacity and reducing power showed a higher biological activity in Magliocco wine, in line with its richest polyphenolic profile (flavonoids, flavonols, total anthocyanins, and polymeric pigments) compared to Nerello and

Gaglioppo. The correlation between the phenolic contents of the three analyzed wines with their antioxidant properties underlined that antioxidant activities increased as a function of the degree of polymerization of polyphenols, consistently with what reported by Li and Sun [2019]. The Magliocco wine was able to counteract hyperglycemia and insulin resistance, the latter a typical feature of prediabetes status, affecting more than 400 million in the world. Projections indicate that, by 2030, more than 470 million people will suffer from prediabetes [Sardu et al. 2019]. Polyphenols, indeed, have been widely studied for their capacity to reduce insulin resistance by the activation of AMPK (AMP-activated protein kinase) or inhibition of the mTORC1 and PI3K/AkT pathways in several experimental models [Park et al. 2012]. Additionally, AMPK activation by polyphenols increases the glucose uptake by positively affecting the endothelial nitric oxide synthase (eNOS) expression and lowering the insulin resistance by inhibiting PI3K/AkT and JNK in the activation of the AMPK-SIRT1-PGC1α axis [Park et al. 2012]. The anti-inflammatory effects of Magliocco are probably ascribable to its quite high content of epicatechin, catechin, and flavanols that beneficially impact the endothelial function and prevent cardiovascular diseases by reducing the proinflammatory milieu in TNF-α-activated endothelial cells [Claude et al 2014]. Moreover, flavanols and flavonols exert their vascular-protective role by reducing the manifestations of age-related vascular injury. Indeed, they reduce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase by affecting MAPK signaling and downregulating the expression of genes via the p38-MAPK and p65-NF-kB pathways [Claude et al 2014]. It is also well-known that excessive ROS production results in the accumulation of DNA damage and induces cellular senescence and that polyphenols can inhibit p53-induced endothelial senescence mediated by TNF-α [Xu et al. 2007]. In line with these observations, our data showed that Magliocco wine counteracted the increased NF-kB protein expression levels that occurred under high-glucose and palmitic acid stimulation in endothelial cells. Additionally, Magliocco wine suppressed ROS production and inhibited p53 and TNF-α expression in endothelial cells under metabolic stress. It is generally accepted that hyperglycemic vascular complications are associated with oxidative stress and that the endothelial loss of SIRT1 and SIRT6 during oxidative damage is related to an increased expression of total and acetylated NF-κB and p53 [D'Onofrio et al. 2018]. The results from this study also revealed that Magliocco wine prevented oxidative stress via the direct/indirect modulation of SIRT1 and SIRT6, key regulators of the metabolism, DNA repair, and

inflammatory response, mainly localized to the nucleus but resulting in cytosolic translocation under stressful conditions.

Polyphenol bioactivities depend on their oral bioavailability, which is usually quite low [Stockley et al. 2012]. Nonetheless, it seems to be high enough to cause polyphenols to exert their health- related beneficial effects altogether. However, as mentioned above, the most abundant grape and wine polyphenols occur as polymeric compounds. In red wines, polymeric polyphenols range from 1000 to 5000 mg/L, as opposed to monomeric compounds such as resveratrol, a widely studied stilbene that is usually present in concentrations hardly above 5 mg/L. The role of resveratrol contained in red wine as an activator in the SIRT1 prevention of endothelial injuries related to atherogenesis and hyperglycemia stress is, to date, widely described [Li and Sun, 2019]. However, when evaluating the health-related benefits of wines, the contribution of polymeric polyphenols cannot be overlooked. Some studies have indeed ascertained that the radical scavenging activity of proanthocyanidins, measured by the DPPH assay, is positively correlated to their degree of polymerization, with polymeric compounds more active than oligomeric ones that, in turn, are more potent scavengers than monomeric molecules, such as catechins [Li and Sun 2019]. The protective effectiveness of Magliocco wine is, presumably, also ascribable to the presence of large quantities of reactive compounds, such as monomeric anthocyanins, catechins, and LMWP in this wine. Furthermore, the contribution of HMWP, of which Magliocco is rich, cannot be ruled out, thus finally showing that wine benefits may be accomplished by the simultaneous mixture of compounds rather than from the action of a single class of polyphenols.

2.2.4 Conclusions

The results of this study supported the relevance of both the content and type of red wine phenols in relation to their in vitro efficacy against endothelial oxidative damages induced by insulin resistance and hyperglycemia. Besides the biological relevance of monomeric anthocyanins, catechins, and LMWP, additional mechanistic in vitro and in vivo studies are necessary to fully define their specific roles and the occurrence of additive or synergistic effects as SIRT1 and SIRT6 activators in the prevention of endothelial damage due to type 2 diabetes.

2.2.5 Considerations

The present activity was conducted in collaboration with the entire Enology group of the Department of Agricultural Sciences, Section of Vine and Wine as well as the Department

of Sciences of Precision Medicine of the University of Campania Luigi Vanvitelli, and the Department of Pharmacy of the University of Naples. The acquired results found editorial placement in an international scientific journal in the form of an original research article entitled "Phenolic Profiles of Red Wine Relate to Vascular Endothelial Benefits Mediated by SIRT1 and SIRT6." The article, to date, has been cited by two groups of Authors [Nemzer et al 2022; Sabetta et al. 2022] for review as well as for the development of a new research article.

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CHAPTER 3

WINE OXIDATION AND AGING: TOWARDS A SUSTINABLE WINEMAKING

The concept of sustainability first emerged in 1987, with the Brundtland Report, and aims at social and economic progress that improves the human way of life without compromising the ability of future generations [Bermejo, 2014; Moscovici and Reed, 2018]. It is one of the main goals of the United Nations 'Sustainable development has been defined as development that meets the needs of the present without compromising the ability of future generations to meet their own needs [UN, 2021]. The wine sector, like other afro-food sectors, must consider sustainability in each phase of wine production and commercialization [De Steur et al., 2019; Santini et al., 2013]. Also contributing to pushing the wine sector towards the sustainability challenge are the demands of consumers, who are increasingly interested in the ecological aspects of winemaking [Fiore et al., 2021]. The wine sector deserves reflection because in its relationship with sustainability it presents elements rich in contradictions. On the one hand, it is strongly associated with environmental sustainability, as viticulture contributes to land conservation trying to adapt to the climate and its variations. However, at the same time, it produces several negative impacts on the environment, including water consumption, pesticide use, wastewater generation, waste, and carbon footprint [Ayuda et al., 2020].

At the beginning of the 21st century, the wine sector began to consider sustainable practices and articles were published analysing the sustainability of the sector by emphasising the concepts of green business, organic, sustainable, or biodynamic [Santini, 2019; De Steur et al., 2019].

One of the most important challenges of sustainable winemaking is to find alternative means of controlling undesirable microorganisms and preventing oxidative processes. To date, wine preservation technologies have been based mainly on the addition of sulphur dioxide (SO_2), because of the broad spectrum of action of this compound, related to the control of undesirable microorganisms and the prevention of oxidative phenomena [Lisanti et al. 2019].

3.1 WINE OXIDATION

Wine oxidation can be divided into enzymatic oxidation and non-enzymatic oxidation. Enzymatic oxidation occurs almost entirely in grape must and is largely related to the

content of hydroxycinnamates, such as caffeoyltartaric acid and para-coumarotartaric acid, and flavan-3-ols. Non-enzymatic oxidation, also called chemical oxidation of wine, prevails in wine and begins with the oxidation of polyphenols containing a catechol or galloyl group catalized by metal ions. These phenolic reactions, both enzymatic and non-enzymatic, result in by-products called quinones.

Quinones, can further stabilise their structure combining with thiols, or undergoing reverse processes mediated by SO_2 to rebuild the original hydroxylated form. Conversely, during chemical oxidation the hydrogen peroxide is also produced in first steps. Further this high reactive peroxide is involved in the Fenton mechanism, initiating the radical chain in the second oxidation stage: the progressive formation of species with increasing reactivity which indiscriminately oxidise major constituents of wines (alcohols, acids) occurred. Thus aldehyde, ketones, by-oxidation adducts and brown pigments were produces responsible for significant variation in the sensory properties of wine were produced. In the next two sections the second stage of non enzymatic wine oxidation is analysed in detail according with formation of radical species, and subsequent alteration of the oenological substrate.

3.1.1 Reactive Oxygen Species ROS and Non-Enzymatic Oxidation

The main mechanism leading to wine oxidation is constituted by the initiation of a radical chain in the presence of critical concentrations of dissolved oxygen in wine and catalysed by the presence of solvated metal ions, mainly copper and iron, at the specific pH of wine [Singleton, 1987; Danilewicz, 2003; Waterhouse & Laurie, 2006].

Reactive oxygen species (ROS) is a collective term used to describe oxygen radical. In wine, ROS can be produced by reduced transition metals ions [e.g. Fe (II)] in the stepwise addition of a single electron to triplet oxygen (O_2). The initial transfer of an electron leads to the formation of superoxide radical anion ($O_2^{\bullet -}$), which at wine pH exists in the protonated form hydroperoxyl radical ($HOO^{\bullet}$). The transfer of a second electron will produce peroxide anion (O_2^{2-}), which at wine pH exists in the protonated form hydrogen peroxide (H_2O_2). The next reduction step creates an even more reactive oxidant, the hydroxyl radical ($HO^{\bullet}$) (Figure 3.1), which can abstract a hydrogen atom from organic compounds to produce water, the final oxygen reduction product [Danilewicz 2003; Waterhouse & Laurie 2006].

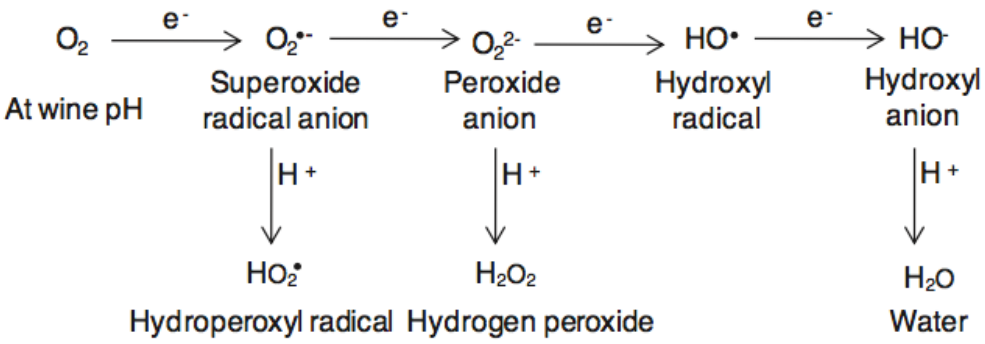

***Figure 3.1**. Oxygen reduction [Waterhouse & Laurie 2006]*

Chemical oxidation of wine occurs at the expense of polyphenols that contain an ortho-dihydroxybenzene part (catechol ring) or a 1,2,3-hydroxybenzene part (galloyl group), such as (+)-catechin/(-)-epicatechin, gallocatechin, gallic acid, 2,3-trihydroxybenzene (galloyl group), such as (+)-catechin/(-)-epicatechin, gallocatechin, gallic acid and its esters, and caffeic acid [Kilmartin, Zou, & Waterhouse, 2001; Li et al. , 2008]. They are oxidized to semichinonic radicals and benzoquinones, while oxygen is reduced to hydrogen peroxide; all are mediated by the Fe_3^+/Fe_2^+ and Cu_2^+/Cu^+ redox cycle (Figure 3.2) [Danilewicz et al. 2008].

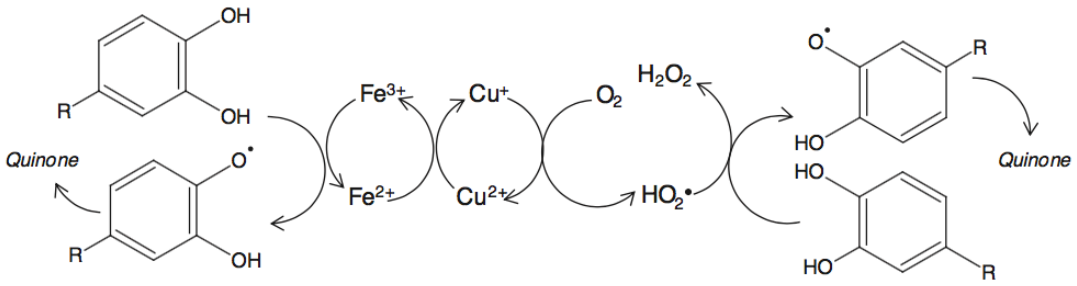

***Figure 3.2** Proposed catalytic action of iron and opper ions in the oxidation of catechols to produce quinones and hydrogen peroxide [Danilewicz et al. 2008].*

In fact, oxygen would not react with phenolic compounds except if there is presence of transition metal ions [Waterhouse and Laurie, 2006]. Beside the polyphenols previously described other compounds with more isolated phenolic groups such as malvidin, para-coumaric acid and resveratrol are oxidized at higher potentials [Kilmartin et al., 2001]. Quinones formed from the oxidation of polyphenols are unstable and can undergo further reactions by spontaneously combining with nucleophilic compounds including some

phenols, thiols and amines. In addition, the dimers or polymers produced can reorganize their structure through an enol-type conversion reaction to form new dihydroxybenzene compounds [Li et al., 2008]. Newly formed dimers or polymers, having lower redox potentials than the initial phenols, are much more easily oxidized and an acceleration of the polymerization process occurred [Boulton et al., 2001; Zhai, Du, Guan, Qiao, & Pan, 2001].

Hydrogen peroxide in association with ferrous ions generates hydroxyl radical (HO-), a reaction known as the Fenton reaction (Fig. 3.3).

Figure 3.3. *Fenton reaction*

The hydroxyl radical is a reduced product of oxygen and is recognized to oxidize almost all organic molecules in wine [Waterhouse & Laurie 2006]. Moreover, because of its nonselectivity, it reacts with the first species it encounters, depending on their concentration [Li et al., 2008], such as ethanol, tartaric acid, glycerol, sugars, and organic acids [Danilewicz, 2003; Waterhouse & Laurie 2006]. Fenton oxidation of ethanol and tartaric acid generates, respectively tartaric acid, acetaldehyde and glyoxylic acid [Danilewicz, 2003; Li et al., 2008]. The α-hydroxy acids in wine, such as L(-)-lactic and L(-)-malic acids, are also oxidized to pyruvic and 2-oxobuta-nedioic acids, respectively. Ortho-dihydroxybenzene rings are oxidized to quinones by sequential transfer of two hydrogen atoms [Danilewicz, 2003]. The rate of reaction of phenolic compounds with ROS depends on their ability to form a stable product radical.

Based on the latter claim, monophenols and their equivalent meta-dihydroxybenzene rings and substituted phenols are not easily oxidized because they do not produce stabilized semichinonic radicals. Similarly, malvidin-3-glucoside, the main anthocyanin found in red wine, is not easily oxidized. While oligomeric and polymeric phenolic compounds (procyanidins and condensed tannins) react with ROS similarly to monomeric catechol derivatives [Waterhouse & Laurie, 2006]. The presence of phenolic radicals in red wine has been evidenced by electron spin resonance spectroscopy studies [Troup et al. 1994]. The first direct evidence of the Fenton reaction in wine was by spin trap studies that detected the 1-hydroxyethyl radical resulting from ethanol oxidation via the hydroxyl radical [Elias, et al. 2009]. Finally, it has been confirmed that the metal-catalyzed

reduction of H_2O_2, via the Fenton reaction, is a key step in the nonenzymatic oxidation of wine that leads to the formation of hydroxyl radicals capable of oxidizing ethanol to acetaldehyde in a model wine system [Elias et al. 2010]. Therefore, Fenton and quinone reaction pathways in wine lead to the production of two important groups of electrophiles: aldehydes and quinones. These two electrophiles react with wine compounds and give rise to new molecular structures that may have sensory significance in wines. On the other hand the same formation of quinones is favoured by nucleophiles capable of reacting with quinones [Lopes et al. 2005]. Such nucleophiles have been identified in wine, including the electron-rich A-rings of flavanols, amino acid residues of peptides or proteins, reducing species (SO_2, ascorbate, glutathione) and volatile thiols.

The nucleophilic attack of the C-8 carbon of a flavanolic unit (catechin or epicatechin) on the B ring of a catechinic quinone proceeds via a Michael-type addition. The resulting flavanolic dimer can undergo intramolecular addition mechanisms following oxidation of the B-ring, giving rise to yellow pigments such as dehydrodicatechin-A [Guyot et al. 1996; Waterhouse and Laurie 2006]. These newly formed dimers or polymers resulting from quinone reactions have lower redox potentials than the original flavanols, increasing the probability of oxidation of the regenerated o-diphenol societies [Boulton et al. 2013; Li et al. 2008]. The reaction of quinones with volatile thiols is a major concern for the preservation of wine aroma [Nikolantonaki and Waterhouse 2012].

3.1.2 Winemaking and exogenous antioxidants

3.1.2.1 Sulphur dioxide and ascorbic acid.

Sulphur dioxide (SO_2) is widely used to protect musts and wines due to its antimicrobial and antioxidant properties. SO_2 is added to grape must or wine as a liquid or gaseous form; in Europe, the SO_2 forms that can be used as food additives are sulfur dioxide, sodium sulfite, sodium hydrogen sulfite, sodium metabisulfite, potassium metabisulfite, calcium sulfite, calcium hydrogen sulfite, and potassium hydrogen sulfite. SO_2 is present in wine not only because of exogenous addition during the winemaking process, but it is also produced by yeast metabolism during alcoholic fermentation, the so-called "biological SO_2". In fact, yeasts use the sulfur present in the must for the synthesis of amino acids, and the production levels of sulfites by yeasts is highly strain-dependent [Noble et al. 2015]. Sulphur dioxide, in wine conditions characterised by an acidic pH, is 94-99% present in ionic form as the bisulphite ion HSO_3^- and only a small percentage is present as free SO_2. Once in solution in the wine, sulphur dioxide can bind to various molecules such as acetaldehyde, anthocyanins, pyruvic acid, glutaric acid, glucose or

phenolic compounds, in particular caffeic acid and para-coumaric acid. Moreover, sulphur dioxide does not react directly with oxygen, but with the reduced form of oxygen, hydrogen peroxide (Figure 3.4). In this way, SO_2 can inhibit the formation of aldehydes by competing for hydrogen peroxide [Elias et al. 2010]. SO_2 also plays an important role in reducing quinones formed during the oxidation process (Fig. 3.4) [Danilewicz, 2007; Danilewicz et al., 2008].

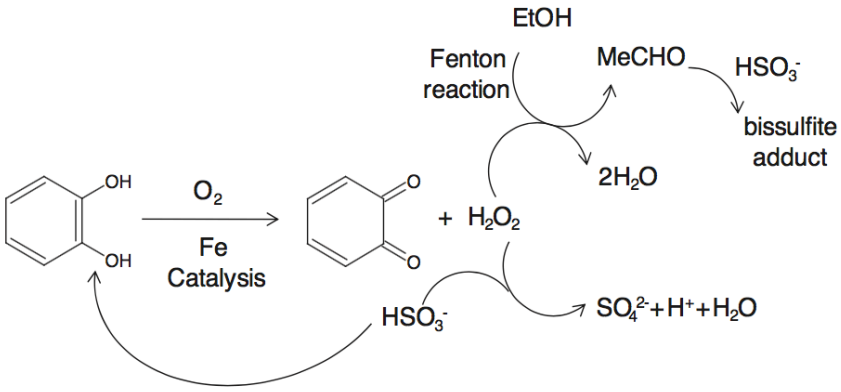

Figure 3.4 *The interaction of SO_2 with hydrogen peroxide and quinones following catechol oxidation, so preventing oxidation of ethanol by the Fenton reaction.*

The molar reaction ratio between oxygen and SO_2 is 1:2, which is consistent with one equivalent mole of SO_2 reacting with hydrogen peroxide and a second with quinone. However, the oxygen/SO_2 molar reaction ratio found in red wines was 1:~1.7, suggesting that some nucleophilic substances might compete with bisulphite for quinones. The reaction speed of oxygen with wine constituents is also accelerated by SO_2 in red wine.

In addition to sulphur dioxide, ascorbic acid represents the other antioxidant substance of interest in winemaking. Ascorbic acid, an important antioxidant, is naturally present in grapes, but is usually consumed quickly after crushing due to its ability to capture oxygen or reduce orthoquinone derivatives formed by the enzymatic oxidation of phenolic compounds. Thus, the ascorbic acid present in wine is mostly due to exogenous additions, prior to bottling or even at various stages of the wine production process. Levels of added ascorbic acid can vary widely but are generally added in percentages ranging from 50 to 150 mg/L [Barril et al. 2009]. Ascorbic acid could effectively eliminate molecular oxygen and is initially converted to dehydroascorbic acid and hydrogen peroxide. Dehydroascorbic acid then undergoes rapid degradation into a variety of species, including numerous carboxylic acids, ketones and aldehydes. In fact, ascorbic acid is

highly reducing and its reduction potential (~210 mV (Ag/AgCl) at pH 3.6) is much lower than that of wine polyphenols [Kilmartin et al., 2001]. Therefore, when adding ascorbic acid, it is important that an adequate amount of SO_2 is present, to remove hydrogen peroxide and react with the various carbonyl compounds that result from the oxidation of ascorbic acid. Some authors have demonstrated the increased production of phenolic pigments in model wine systems from ascorbic acid and (+)-catechin and have shown that ascorbic acid is able to react with (+)-catechin and form coloured xanthylium cations [Barril et al., 2009; Barril et al. 2008].

3.1.3 Reactive Oxygen Species ROS and Non-Enzymatic Oxidation

Numerous factors, such as increasing consumer attention to health concerns and restrictive legislation on preservatives, together with the demand for environmentally friendly production, have driven the interest of the scientific community and manufacturers in alternative methods to traditional protective and antioxidant agents. In addition, the massive employment of SO_2 is not always compatible with the production of high-quality wines, as it could cause organoleptic alterations in the final product, neutralizing the aroma, and producing undesirable aromas of the sulfurous gas. [Avramona et al. 2018]. Furthermore, the World Health Organisation (WHO) has recommended the reduction of SO_2 in sectors, such as wine, that contribute significantly to daily accumulation and intake. In detail, alternative methods based on both chemical inhibitors and physical methods have been studied. Some of these have been approved by the EU for use in winemaking, while others still lack legislative or regulatory approval.
Chemical methods, such as the addition of bacteriocins and lysozyme, have the advantage of ease of use and do not require as specific and expensive equipment as physical methods. However, chemical additives are not always free of health risks and the use of new products requires a lengthy authorisation process, including the setting of dose limits. Moreover, some of them may affect sensory characteristics of wine and their evolution over time. Several studies have focused on the possible application of innovative physical methods. Most of them, such as high pressure, pulsed electric fields and microwaves, have shown good efficacy, but their validation on an industrial scale and on different wine styles is lacking.

Nowadays, all these methods still lack effective protection against must and wine oxidation (both enzymatic and chemical) and therefore require the complement of SO_2 to be used effectively. The challenge for oenological research is therefore to develop strategies involving the integrated use of different methodologies with reduced doses of SO_2. In this regard, several studies are of interest that have reported beneficial effects due to the addition of chitosan in wine due to its antimicrobial and antioxidant activity linked to to a direct radical scavenging mechanism or to a metal chelation capability, which blocks the generation of radical species [Brasselet et al. 2019; Park et al. 2004] [Castro Marín et al. 2019]. All alternative methods, however, need effective validation on an industrial scale and their consideration in relation to ageing.

3.1.4 Monitoring and determination of wine oxidation

The monitoring of wine oxidation is an urgent issue for wine scientists and industry. Until now numerous are the methods developed to evaluate the antioxidant activity of wine but how it is correlated with wine oxidation is not well known. In this paragraph the main methods to assess the antioxidant activity in biological systems and foodstuffs are discussed. Over the years, methods involving potentiometric titrations have been used to measure oxidation resistance under normal and forced ageing conditions [Oliveira, et al. 2002]. Alongside these methods, cyclic voltammetric measurements have been introduced that allow quantitative and qualitative information on antioxidants to be combined [Roginsky et al., 2006; Makhotkina & Kilmartin, 2009]. As an example of its use, the influence of sulphur dioxide, glutathione and ascorbic acid on the cyclic voltammograms of four representative polyphenols in wine was investigated using a glassy carbon electrode [Makhotkina & Kilmartin, 2009] and the results showed that sulphur dioxide increased the anodic current and decreased the cathodic current for all four polyphenols and all wines, indicating a rapid interaction of SO_2 with the oxidised polyphenolic quinones. A similar trend was observed for glutathione, except in the case of quercetin, where the addition of glutathione led to the formation of a second series of voltammetric peaks, corresponding to the redox activity of a glutathione derivative [Makhotkina & Kilmartin, 2009]. In addition, it should be emphasised that a simple method based on a single-line FIA (Flow Injection Analysis) system with amperometric detection [Mannino et al. 1998] has been developed over the past decades to assess the antioxidant capacity of red and white wines. In this case, the antioxidant capacity of white wines correlated well with the total phenol content obtained by the traditional Folin-

Ciocalteu (FCR) assay. The FCR-based assay has gained popularity and is commonly known as the total phenolics (or phenolic) assay. However, there is not such a good correlation for red wines, as some red wines showed high antioxidant capacity despite having low concentrations of total phenols. To date, the most common methods used to determine the antioxidant capacities of wines, based on chemical reactions, are the electron transfer (ET) based assays. These methods involve one redox reaction with the oxidant as an indicator of the reaction endpoint [Fernández- Pachón, Villaño, García-Parrilla, & Troncoso, 2004; Lachman, Šulc, & Schilla, 2007; Oliveira, Silva Ferreira, Pinho, & Silva, 2008]. Due to its operational simplicity, the Trolox Equivalence Antioxidant Capacity (TEAC) assay has been used in many research laboratories for studying wine antioxidant capacity. In the same way, DPPH (diphenyl-1-picrylhydrazyl) assay involves a stable and commercially available organic nitrogen radical and provides a technically simple assay for evaluation the wine antioxidant capacity [Fernández-Pachón et al., 2004; Lachman et al., 2007; Oliveira et al., 2008]. Other methods are also used, namely, Ferric Ion Reducing Antioxidant Power (FRAP), "Total Antioxidant Potential" assay, using a Cu^{2+} complex as an oxidant, and the Folin Ciocalteu Reagent (FCR). Also, the total antioxidant capacity (TAC) of five different wines (four red and one white) was determined in five different steps of winemaking carried out in a commercial wine cellar by a chemiluminescence (CL) assay. The CL method is suitable to determine the antioxidant capacity of beverages, and preliminary trials showed that the TAC immediately after the bottle was opened was greater than the day after (about 25% decrease). The wines were characterized by different levels of total phenolic compounds and TAC, and the differences were related to grape composition and winemaking technologies [Girotti et al., 2006]. Finally, the level of wine oxidation can be followed by measuring the formation of primary or secondary oxidation products. With Electron Paramagnetic Resonance Spin Trapping ESR it is possible to study the radicals that are involved in the oxidative reactions directly, which makes it possible to determine the level of oxidation more directly during forced aging experiments [Elias et al., 2009a; Elias et al., 2009b; Elias et al., 2010].

In spite of all thes methods aimed at understanding the antioxidant capability of wine, no or few is known about methods useful to predict wine oxidation.

3.2 CONSIDERATION AND CASE STUDIES

Based on these considerations, two case studies are presented in this chapter. The first case study is based on the need for information on the oxidative evolution of red wines by means of accelerated ageing tests. As already exposed in the previous chapters, phenols in wines vary in quality and quantity. It follows that it is of fundamental importance to assess how different types of red wines react to oxidation. This chapter reports the results of the study that aimed to evaluate the effect of three different oxygen addition systems on different red wines (Aglianico, Barbera, Magliocco, Gaglioppo and Nerello) with the aim of developing a tool for predicting the evolution of phenolic compounds in a short time. The second case study aimed to investigate a sustainable winemaking strategy, such as the use of chitosan, on the evolution of the main product of wine oxidation, the acetaldehyde, after exposure to an oxidative stress designed to simulate wine ageing. In addition, the evolution of wine tannins by assessing their reactivity towards salivary proteins, one of the mechanisms underlying the sensation of astringency in red wines, was studied.

3.3 THE CASE STUDY: THREE ACCELERATED OXIDATION TESTS APPLIED TO RED WINES WITH DIFFERENT CHEMICAL COMPOSITION

Wine quality is strongly influenced by ageing, a period during which the polyphenolic component of wine undergoes changes and interactions in which oxygen plays an essential role. In detail, oxygen, during the storage and ageing process, interacts with phenolic compounds resulting in important changes in the sensory profile of wines, particularly in terms of colour, bitterness and astringency. It is well known. in this regard that exposure to excessive amounts of oxygen gives red wines undesirable aromatic profiles, such as oxidative off-flavours and colours that include orange hues [Waterhouse et al. 2006].

Thus, exposure to oxygen, which is critical to the ageing process of wines, is an important issue for oenologists. The scientific literature is full of articles on the reactions that occur when wine is exposed to oxygen. As also set out in previous sections, once oxygen diffuses into the wine, it oxidises Fe(II) to generate Fe(III) and hydrogen peroxide, further catalysed by the presence of copper. Subsequently, Fe(III) oxidises the ortho-diphenol units, typical of some phenolic compounds in wine, to quinones. Hydrogen peroxide (H_2O_2) together with Fe(II) and/or Cu(I) initiates the Fenton reaction.

A critical product of the latter reaction is the hydroxyl radical (HO-), a powerful oxidant that can indiscriminately oxidise many organic compounds. The main product of the Fenton reaction is therefore acetaldehyde resulting from the oxidation of ethanol. In this chemical context, sulphur dioxide plays a key role, as it removes hydrogen peroxide, thus preventing the Fenton reaction from occurring. In addition, SO_2 reduces quinones to ortho-diphenols (catechol) and thus prevents quinones from reacting further. Finally, it binds reversibly (1:1) to acetaldehyde.

The oxidative cascade in red wine is thus characterised by three highly reactive compounds: oxygen, hydrogen peroxide and acetaldehyde, but the phenolic composition of red wines must be considered. Phenols in wines vary in terms of quality and quantity of low or high molecular weight molecules and on the one hand act as natural antioxidants by quenching free radicals, but on the other hand they reduce Fe(III) to Fe(II), thus causing oxidation to proceed. It follows that it is of fundamental importance to assess how different types of red wines react to oxidation.

There are numerous studies in the literature on the oxidative evolution of red wines by means of very different accelerated ageing tests. Some are based on exposure to high temperatures, others on enzymatic tests, the addition of hydrogen peroxide [Picariello et al. 2017; Deshaies et al. 2020] and the addition of exogenous acetaldehyde [Sheridan and Elias, 2015; Teng et al. 2019]. Among these, one of the most widely used is the evaluation of oxygen consumption after subsequent saturation to study the behaviour of pigments, tannins and aromatic compounds in wine [Gambuti et al. 2018; Ferreira et al. 2015]. To the best of our knowledge, only recently has a comparison of three of these tests been carried out on Shiraz wine [Deshaies et al. 2020], and different red wines have never been considered.

In this work, we report on the effect of three different systems of oxygen addition to wine with the aim of developing a tool to predict the evolution of phenolic compounds due to moderate oxidation in wines. The effect of direct oxygen consumption after three cycles of saturation (sat) and the addition of hydrogen peroxide (H_2O_2) and acetaldehyde (AtCH) was evaluated in five red wines (Aglianico, Barbera, Magliocco, Gaglioppo and Nerello) characterised by very different initial phenolic compositions in terms of monomeric and polymeric pigments and uncoloured phenols.

3.3.1 Materials and Methods

3.3.1.1 Wines

Five red wines of southern Italy made from different indigenous grape varieties—Aglianico, Barbera, Gaglioppo, Magliocco, and Nerello—were used in the study. Mono-varietal wines were produced with Nerello Mascalese and Magliocco grapes produced in the Calabria region (Crotone, Italy) by Marrelli Wines Cantina e Vigneti (Crotone, Italy) with a standard industrial process during 2019 vintage. The blend Gaglioppo-Magliocco (50%−50%) was produced in the same area in 2018 vintage. Aglianico wine was produced in 2019 vintage by Masseria Della Porta, and Barbera wine was produced in 2019 by A'cancellera winery (BN, Italy). Table 3.1 shows the basic parameters of wines before treatments, after centrifugation and 0.45 mm filtration. Microbiological plating showed that wines were not containing yeasts and bacteria after filtration. As the aim of this study was to simulate the possible application of these tests in a winery in the pre-bottling phase, values of free SO_2 were in the range 10 ± 8 mg/L as it often occurs in this phase. Wine parameters were determined by OIV methods of analysis [2020].

3.3.1.2 Accelerated Oxidation Test

Three accelerated oxidation tests were compared: oxygen saturation (sat), hydrogen peroxide addition (H_2O_2), and acetaldehyde addition (AtCH). Saturation test (sat): The oxidation test consisted of three consecutive air saturation cycles. The chemical composition of wines before and after the oxidation was extensively characterized. The procedure used was the same of Gambuti et al. [2018]. Briefly: Two 1 L bottles of each wine containing PSt3 oxygen sensors (Nomacorc SA, Thimister-Clermont, Belgium) were saturated by adding a gentle flow of air through a mini-compressor for 15 min until the oxygen level of the wine reached 6.6 mg/L. Bottles were previously autoclaved. Wines were stored in an incubator in the dark at 25 ◦C, and the dissolved oxygen level was monitored at least once a day with a Nomasense oxygen analyzer from Nomacorc S.A. (Thimister-Clermont, Belgium). The oxidation cycle was considered finished once O_2 levels dropped to 10% of the initial concentration (Figure 3.5A). Hydrogen peroxide test (H_2O_2): Treated wines were obtained by adding 19 mg/L of H_2O_2 (eq. to 18 mg/L O_2) (30% Fluka, Sigma Aldrich Chemie GmbH Steinheim, France) at 20 ◦C, considering a 1:1 stoichiometry of oxygen to hydrogen peroxide. Acetaldehyde test (AtCH): Treated wines were obtained by adding 24.7 mg/L (eq. to 18 mg/L O_2) of acetaldehyde (ACS reagent ≥99.5%. Sigma Aldrich Chemie GmbH, Steinheim, France) at 20 ◦C.

Furthermore, in this case, a 1:1 stoichiometry of oxygen to acetaldehyde generation was considered. H_2O_2 and AtCH were performed by transferring the red wines in autoclaved pyrex tubes previously saturated with nitrogen. Just after filling the tubes with wine, they were closed with a cork by using sealing wax. When AtCH and H_2O_2 tests were carried out, the solutions containing the two reagents (respectively acetaldehyde and hydrogen peroxide) were added to the wines through the corks by using a sealed hypodermic syringe attached to them. All the tests were performed to simulate a further contact of wine with 18 mg/L of oxygen and assuming that the consequent oxidation process produced exclusively hydrogen peroxide or acetaldehyde. Analyses of treated wines were carried out 15 days after the tests were performed. All tests were made in duplicate.

3.3.1.3 Spectrophotometric Analyses

Color intensity (Abs 420 nm + Abs 520 nm + Abs 620 nm) and hue (Abs 420 nm/Abs 520 nm) were determined spectrophotometrically using a UV spectrophotometer. All analyses were carried out in duplicate. The CIELAB parameters (L*, a*, b*) were determined by using the software Panorama (PANORAMA SOFTWARE UPGRADE PATH), following the recommendations of the Commission Internationale de L'Eclariage (CIE). Color differences (ΔE⁄ab) were calculated as the Euclidean distance between two points in the 3D space defined by L*, a*, and b*. Total anthocyanins, bovine serum albumin (Sigma Aldrich SRL, Milano, Italy), reactive tannins (BSA reactive tannins), short polymeric pigments (SPP), and large polymeric pigments (LPP) were determined by the Harbertson–Adams assay [Harbertson et al. 2003]. Briefly, pH changes permitted the evaluation of total anthocyanins, and small polymeric pigments (SPP) and large polymeric pigments (LPP) were obtained by combining analysis of supernatant obtained after protein precipitation using bovine serum albumin BSA (Spectrum Chemical, Gardena, CA, USA) with the bisulfite bleaching of pigments. Vanillin reactive flavans (VRF) were determined as described by Gambuti et al. [2018]. Briefly, 125 µL of wine previously diluted 1 to 10 with methanol were added with 750 µL of a solution of vanillin (4% in methanol). After 5 min, 375 µL of concentrated hydrochloric acid was added at 4 ◦C. After a 15-min incubation of the mixture at room temperature (20 ◦C), the absorbance was determined at 500 nm against a blank in which pure methanol was used instead of the solution of vanillin. All experiments were carried out in duplicate and two analytical replicas were performed.

3.3.1.4 High-Performance Liquid Chromatography Determination of Acetaldehyde

Acetaldehyde was determined by HPLC after derivatization reaction with 2.4- dinitrophenylhydrazine reagent (Aldrich chemistry) according to the OIV method of analysis [2020]. Wine sample aliquots (100 µL) were dispensed to a vial, followed by the addition of 20 µL of freshly prepared 1120 mg/L SO2 solution, 20 µL of 25% sulfuric acid (Carlo Erba reagent 96%), and 140 µL of 2 g/L 2.4-dinitrophenylhydrazine reagent. After mixing, the solution was left to react for 15 min at 65 ◦C and then promptly cooled to room temperature. Carbonyl hydra- zones were analyzed by HPLC using a HPLC Shimadzu LC10 ADVP apparatus (Shimadzu Italy, Milan), consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, a SPD-M 10 AVP detector, and an injection system full Rheodyne model 7725 (Rheodyne. Cotati. CA) equipped with a 50 µL loop. The separation was carried out on a Waters Spherisorb column (250 × 4.6 mm, 4µm particles diameter) equipped with a guard column. Optimum efficiency of separation was obtained using a flow rate of 0.75 mL/min, and the column temperature was of 35 ◦C. Mobile phase solvents were (A) 0.5% formic acid (Sigma Aldrich ≥ 95%) in water milli-Q (Sigma Aldrich) and (B) acetonitrile (Sigma Aldrich ≥ 99.9%); gradient elution protocol was 35% B to 60% B (t = 8 min), 60% B to 90% B (t = 13 min), 90% B to 95% B (t = 15 min. 2-min hold), 95% B to 35% B (t = 17 min, 4-min hold), total run time, 21 min. Eluted peaks were compared with derivatized acetaldehyde standard. All experiments were carried out in duplicate and two analytical replicas were performed.

3.3.1.5 High-Performance Liquid Chromatography Analyses of Anthocyanins

The separation of the monomeric anthocyanins was performed according to the OIV method of analysis [2020] in the HPLC system previously described by using a column heating device set at 40 ◦C, with a C18 column, Waters Spherisorb column (250 × 4.6 mm, 4 µm particles diameter) with pre-column. All the samples were filtered through 0.45 µm filters (Durapore membrane filters, Millipore—Ireland) into glass vials and immediately injected into the HPLC system. A 50 µL loop was used. Elution was carried out by using a flow rate of 0.80 mL/min. Eluents were solvent A consisting of water milli-Q (Sigma Aldrich)/formic acid (Sigma Aldrich ≥ 95%)/acetonitrile (Sigma Aldrich ≥ 99.9%) (87:10:3) v/v/v, and solvent B consisting of water/formic acid/acetonitrile (40:10:50) v/v/v. The following gradient was used: zero-time conditions 94% A and 6% B. after 15 min the pumps were adjusted to 70% A and 30%B, at 30 min to 50% A and50% B, at 35 min to 40% A and 60% B, at 41min end of analysis, to 94% A and 6%

B. For calibration, the external standard method was used: the calibration curve was plotted for the malvidin-3-monoglucoside (Extrasynthese, Lyon, France) based on peak area. The concentration of the following monomeric anthocyanins was determined: delphinidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-monoglucoside, mal-vidin3-glucoside, malvidin3-(6II-acetyl)-glucoside, malvidin3-(6II-coumaroyl)-glucoside. The concentration was expressed as mg/L of malvidin-3-monoglucoside. All experiments were carried out in duplicate and two analytical replicas were performed.

3.3.1.6 Statistical Analysis

Data were expressed as the average and standard deviation of measurements performed for the different wines and treatments. The influence of treatments was analyzed by a non-parametric procedure by aligned ranks transformation ANOVA to determine whether there was an overall significant difference in group means. Post hoc pairwise comparisons of levels within single factors were conducted among all levels within each factor. All statistical analyses were implemented in R environment [R Core Team 2020], using ARTool R package [Kay and Wobbrock, 2011]. The package factoextra was used for the principal component analysis (PCA). Graph and plot reports were created with the ggplot2 package [Kassambara, 2017].

Five red wines of southern Italy made from different indigenous grape varieties—Aglianico, Barbera, Gaglioppo, Magliocco, and Nerello—were used in the study. Mono-varietal wines were produced with Nerello Mascalese and Magliocco grapes produced in the Calabria region (Crotone, Italy) by Marrelli Wines Cantina e Vigneti (Crotone, Italy) with a standard industrial process during 2019 vintage. The blend Gaglioppo-Magliocco (50%−50%) was produced in the same area in 2018 vintage. Aglianico wine was produced in 2019 vintage by Masseria Della Porta, and Barbera wine was produced in 2019 by A'cancellera winery (BN, Italy). Table 3.1 shows the basic parameters of wines before treatments, after centrifugation and 0.45 mm filtration. Microbiological plating showed that wines were not containing yeasts and bacteria after filtration. As the aim of this study was to simulate the possible application of these tests in a winery in the pre-bottling phase, values of free SO_2 were in the range 10 ± 8 mg/L as it often occurs in this phase. Wine parameters were determined by OIV methods of analysis [2020].

3.3.2 Results

Wine oxidations are related to a complex series of chemical modifications, mainly involving phenolic components. In this work, we evaluated the effect of oxidative events

in five red wine samples, which were selected as to cover a wide range of wine types characterized by different phenol compositions. The primary purpose of the research was to assess the response of wine to oxidative injury based on the distribution of wine phenolics. To this aim, we simulated the oxidative evolution by resorting to air saturation as well as to the addition of acetaldehyde or H_2O_2. The response of the wine phenolic compounds to oxidation was determined by evaluating the variation of anthocyanins, polymeric pigments, chromatic features, the protein reactive tannins, and vanillin reactive flavans. In regard to the sulphite-driven protection of red wines assayed, we used samples poorly protected as it often occurs in wineries after the centrifugation and filtration treatments and before the bottling. On the basis of the different values of sulfur dioxide in wines (Table 3.1), we could refer to all wines as poorly protected by SO_2 because the official method of analysis used to determine the free SO_2 in red wines systematically overestimates both the molecular and free SO_2 in red wines due to the presence of anthocyanins [Jenkins et al. 2020]. However, it is worth emphasizing that the complex between SO_2 and anthocyanins readily dissociates thus making such initially bound SO_2 available during oxidation. This is especially true for Barbera that is the richest in anthocyanins (Figure 3.5A).

Table 3.1 *Base parameters of Aglianico, Barbera, Gaglioppo, and Nerello wines before the treatments*

Total Acidity (mg/L)	Aglianico	Barbera	Gaglioppo	Magliocco	Nerello
EtOH (%)	15.11 ± 0.14	13.34 ± 0.06	13.05 ± 0.01	10.68 ± 0.02	11.47 ± 0.02
pH	3.25 ± 0.02	3.77 ± 0.01	3.55 ± 0.01	3.84 ± 0.08	3.77 ± 0.01
free SO_2 (mg/L)	12.65 ± 0.44	19.77 ± 0.80	11.1 ± 0.60	7.15 ± 0.05	2.40 ± 0.01
total SO_2 (mg/L)	47.05 ± 0.66	37.45 ± 0.10	61.60 ± 0.46	26.4 ± 0.16	16.75 ± 1.90
total acidity (mg/L)	7.17 ± 0.04	4.59 ± 0.05	5.57 ± 0.10	4.65 ± 0.01	4.20 ± 0.05
volatile acidity (mg/L)	0.51 ± 0.02	0.69 ± 0.02	0.93 ± 0.02	0.61 ± 0.01	0.64 ± 0.01
sugar (mg/L)	1.95 ± 0.12	2.3 ± 0.11	1.92 ± 0.09	1.39 ± 0.01	1.7 ± 0.14
monomeric anthocyanins (mg/L)/BSA Reactive Tannins (mg/L)	0.31	2.40	0.03	0.22	0.05

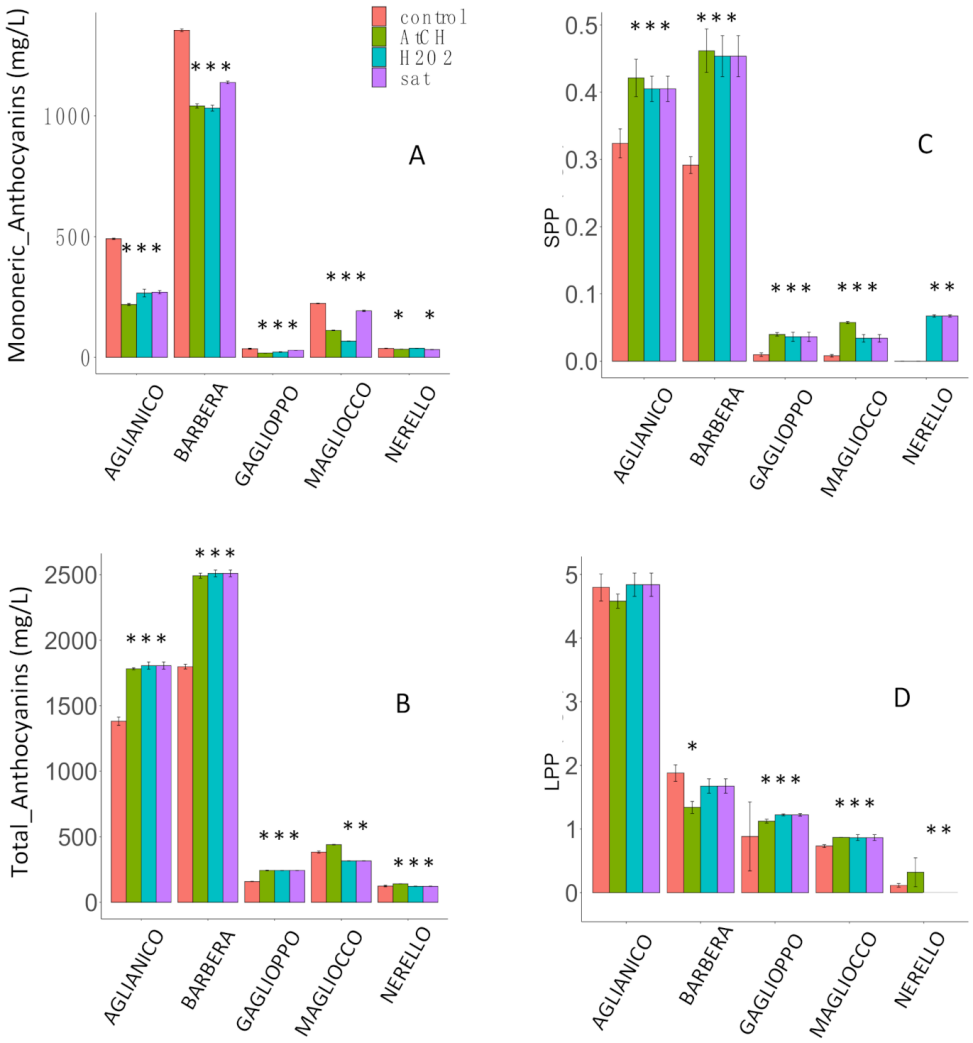

Figure 3.5. *Variation of pigments in response to oxidative tests in different wines (Aglianico; Barbera; Gaglioppo; Magliocco; Nerello; Mascalese). (**A**) Sum of monomeric anthocyanins; (**B**) total anthocyanins; (**C**) polymeric pigments with a low degree of polymerization; (**D**) polymeric pigments with a high degree of polymerization. Asterisks (*) indicate significant differences between treated wine and its control (p-value less than 0.05).*

3.3.2.1 Variation of Pigments and Chromatic Characteristics

As expected, oxidation events induced drastic chromatic variations in wines due to changes in their pigment compositions (Figure 3.5 and Table 3.2). The total content of monomeric anthocyanins, determined by HPLC-based analyses, significantly decreased in all the wine samples compared to their respective controls (Figure 3.5A and Table 3.2). Slight differences among the oxidative treatments were detected. In contrast, oxidative treatments induced an increase in Aglianico and Barbera wines of the total anthocyanins, determined by the Harbertson–Adams assay [Harbertson et al. 2003]. This was likely the consequence of the formation of new pigments exhibiting absorption bands close to λmax of anthocyanins (520 nm) (Figure 3.5B) [Waterhouse and Zhu, 2020]. The amount of the

newly formed pigments overcame the decrement of native anthocyanins. Total pigments increased at comparable levels due to oxidative treatments and appeared to be independent of the polyphenolic composition of wines. In the Aglianico and Barbera wines, the increased concentration of total pigments was more evident than for the other wines because of the relatively higher number of native anthocyanins. In fact, the most important anthocyanin derivatives are new polymeric pigments generated either by direct condensation reaction between anthocyanins and flavanols or by the ethanal action forming flavanol-ethyl-anthocyanin adducts [Waterhouse and Zhu, 2020]. In this study, polymeric pigments were determined as two different classes, namely, large polymeric pigments (LPP) and small polymeric pigments (SPP), separately determined by the Harbertson–Adams assay [Harbertson et al. 2003]. SPP increased in all the treated wine samples apart from $\text{Nerello}_{\text{ATCH.}}$ (Figure 3.5C). Given that Nerello turned out to be quite poor in both flavanols and anthocyanins, the formation of ethylidene-bridged pigments was not significant, while in $\text{Nerello}_{\text{H2O2}}$ and $\text{Nerello}_{\text{sat}}$ the oxidative environment may have favored the direct condensation between anthocyanins and flavanols. Additionally, except $\text{Barbera}_{\text{ATCH}}$, the level of LPP turned out to be practically unaffected by oxidative tests (Figure 3.5D). The evidence that newly formed pigments were small-sized condensation products, which are not precipitated by BSA, would suggest that kinetically the formation of small tannins would be favored over the time the experiments were carried out. The decrease of LPP in $\text{Barbera}_{\text{ATCH}}$ could be since molecules with higher polymerization degrees are not detected by the BSA-based assay, even though their formation cannot be ruled out altogether. The CIEL*a*b* colorimetric model defines a panel of descriptors (coordinates) for the chromatic variation of wines. The values of the different color coordinates measured for the oxidative treatments are shown in Table 3.2. Lightness (L*) decreased for oxidized wines due to a progressive darkening. The a* coordinate (green-red color component) increased for all wines, pointing out that wines acquired more intense red shades, likely related to the neoformation of red pigments [Waterhouse and Zhu, 2020]. The b* coordinate (blue-yellow color components) increased in Aglianico, Barbera, and Nerello giving more orange-yellow pigments while it decreased in Gaglioppo and Magliocco, suggesting that, for these wines, there was no appreciable formation of orange-yellow pigments resulting from oxidation of flavanols or from anthocyanin degradation [Pérez-Magarino and Gonzàlez-San, 2004]. Chromacity (C) increased after oxidation for all wines. A similar

trend was even detected by Avizcuri et al. [2016] after aging in bottle of sixteen commercial red wines. During the oxidative tests, it is possible that a change in color occurs due to the removal of sulfur dioxide from the colorless derivatives previously formed by sulfite and anthocyanins [Howe et al. 2018]. Furthermore, the formation of new pigments could be responsible for the observed effects [Waterhouse and Zhu, 2020]. ΔE provides a useful indicator of the effects of oxidation on human visual perception [Mokrzycki and Tatol, 2011]. In our case, this parameter confirmed that oxidation tests produced noticeable (Nerello wine) or remarkable (Aglianico, Barbera, Gaglioppo, and Magliocco wines) color variations. Data on color intensity and hue confirmed the trend detected for CIELab coordinates (Figure 3.5A). The color alteration was particularly high for Barbera wine (ΔE = 16.49–18.56).

Table 3.2. Main CIEL*a*b* values of wines in response to oxidative tests *

Name	L*	a(u)*	b(v)*	Chromacity	Hue Angle	ΔE
Aglianico						
control	41.5 ± 0.31	40.15 ± 0.1	9.325 ± 0.05	41.25 ± 0.01	41.25 ± 0.02	
sat	37.67 ± 0.05	42.57 ± 0.05	11.72 ± 0.05	44.17 ± 0.05	15.42 ± 0.05	5.13 ± 0.19
H_2O_2	36.42 ± 0.09	43.25 ± 0.05	11.37 ± 0.05	44.75 ± 0.05	14.75 ± 0.05	6.29 ± 0.18
AtCH	36.57 ± 0.20	43.75 ± 0.70	11.15 ± 0.17	45.12 ± 0.72	14.3 ± 0.00	6.41 ± 0.14
Barbera						
control	51.8 ± 0.12	28.775 ± 0.05	10.7 ± 0.08	30.7 ± 0.08	20.4 ± 0.08	
sat	40.9 ± 0.2	41.025 ± 0.12	12.475 ± 0.05	42.87 ± 0.09	16.95 ± 0.1	16.49 ± 0.18
H_2O_2	39.52 ± 0.09	41.72 ± 0.05	11.22 ± 0.05	43.17 ± 0.09	15.05 ± 0.05	17.85 ± 0.09
AtCH	39.67 ± 0.20	42.8 ± 0.11	11.52 ± 0.09	44.35 ± 0.05	15.07 ± 0.15	18.56 ± 0.11
Gaglioppo						
control	71.25 ± 0.45	9.3 ± 0.10	5.63 ± 0.20	10.86 ± 0.05	31.12 ± 1.19	
sat	70.27 ± 0.05	11.17 ± 0.05	9.67 ± 0.05	14.77 ± 0.05	40.77 ± 0.05	4.59 ± 0.16
H_2O_2	70.37 ± 0.05	11.0 ± 0.8	9.3 ± 0.00	14.4 ± 0.8	40.2 ± 0.14	4.18 ± 0.13
AtCH	69.85 ± 0.75	11.27 ± 0.05	9.52 ± 0.32	14.8 ± 0.18	40.17 ± 1.01	4.63 ± 0.12
Magliocco						
control	71.42 ± 0.12	9.35 ± 0.05	8.52 ± 0.09	12.65 ± 0.19	42.3 ± 0.21	
sat	67.45 ± 0.05	14.5 ± 0.00	6.05 ± 0.1	15.67 ± 0.05	22.725 ± 0.29	6.96 ± 0.13
H_2O_2	69.22 ± 0.32	12.42 ± 0.32	6.65 ± 0.05	14.1 ± 0.29	28.17 ± 0.74	4.24 ± 0.43
AtCH	65.27 ± 0.98	15.92 ± 0.26	5.1 ± 0.32	16.72 ± 0.36	17.65 ± 0.83	9.68 ± 0.78
Nerello						
control	75.35 ± 0.7	4.2 ± 0.27	4.975 ± 0.05	6.575 ± 0.15	50.075 ± 2.11	
sat	74.15 ± 0.13	6.5 ± 0.00	5.7 ± 0.00	8.62 ± 0.05	41.37 ± 0.09	2.83 ± 0.8
H_2O_2	74.5 ± 0.18	5.92 ± 0.05	5.65 ± 0.12	8.17 ± 0.09	43.65 ± 0.23	2.16 ± 0.46
AtCH	73.8 ± 0.5	6.27 ± 0.09	5.55 ± 0.17	8.37 ± 0.20	41.62 ± 0.55	2.82 ± 0.80

3.3.2.2 Variation of Protein Reactive Tannins and Vanillin Reactive Flavans

Protein (BSA) reactive tannins, determined by the Harbertson–Adams assay [Harbertson et al. 2003], were scarcely affected by oxidative treatments in all wines (Figure 3.6A). This finding suggests that accelerated oxidation is not able to induce condensation between flavan-3-ols to generate new oligomeric tannins. Following the oxidation tests, we observed that mainly short polymeric pigments were affected. In our opinion, the above results confirmed that anthocyanins are indeed the compounds crucially involved in modulating the response of red wines to moderate oxidation [Gambuti et al. 2018]. Slight differences between control wines and treated ones were observed. Wine A was the only wine where a small decrease of tannins was detected after oxidation. This was

likely due to its peculiar A/T ratio as already observed in a previous study [Gambuti et al. 2018]. For Gaglioppo and Magliocco, only the acetaldehyde addition determined a decrease of the BSA reactive tannins.

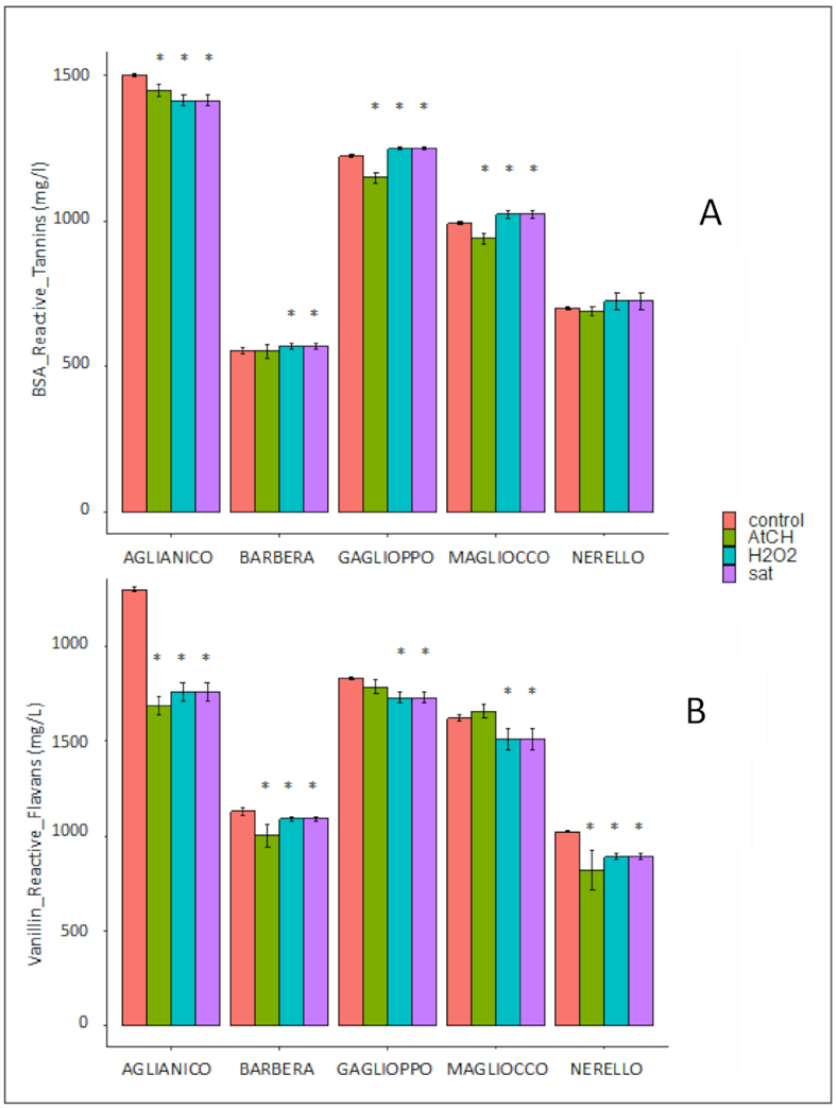

Figure 3.6 *Tannins content in treated wines (Aglianico; Barbera; Gaglioppo; Magliocco; Nerello; Mascalese); (**A**) BSA reactive tannins (mg/L), (**B**) vanillin reactive flavans (mg/L) content in wines before and after treatments. Asterisks (*) indicate significant differences between treated wine and its control (p-value less than 0.05).*

In order to understand the effect of the different treatments on the acetaldehyde content of wines, this molecule was determined by HPLC analysis after derivatization of wine samples with DNPH. No clear trend either upward or downward was shown (Figure 3.6A) probably owing to the great reactivity of this electrophile compound in such complex media which are red wines. It should also be considered that acetaldehyde instantly reacts with all the free SO_2 present in wine [Sacks et al. 2020]. Further studies in either model

solutions or real wines could help the understanding of this important issue. Vanillin reactive flavans (VRF) give an indirect indication about the variation of the polymerization degree of condensed tannins [Vrhovsek et al. 2001]. Vanillin is reactive towards the C6 and C8 positions of free flavan-3-ols, whereas it is less correlated with long polymeric flavan-3-ols (mean polymerization degree mPD > 4), which have the C6 and C8 carbon positions already involved in inter-monomers covalent bonds. We observed a reduction of VRF in all the examined wines; this was particularly remarkable for the Aglianico wine probably due to a lower pH value compared to the other wines. In fact, it is well known that a low pH favors any carbonyl functionality reactivity, including acetaldehyde, with nucleophilic positions of wine phenolics (Figure 3.6B).

The reduction of VRF, the increased amount of SPP, the slight variation in BSA reactive tannins observed in the oxidized wines confirmed the hypothesis that condensation between anthocyanins and flavan-3-ols together with the slight increase of degree of polymerization would be occurring, rather than an ex novo polymerization of catechins.

3.3.2.3 Application of Accelerated Oxidation Tests

A principal component analysis was performed to assess the effects of oxidative treatments on phenolic composition and chromatic characteristics of each monovarietal wine considered (Figure 3.7). For all wines, SPP and Abs 520 nm are the common drivers for PC1. For each kind of red wine, different drivers are associated to PC2, for Aglianico they are LPP and total polyphenols, BSA reactive tannins for Barbera, monomeric anthocyanins for Gaglioppo, VRF and total anthocyanins for Magliocco and Nerello. For Aglianico, Barbera, and Gaglioppo, the control wine was always well separated from the treated ones along the first component. The PCA showed that, for these wines, values associated with oxidized wines are more closely associated with SPP and total anthocyanins. During oxidation of red wine, the formation of more stable complex pigments occurs [Waterhouse and Zhu, 2020; Timberlake and Bridle, 1976], the good separation along the first component (from 59.6% to 76.1% of total variance explained) is therefore easily justified. For Aglianico wines, those treated with sat and H_2O_2 treated are well grouped and are associated with LPP. This result is not surprising because a shift towards more polymerized structures during oxidation is expected [Poncet-Legrand et al. 2010].

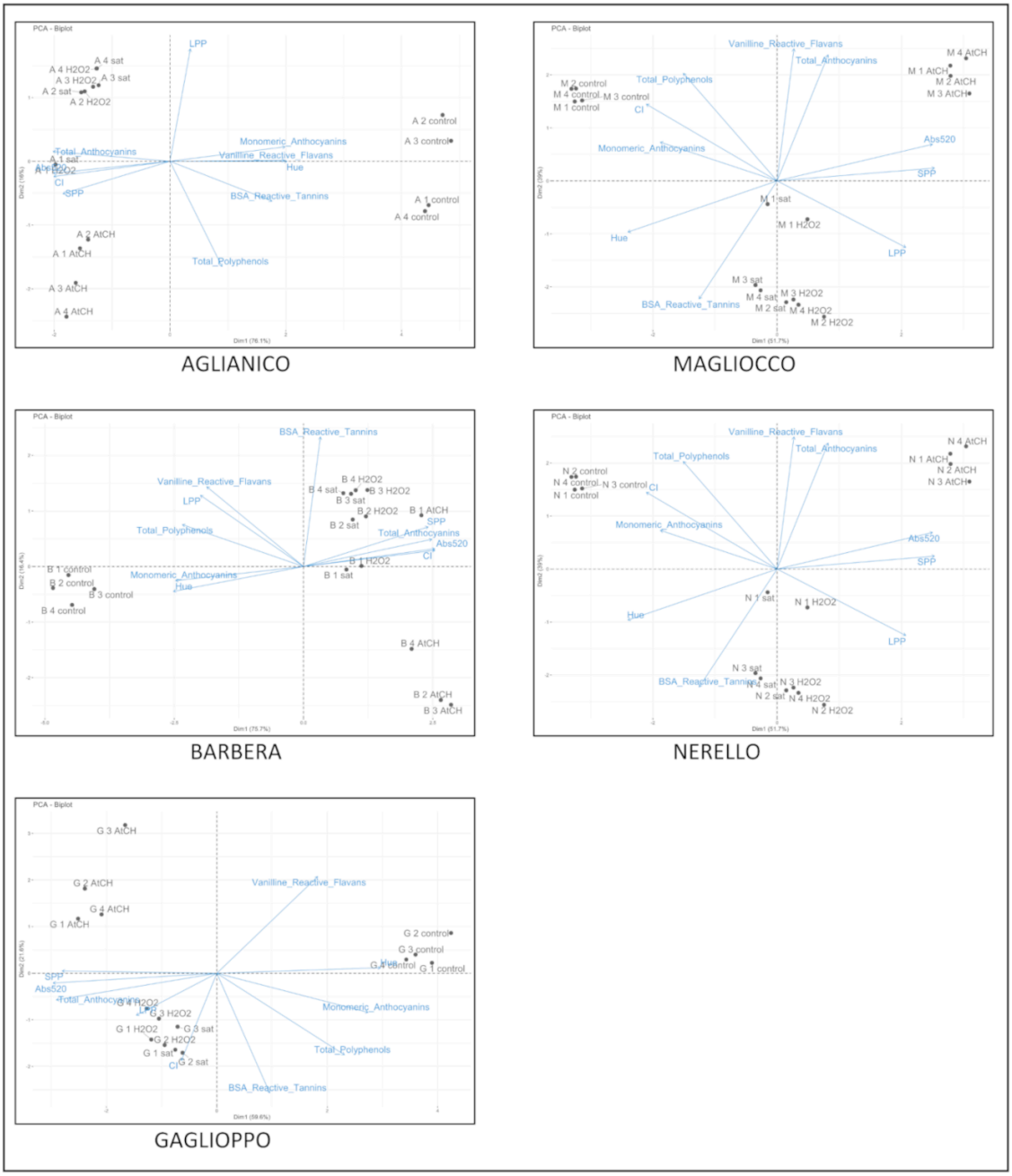

Figure 3.7 *PCA score biplot of the first two PCs of data set of all phenolic compounds and chromatic characteristics in control and oxidized samples for each monovarietal wine (Aglianico, Barbera, Gaglioppo, Magliocco, Nerello).*

The analysis of PCA allowed also to discriminate accelerated oxidation tests from control wines in the case of Magliocco and Nerello wines but the separation was not so high as for Aglianico, Barbera where the 90.7% of total variance was explained. In contrast, AtCH tests differ from other tests and are associated with Abs at 520 nm. This is not surprising because acetaldehyde is highly reactive towards monomeric anthocyanins and the high correlation between AtCH tests and Abs at 520 nm confirms previous studies in which an increase in color intensity in red wines due to the effect of acetaldehyde was

observed in model solutions [Timberlake and Bridle, 1976; Forino et al. 2020] and red wine [Sheridan and Elias, 2015]. The chemical reasons for this behavior are linked to the fact that the main products of the reaction between acetaldehyde and anthocyanins are ethyl-linked anthocyanin-flavanols adducts (A-e-F) and vitisin B [Waterhouse and Zhu, 2020]. Several authors have in fact found that the formation of ethylene linked Mv3gl-catechin dimers caused a shift towards blue tint of solution containing anthocyanins [Rivas-Gonzalo et al. 1995]. Nonetheless, it should also be considered that these reactions are not the only occurring during oxidative processes linked to wine aging. Recently, in a study in which anthocyanins and their derivatives of 234 bottled commercial wines from 1 to 23 years aged were analyzed, a great variation in the evolution pattern among anthocyanin derivative classes was detected [Zhang et al. 2020]. In particular, vitisin B and A-e-F products degraded in almost five years. Moreover, Escribano- Bailón et al. [Esribano-Bailòn et al. 2001] showed that A-e-F are more likely to degrade in aqueous solution than anthocyanins due to the facile cleavage of the ethyl bridge. In addition, previous studies have shown that SO_2 including that deriving from the acetataldehyde-bisulfite adducts is eventually consumed through oxidation reactions [Sacks et al. 2020], hence an increase of acetaldehyde in solution must be also considered. Based on what was reported above and of our results, we can conclude that the use of AtCH addition to predict wine oxidation is less accurate than the addition of H_2O_2 for a correct estimation of pigments evolution and, in general, of wine evolution under moderate oxidation. Therefore, given the consistence of the response to H_2O_2 oxidation test with those based on air saturation, we propose the employment of the H_2O_2 test to predict the phenolic and color evolution of red wines under moderate oxidation since it is quite easy and fast to perform compared to air saturations and handling of acetaldehyde. Hence, it could be regarded as a preferential tool to assess wine response to oxidations. The application of accelerated oxidation tests to the five monovarietal wines considered was instrumental to understand as to whether the oxidation could determine changes in the phenolic composition of red wines to such an extent to limit the possibility of distinguishing the wines from each other. A principal component analysis was then performed and, as it can be seen from the PCA score plot (Figure 3.8), wine samples tended to clusterize regardless of the oxidation treatment applied.

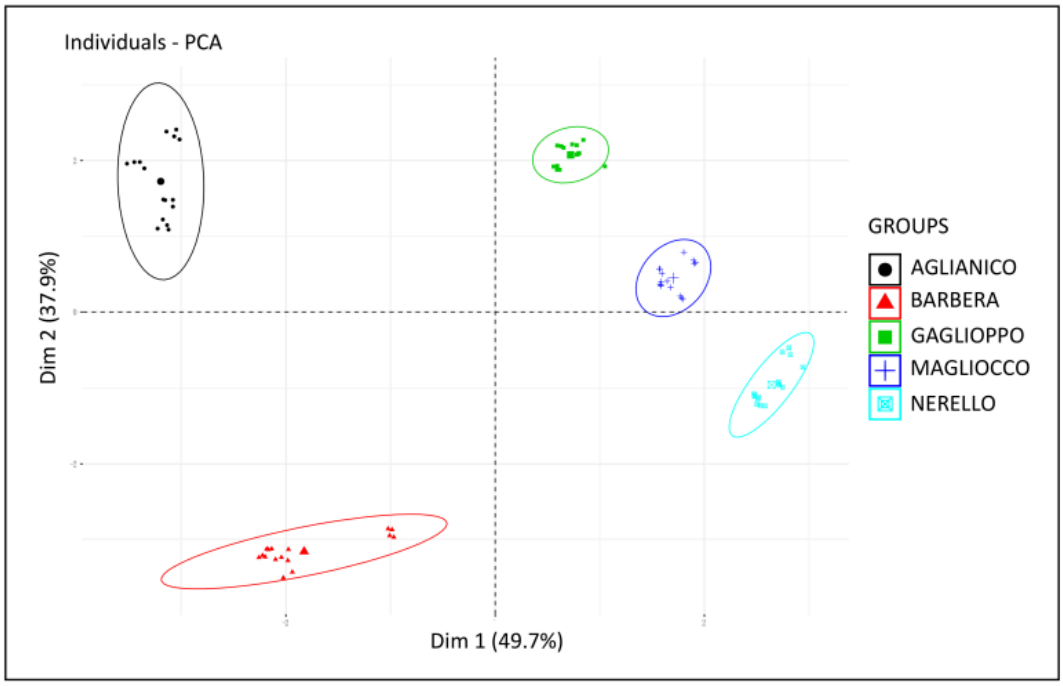

Figure 3.8 *PCA score plot of the first two PCs of whole phenolics and chromatic characteristics data set.*

Anthocyanin-poor wines (Gaglioppo, Magliocco, and Nerello) turned out to be clustered on the same side of the plot and they were differentiated by the variables VRF, BSA reactive tannins, total polyphenols, and acetaldehyde. In turn, Barbera and Aglianico occurred on opposite sides with respect to dimension 2 (characterized by parameters such as SPP, total anthocyanins, monomeric anthocyanins, CI, and Abs 520). It is interesting to observe that Gaglioppo and Magliocco wines showed a lower variability before and after the oxidative tests while Barbera, Aglianico, and, to a lesser extent, Nerello changed after the oxidation. This could indicate a reactivity of Barbera and Aglianico towards oxygen that Gaglioppo and Magliocco do not have anymore. As when red wines age, they possess lower quantities of reactive phenolic compounds [Zhang et al. 2020], it is possible to adopt these accelerated oxidation tests to predict the "possible evolution of color and pigments" of a red wine. In particular, the changes in hue, total anthocyanins, and VRF should be considered. The more a red wine treated with H_2O_2 changes these parameters increasing total anthocyanins and preserving a hue below 1 (Abs520 nm > Abs420 nm), the more the moderate exposure to oxygen is not detrimental for this wine. A concomitant significant decrease of VRF could be also associated to a possible variation in the structure of polymeric flavonoids affecting mouthfeel. Thus, the enologists could adopt aging strategies (micro-oxygenation, closures with specific oxygen permeating capacity,

and SO_2 protection) more adapted to the phenolic composition of this red wine. In conclusion, oxygen saturation cycles and hydrogen peroxide addition tests have the same impacts on most of the wine chromatic properties and phenolic composition of the treated wines. Thus, both can be used as suitable methods to simulate red wine oxidation and to allow a discrimination among wines based on their possible evolution under oxygen exposure.

3.3.3 Conclusions

In conclusion, oxygen saturation cycles and hydrogen peroxide addition tests have the same impacts on most of the wine chromatic properties and phenolic composition of the treated wines. Thus, both can be used as suitable methods to simulate red wine oxidation and to allow a discrimination among wines based on their possible evolution under oxygen exposure.

3.3.4 Considerations

The present activity was conducted in collaboration with the entire Enology group of the Department of Agricultural Sciences. The results acquired found editorial placement in an international scientific journal in the form of an original research article entitled "Comparison of three accelerated oxidation tests applied to red wines with different chemical composition." The article, to date, has been cited by different groups of Authors including [Deshaies et al 2021; Carrasco-Quirozet al. 2023] for the development new original research articles.

3.4 THE CASE STUDY: EFFECT OF CHITOSAN ON THE REMOVAL OF DIFFERENT TYPES OF TANNINS FROM RED WINES

Chitosan is a natural polysaccharide derived from chitin with polycationic character and a solubility variable in acidic media due to its deacetylation degree and molecular weight [Friedman et al. 2010]. The use of C has been authorized in must and wine for microbial stabilization or metal and protein removal. The admitted range for the addition of chitosan is 10–500 g/hL according to the aim [Commission regulation, EU, 2001]. Previous studies reported several beneficial effects due to chitosan additions in wine, such as antimicrobial [Brasselet et al. 2019] and antioxidant activity due to the direct radical scavenging mechanism or indirectly, via metal chelation, which blocks the generation of radical species [Castro Marín et al. 2019]. Chitosan is also of great relevance because it is effective in controlling *B. bruxellensis* during winemaking [Taillandier et al. 2015]. Given all these positive properties, C can be used on finished red wines to preserve them from microbial and oxidative spoilage. However, the possible effect on compounds responsible

for wine sensory quality should also be considered. Recently, Castro Marín and colleagues [Castro Marín et al. 2021] showed that a post-fermentative treatment with 0.5 g/L of C on a Sangiovese wine slightly affected aroma compounds and did not severely impair the quality parameters of the final wines. However, in this same work authors also observed that ellagic acid, a tannic molecule released in wines after contact with wood, was adsorbed onto chitosan and it reduced this content by 40%. In a previous study, we also detected a decrease in BSA reactive tannins after a treatment of an Aglianico wine with Chitosan [Picariello et al. 2020]. Tannins are very important for red wine quality as they are responsible for their high dry extract and longevity but also for the sensory attributes linked to their ability to precipitate salivary proteins during tasting, causing an astringent sensation. This sensory attribute is typical of red wine but, if present at high intensity, it results in negative consumer reactions. Because most of premium red wines are rich in tannins and often are previously aged in barrels or in contact with tannins extracted from grapes and wood, it is interesting to determine if C could influence their content and reactivity towards proteins. During winemaking and storage, the addition of commercial tannins is definitively a practice often used to improve the wine quality. Tannins of grape origin, as well as of wood origin can be used [Versari et al. 2013]. Wood tannins are the hydrolysable tannins, gallotannins, and ellagitannins, which are extracted from different botanical species. Ellagitannins originate from oak and chestnut wood, while gallotannins mainly originate from galla and tara nuts [Ribéreau-Gayon et al. 2006]. Grape tannins are condensed flavanols mainly present in berry skins, seeds, and stalks. One of the effects of the addition of tannins is the variation in the pigments and chromatic characteristics of wine, but important is also the effect on sensory attributes such as the astringency and astringency subqualities [Picariello et al. 2020]. This is of particular importance especially considering that red wines rich in tannins change their chromatic characteristics and astringency during aging and exposition to controlled moderate amounts of oxygen [Rinaldi et al. 2021]. Taking into consideration all these aspects, it is worth investigating the possible effect of C on tannins (condensed and hydrolysable) in addition to their reactivity towards salivary proteins, one of the mechanisms at the base of the sensation of astringency of red wines [Gambuti et al. 2020]. Apart from the evaluation of the reactivity of tannins towards BSA, one of the most objective methods to evaluate their possible astringency is the evaluation of their reactivity towards proteins by means of the determination of the saliva precipitation index (SPI), an index pointed out by means of SDS-page analysis of salivary proteins before and after the binding with

wine in lab conditions that simulate the phenomenon occurring during wine tasting and aging [Gambuti et al. 2020]. The aim of this work is to determine the influence of a treatment with C on red wines with condensed tannins, ellagitannins, and gallotannins previously added. The effect is evaluated before and after exposure of red wines to an oxidative stress aimed at simulating wine aging.

3.4.1 Material and Methods

3.4.1.1 Experimental Plan

The experimental plan was performed on an Aglianico red wine produced in South Italy named W. The main oenological parameters of wines (free sulphur dioxide, total sulphur dioxide, residual sugar, malic acid, and lactic acid) were determined using a Y15 BioSystems analyser (BioSystem, Barcelona (Spain) for Sinatech, Montegranaro (FM), Italy). Alcohol, titratable acidity, volatile acidity, and pH were measured according to the OIV Compendium of International Methods of Wine and Must Analysis [OIVV, 2019]. The main parameters were alcohol 14.88 ± 0.33% v/V, titratable acidity 5.23 ± 0.03 g/L of tartaric acid, pH 3.12 ± 0.01, volatile acidity 0.16 ± 0.03 g/L of acetic acid, malic acid 0.90 ± 0.01 g/L, and lactic acid 0.09 ± 0.01 g/L. Wine was divided in 16 aliquots of 500 mL each to have 4 sets of samples, the first set of samples was prepared to evaluate the effect of tannins addition, the second set to evaluate the effect of C addition, and the third and fourth sets to evaluate the effect of forced oxidation.

3.4.1.2 Tannins and Chitosan Addition

The first set of 4 samples contained: the untreated wine W and samples treated by adding 400 mg /L of three different types of oenological tannins: Oligomeric Tannins (VR Grape Laffort Oenologie, Bordeaux, France) named CT, Gallotannins (Galalcool Laffort Oenologie, France) named GT, and ellagitannins (Quertanin Laffort Oenologie, Bordeaux France) named ET. Thus, the first four wines were obtained: W, CT, ET, and GT. The chemical characterisation of the commercial tannins formulations was previously reported [Picariello et al. 2020]. To obtain the second set of 4 samples, wines prepared as for W, CT, ET, and GT were successively treated with 500 mg/L of C (Sigma-Aldrich CAS: 9012-764) to have the four experimental samples W-C, CT-C, ET-C, and GT-C. Seven days after the treatment, the first and the second sets of wines were centrifuged and filtered (0.45 micron) and then analysed

3.4.1.3 Forced Oxidation Tests

The oxidative response of the different wines was assessed by applying an accelerated oxidation test recently published by Coppola et al. [2021]. Wines prepared as previously described for the preparation of the first and second sets of wines (W, CT, ET, GT, and W-C, CT-C, ET-C, and GT-C) were treated by adding 19 mg/L of H_2O_2 (equivalent to 18 mg/L O_2) (30% Fluka, Sigma-Aldrich Chemie GmbH Steinheim, France). The forced oxidation tests were performed at 18 °C, considering a 1:1 stoichiometry of oxygen to hydrogen peroxide. Two other sets of samples were obtained: oxidized wine named Wo, oxidized wine treated with condensed tannins named CTo, oxidized wine treated with ellagitannins named ETo, oxidized wine treated with gallotannins named GTo, oxidized wine treated with chitosan named W-Co, oxidized wine treated with condensed tannins and chitosan named CT-Co, oxidized wine treated with ellagitannins, and chitosan named ET-Co, and oxidized wine treated with gallotannins and chitosan named GT-Co. After fifteen days, the samples were centrifuged and filtered (0.45 micron) and then analysed. All the samples were prepared in duplicate.

3.4.1.4 High-Performance Liquid Chromatography Determination of Acetaldehyde

Acetaldehyde was determined by HPLC analysis as described by Han et al. [2015]. The derivatization of samples was performed by adding 20 µL of freshly prepared 1120 mg/L SO_2 solution to an aliquot of wine sample (100 µL). The samples were then acidified with 20 µL of 25% sulfuric acid (Carlo Erba reagent 96%), and then 140 µL of 2 g/L 2,4-dinitrophenylhydrazine reagent (Aldrich chemistry) were added. After mixing, the solution was allowed to react for 15 min at 65 °C and then promptly cooled to room temperature. Analysis of carbonyl hydrazones was conducted by an HPLC (HPLC Shimadzu LC10 ADVP apparatus (Shimadzu Italy, Milan)), consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, a SPD-M 10 AVP detector, and an injection system full Rheodyne model 7725 (Rheodyne, Cotati, CA) equipped with a 50 µL loop. A Waters Spherisorb column (250x4.6 mm, 4 mm particles diameter) was used for separation. The chromatographic conditions were: sample injection volume, 15 mL; flow rate, 0.75 mL/min; column temperature, 35 °C; mobile phase solvents, (A) 0.5% formic acid (Sigma-Aldrich 95%) in water milli-Q (Sigma-Aldrich) and (B) acetonitrile (Sigma-Aldrich 99.9%); and gradient elution protocol, 35% B to 60% B (t = 8 min), 60% B to 90% B (t = 13 min), 90% B to 95% B (t = 15 min, 2-min hold), 95% B to 35% B (t = 17 min, 4-min hold), total run time, 21 min. Eluted peaks were compared with derivatized

acetaldehyde standard. All analyses were conducted through two experimental replicas and two analytical replicas. Data are the means of four values.

3.4.1.5 High-Performance Liquid Chromatography (HPLC) Analyses of Monomeric and Polymeric Phenolics

HPLC analysis was performed for the separation and quantification of monomeric and polymeric phenolics according to [Waterhouse et. 1999]. A HPLC Shimadzu LC10 ADVP apparatus (Shimadzu Italy, Milan, Italy) equipped with a SCL-10AVP system controller, two LC-10ADVP pumps, a SPD-M 10 AVP detector, and an injection system full Rheodyne model 7725 (Rheodyne, Cotati, CA, USA) was used. An Agilent PLRP-S 100- Å reversed phase polystyrenedivinyl benzene column (4.6 × 150 mm, 3 μm particle size) protected with a guard cartridge with the same packing material (PLRP-S, 5 × 3 mm) kept at 35 °C was used as the stationary phases. The HPLC solvents were: solvent A: 1.5% v/v ortho-phosphoric acid (EMP Chemicals, Gibbstown, NJ, USA) and solvent B consisting of 80% acetonitrile (HPLC grade, Honeywell, Muskegon, MI, USA) with 20% of solvent A. The following gradient was established: 0-time conditions, B 6%; 73 min, B 31%; 78 min, B 62%, staying constant until 86 min; and 90 min, B 6%. This zero-time solvent mixture was followed by a 15 min equilibrium period prior to injecting the next sample. The flow rate of the mobile phase was 1 mL/min. Twenty μL of wine or calibration standards were injected onto the column. All the samples were filtered through 0.20 μm Microliter PTFE membrane filters (DWK Life Sciences, Wheaton, Milville, NJ 08332 USA) into dark glass vials and immediately injected into the HPLC system. Detection was carried out by monitoring the absorbance signals at 520 nm for monomeric anthocyanins and polymeric anthocyanins. Eluted peaks were compared with malvidin-3-monoglucoside (Extrasynthese, Lyon, France) on the basis of peak area. All analyses were conducted through two experimental replicas and two analytical replicas. Data are the means of four values.

3.4.1.6 Spectrophotometric Analyses

The chromatic characteristics and spectrophotometric measures were determined using a spectrophotometer (Jenway 7305 Spectrophotometer). Colour intensity (Abs 420 nm + Abs 520 nm+ Abs 620 nm) and hue (Abs 420 nm/Abs 520 nm) were evaluated according to the Glories methods [Glories, 1984]. The CIELAB parameters (L∗, a∗, b∗) were determined by using NomaSense Color P100 Equipped with a 0.5 cm cell, the colour differences were calculated as the Euclidean distance between two points in the 3D space

defined by L∗, a∗, and b∗, as reported by the Commission Internationale de L'Eclariage (CIE). Total anthocyanins, short polymeric pigments (SPP), large polymeric pigments (LPP), and bovine serum albumin (Sigma-Aldrich SRL, Milano, Italy) reactive tannins (BSA reactive tannins) were determined by the Harbertson–Adams assay [Harbertson et al. 2003]. Vanillin reactive flavans (VRF) were determined as reported in [Gambuti et al. 2015]. All analyses were conducted through two experimental replicas and two analytical replicas.

3.4.1.7 Saliva Precipitation Index

For the binding assay, 50 µL of saliva was mixed with 25 µL of diluted wine (1:4). Human saliva used for binding reactions was obtained by mixing resting saliva samples from different individuals. Saliva collection was performed between 10 and 11 a.m. Subjects were asked to follow teeth cleaning instructions in the early morning and not to consume any food and beverage for 2 h before saliva collection. Saliva was collected from six non-smoking volunteers (three males and three females) by expectorating saliva into a pre-weighted ice-cooled tube for 5 min. The resulting mix was centrifuged at 10,000× g for 10 min to remove any insoluble material and the supernatant was used for analysis. Binding assays were performed in Eppendorfs maintained at 37 °C for 5 min. The mixture was then centrifuged for 10 min at 10,000 g. The electrophoretic analysis was performed on the resulting supernatant, representing the salivary proteins that remained in solution after the precipitation by wine tannins. Two binding assays were performed on the two wine replicates. Saliva samples (before and after the binding assay) mixed with an equal volume of 2xelectrophoresis sample buffer (0.125 M Tris–HCl, 4% SDS; 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) and heated at 95 °C for 5 min were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) using 14% acrylamide resolving gels. The stacking gel was 4% acrylamide (Bio-Rad). Electrophoresis was performed on a Bio-Rad MiniProtean Cell electrophoresis apparatus (Bio-Rad, Milan, Italy) and a PowerPac 1000 Bio-Rad power supply set a 150 V for the stacking and resolving gels. The gels were fixed with a mixture of ethanol, acetic acid, and deionized water (40:10:50) for 1 h. After washing in water for 5 min, the gels were stained with Coomassie Brilliant Blue R250 staining solution (Bio-Rad, Milan, Italy). The destain step was performed by incubation in the destain solution Coomassie Blue R250 (Bio-Rad, Milan, Italy). Molecular weights were estimated by comparison with the migration rates of Precision Plus Protein All Blue protein standards (Bio-Rad,

Milan, Italy 3). Densitometric tracing of minigels was performed with a Biorad GS800 Densitometer. The SPI was calculated by the percentage decrease in the bands of saliva before and after the binding assay and expressed in g/L of gallic acid equivalent (GAE), as previously reported [Rinaldi et al. 2010, 2012].

3.4.1.8 Statistic Analysis

These analyses were performed using XLSTAT (software Addinsoft, 2017.1). The effect of treatments were evaluated by the analysis of the variance (ANOVA) using the Tukey method for the significant differences procedure ($p < 0.05$). All data are means of four values (2 experimental replicates × 2 analytical replicates).

3.4.2 Results and Discussion

The study of the effect of C on wines containing different oenological tannins is divided in two parts: the first aimed at evaluating the effect of tannins and C addition on phenolic compounds, pigments, and chromatic characteristics of treated wines; the second focussed on understanding if changes detected just after the addition of C also persisted after an oxidation treatment aimed at simulating wine evolution.

3.4.2.1 The Effect of Chitosan on Wines Rich in Tannins of Different Origin

The effect of tannins addition and C on main phenolic parameters of wines is reported in Figure 3.9. As expected, the addition of tannins increased the concentration of total phenols and BSA reactive tannins compared with the control wine. The highest significant increase in total phenolics was detected only for GT. As the method we used to determine total phenols evaluated their reactivity towards iron, it is expected that gallotannins, molecules that form water-insoluble complexes with iron [Haslam, 1996], gave a higher absorbance after the reaction of GT with the ferric chloride used in this assay. Similar results were showed by [Harbertson et al. 2012] in a study in which several enological tannins were assessed for their abilities to precipitate BSA protein and to react with ferric chloride.

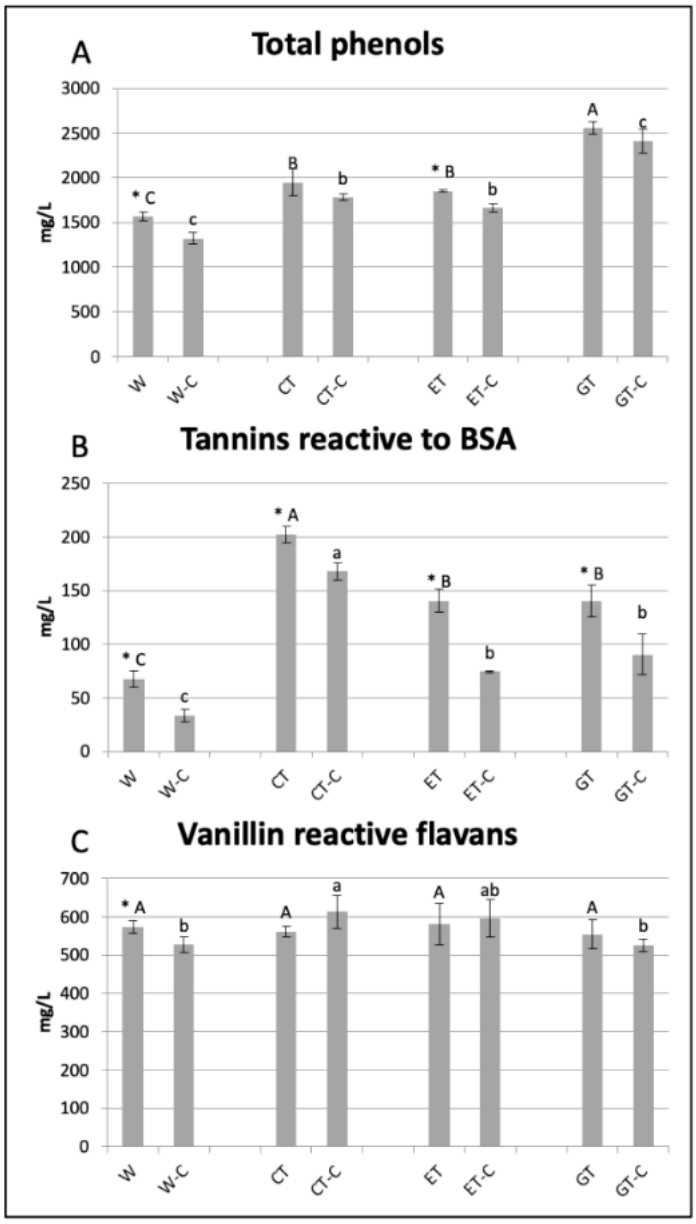

Figure 3.9 Fining effect on total phenols (**A**), tannins reactive to BSA (**B**), and vanillin reactive flavans (**C**). Different letters indicate a statistically significant difference among treated wines, according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannin addition is expressed with a capital letter on wines W, CT, ET, and GT (A, B, C), and the effect of chitosan addition is expressed with a lowercase letter on wines W-C, CT-C, ET-C, and GT-C (a, b, c). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. All data are expressed as means ± standard deviation.

In agreement with a previous study [Picariello et al. 2017], the CT sample showed the greatest increase in BSA reactive tannins compared with the treated wines. The increase is due to the molecular structure of condensed tannins which mainly are constituted of proanthocyanidins with a polymerization degree and composition in monomers highly reactive towards BSA [36]. No effect of enological tannins was detected for vanillin

reactive flavans, VRF, probably because vanillin is mainly reactive towards low molecular weight proanthocyanidins [Vrhovsek et al. 2001]. Concerning the effect of C addition, a slight decrease in total phenols in W and ET (−15.68% and −10.03%, respectively) was determined. According to Bassi et al. [2000], C does not directly interact with phenolics and the decrease in phenolics compounds is more ascribable to a sedimentation effect [Diblan and Ozkan, 2021]. In agreement with recent results [Picariello et al. 2020], a decrease in BSA reactive tannins after the addition of C was detected for all wines. A slight VRF removal was instead detected only for W. In a previous study on white wines, Spagna et al. [Spagna et al. 1996] detected a decrease in VRF after a treatment with C of a Sauvignon Blanc in which the levels of VRF were largely accountable for the presence of monomer catechins. In red wines, the amount of catechins is low compared with the structures with a higher polymerization degree and this could justify the absence of an effect of the treatment with C on the reactivity of wines towards vanillin, especially in wines treated with commercial tannins. A possible competition for the adsorption on C of different tannic structures is not ruled out. If this is the case, the adsorption of phenolic structures highly reactive towards BSA is favoured. As reported in Table 3.3, after the addition of tannins, a decrease in individual anthocyanins analysed by HPLC was detected and the decrease was higher for ET. Simultaneously, the polymeric pigments increased in all experimental samples, probably due to the involvement of native anthocyanins in the formation of new pigments at higher polymerization degree, as already observed in previous experiments [Picariello et al. 2018].

Table 3.3. *Effect of tannins and C on total anthocyanin, polymeric pigments, and acetaldehyde determined by HPLC methods.*

	Total Native Anthocyanins (mg/L)		Polymeric Pigments (mg/L)		Acetaldehyde (mg/L)	
W	1330.18 ± 20.44	* A	137.30 ± 9.39	C	11.74 ± 0.10	AB
W-C	1231.73 ± 13.07	a	136.74 ± 24.49	c	11.81 ± 0.23	a
CT	1235.17 ± 27.14	B	204.92 ± 34.33	B	11.75 ± 0.07	B
CT-C	1137.24 ± 74.98	a	207.01 ± 18.26	b	11.83 ± 0.05	a
ET	1173.72 ± 44.15	C	216.44 ± 2.40	* B	11.90 ± 0.09	AB
ET-C	1147.02 ± 29.01	a	199.27 ± 3.60	b	11.76 ± 0.06	a
GT	1185.37 ± 16.59	* BC	264.72 ± 6.49	* A	11.92 ± 0.02	A
GT-C	1132.69 ± 25.32	a	246.25 ± 4.40	a	11.88 ± 0.04	a

Different letters indicate a statistically significant difference among treated wines, according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins addition is expressed with a capital letter on wines W, CT, ET, and GT before the addition of C (A, B, C), and the effect of chitosan addition on wines W, CT, ET, and GT is expressed with

a lowercase letter on wines W-C, CT-C, ET-C, and GT-C (a, b, c). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. All data are expressed as means ± standard deviation. Concerning C, in agreement with literature [14,43] the effect on pigments was limited but significant. The removal of total monomeric anthocyanins was significant for W and GT and those of polymeric pigments for ET and GT. However, the loss was never higher than 7.40%. Looking at the individual anthocyanins (Table 3.4), only in W the addition of C determined a loss of almost all anthocyanins suggesting that in wines enriched with enological tannins, a possible competition for the adsorption on C occurred. The effect of tannins and C on acetaldehyde content of wines was not significant.

Table 3.4. *Effect of tannins and C on native anthocyanins concentrations (mg/L).*

	Delphinidin 3-Glucoside (mg/L)		Cyanidin 3-Glucoside (mg/L)		Petunidin 3-Glucoside (mg/L)		Peonidin 3-Glucoside (mg/L)		Malvidin 3-Glucoside (mg/L)		Malvidin 3-(6II-Acetyl)-Glucoside (mg/L)		Malvidin 3-(6II-Coumaroyl)-Glucoside (mg/L)	
W	248.92 ± 14.36	*A	10.97 ± 0.76	AB	174.10 ± 4.40	*A	91.77 ± 0.64	B	609.42 ± 16.31	*A	150.20 ± 2.50	*A	44.80 ± 1.48	AB
W-C	204.16 ± 6.36	a	9.96 ± 0.54	b	162.20 ± 4.06	a	89.67 ± 3.78	a	577.55 ± 8.49	a	144.79 ± 1.41	a	43.40 ± 1.85	a
CT	242.62 ± 20.99	AB	7.85 ± 1.08	C	169.50 ± 4.96	*AB	98.58 ± 3.27	*A	520.92 ± 17.89	B	148.40 ± 3.00	A	47.28 ± 2.03	A
CT-C	214.95 ± 24.50	a	9.80 ± 0.83	b	154.95 ± 4.54	ab	92.38 ± 1.22	a	470.10 ± 31.68	c	140.01 ± 6.77	a	55.04 ± 17.57	a
ET	205.24 ± 22.95	BC	9.48 ± 1.14	BC	155.15 ± 9.65	C	90.33 ± 2.78	B	549.18 ± 6.10	*B	126.45 ± 14.41	B	37.89 ± 1.10	C
ET-C	201.81 ± 22.04	a	10.05 ± 0.70	b	146.95 ± 4.12	b	88.39 ± 2.16	a	527.28 ± 5.00	ab	131.95 ± 8.51	ab	40.59 ± 3.61	a
GT	203.45 ± 4.72	*C	11.69 ± 0.21	A	158.82 ± 3.18	*BC	93.90 ± 1.59	AB	544.34 ± 26.09	B	129.31 ± 7.75	B	43.87 ± 0.99	B
GT-C	194.60 ± 3.22	a	12.68 ± 1.07	a	152.31 ± 1.29	b	90.03 ± 4.73	a	516.52 ± 26.71	bc	124.36 ± 6.41	b	42.19 ± 2.51	a

Different letters indicate a statistically significant difference among treated wines, according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins addition is expressed with a capital letter on wines W, CT, ET, and GT (A, B, C), and the effect of chitosan addition is expressed with a lowercase letter on wines W-C, CT-C, ET-C, and GT-C (a, b, c). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. All data are expressed as means ± standard deviation. The effect detected for monomeric and polymeric anthocyanins is evident also for total anthocyanins and for main chromatic characteristics (Table 3.5). In agreement with literature [Castro Marin and Chinnici, 2020], colour intensity and tonality slightly increased after the addition of tannins (Table 3.5). However, for all wines the addition of chitosan determined a slight decrease in CI and, for CT and ET, also in hue. Because CT and ET showed a lower content of polymeric pigments compared with GT, it is likely that, in wine treated with condensed tannins and ellagitannins, anthocyanins

are more involved in copigmentation reactions influencing colour intensity [Boulton, 2001].

Table 3.5. *Effect of tannins and C on total anthocyanins, colour intensity, and tonality (evaluated by using spectrophotometric methods).*

	Total Anthocyanins (mg/L)		Colour Intensity (420 nm + 520 nm + 620 nm abs units)		Tonality (420 nm/520 nm)	
W	301.32 ± 2.77	* AB	4.11 ± 0.10	* B	0.54 ± 0.01	B
W-C	288.90 ± 3.00	a	3.83 ± 0.07	c	0.53 ± 0.00	a
CT	308.01 ± 4.38	* A	4.38 ± 0.27	* A	0.61 ± 0.05	* A
CT-C	292.13 ± 1.91	a	3.96 ± 0.03	ab	0.56 ± 0.00	a
ET	307.79 ± 5.20	* A	4.25 ± 0.03	* AB	0.57 ± 0.00	*AB
ET-C	289.63 ± 2.37	a	4.02 ± 0.17	a	0.53 ± 0.02	a
GT	298.01 ± 1.56	* B	4.19 ± 0.08	* B	0.53 ± 0.00	* B
GT-C	285.96 ± 4.37	a	3.87 ± 0.06	bc	0.53 ± 0.00	a

Different letters indicate a statistically significant difference among treated wines according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins addition is expressed with a capital letter on wines W, CT, ET, and GT (A, B), and the effect of chitosan addition is expressed with a lowercase letter on wines W-C, CT-C, ET-C, and GT-C (a, b, c). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. All data are expressed as means ± standard deviation. A parameter useful to understand the effect of a treatment on human visual perception was given by CieLAB analysis and it is the ΔE. The ΔE value is used to express the overall colour difference between a sample and the control. According to Mokrzycki et al. [1999], two samples show a visible difference if ΔE calculated between them is higher than 1.5. In this study, we performed the calculation of ΔE with the aim to understand if the effect of C addition was significant (Table 3.6).

Table 3.6. *Effect of C on ΔE calculated between samples treated with C and related controls.*

	Total Anthocyanins (mg/L)		Colour Intensity (420 nm + 520 nm + 620 nm abs units)		Tonality (420 nm/520 nm)	
W	301.32 ± 2.77	* AB	4.11 ± 0.10	* B	0.54 ± 0.01	B
W-C	288.90 ± 3.00	a	3.83 ± 0.07	c	0.53 ± 0.00	a
CT	308.01 ± 4.38	* A	4.38 ± 0.27	* A	0.61 ± 0.05	* A
CT-C	292.13 ± 1.91	a	3.96 ± 0.03	ab	0.56 ± 0.00	a
ET	307.79 ± 5.20	* A	4.25 ± 0.03	* AB	0.57 ± 0.00	*AB
ET-C	289.63 ± 2.37	a	4.02 ± 0.17	a	0.53 ± 0.02	a
GT	298.01 ± 1.56	* B	4.19 ± 0.08	* B	0.53 ± 0.00	* B
GT-C	285.96 ± 4.37	a	3.87 ± 0.06	bc	0.53 ± 0.00	a

W-WC: the ΔE value related to the overall colour difference between W and WC; CT-CT C: the ΔE value related to the overall colour difference between CT and CT C; ET-ET C: the ΔE value related to the overall colour difference between ET and ET C; GT-GT C: the ΔE value related to the overall colour difference between GT and GT C.

Different letters indicate a statistically significant difference among treated wines according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins or chitosan addition is expressed with letters (a, b). All data are expressed as means ± standard deviation. All wines treated with C showed a ΔE higher than 1.5 when compared with the respective untreated sample and the higher difference was observed for ET. This suggests a possible well visible impact of C especially on wines rich in ellagitannins.

3.4.2.2 The Forced Oxidation Trial

Several studies showed the antioxidant activities of tannins [47] and chitosan, but no study addresses the effect of both treatments on the main phenolic parameters of red wines after an exposure at controlled amounts of oxygen. To obtain this information, all experimental wines underwent a controlled oxidative stress [26]. A significant variation in the reactivity of tannins after oxidation was detected (Figure 3.10). For all wines, the BSA reactive tannins increased (Figure 3.10B) and for W-Co, CT-Co, ET-Co, and GT-Co a decrease in VRF occurred (Figure 3.10C). Data on total phenols did not follow a clear trend, which can be justified considering all factors influencing the formation of coloured complexes among phenolic structures and iron chloride used for this assay [Harbertson et al. 2012]. Concerning the treatment with C, data highlighted that, after oxidation, the effect of this treatment on BSA reactive tannins and VRF is still evident but less enhanced.

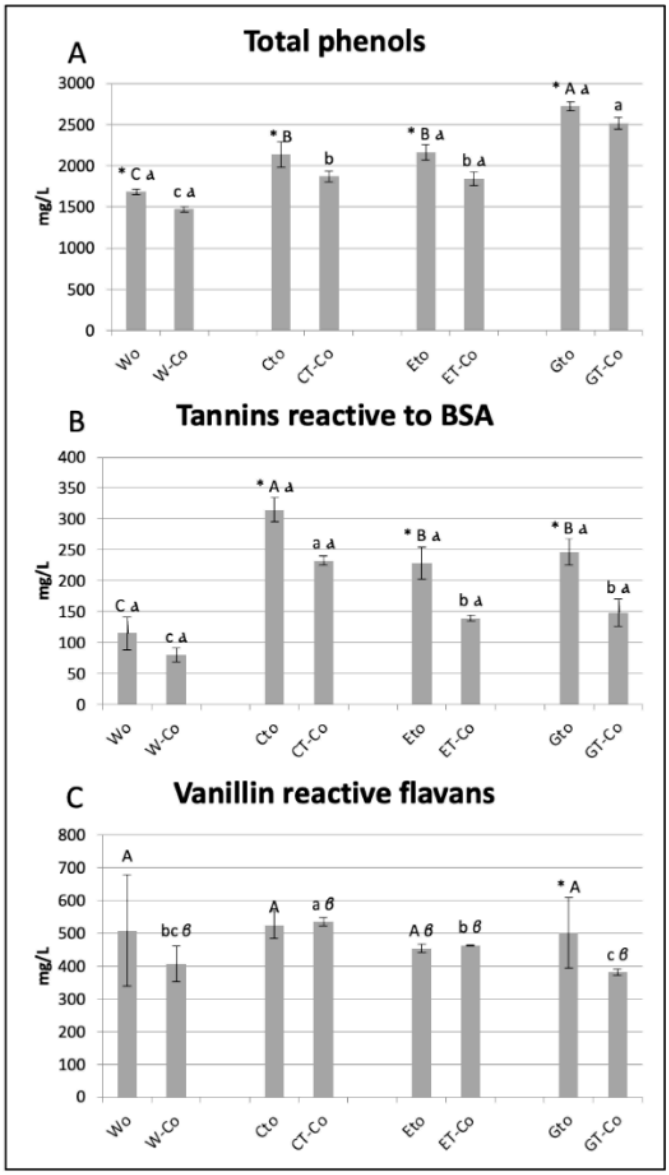

Figure 3.10. *Oxidation effect on total phenols (**A**), tannins reactive to BSA (**B**), and vanillin reactive flavans (**C**) of treated wines. Different letters indicate a statistically significant difference among treated wines according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins addition is expressed with a capital letter on wines Wo, CTo, Eto, and Gto (A, B, C), and the effect of chitosan addition is expressed with a lowercase letter on wines W-Co, CT-Co, ET-Co, and GT-Co (a, b, c). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. The Greek symbol (ɑ) is used if the value is significantly higher than the non-oxidized sample, and the symbol (β) is used if the value is significantly lower than the non-oxidized sample. All data are expressed as means ± standard deviation.*

As expected, after oxidation total individual anthocyanins dramatically decreased while polymeric pigments and acetaldehyde increased (Table 3.7). The highest increase in polymeric pigments was detected for CT as already observed in a comparative experiment performed on red wines added with the same kind of tannins [Picariello et al. 2017]. The effect of C on the content of polymeric pigments was higher than those observed before oxidation (between 10.02% and 19.70%). This is not surprising because, as observed for

BSA reactive tannins, in this case it is also possible that the removal of molecules with a molecular size ranging from trimer to octamer, showed to be more reactive to BSA, is favoured.

Table 3.7. *Oxidation effect on acetaldehyde, total anthocyanins, native anthocyanins, and polymeric pigments of treated wines*

	Total Native Anthocyanins (mg/L)		Polymeric Pigments (mg/L)		Acetaldehyde (mg/L)	
Wo	58.10 ± 4.02	A β	367.88 ± 13.67	* C □	66.10 ± 0.30	* A □
W-Co	46.77 ± 6.66	a β	331.01 ± 5.39	b □	62.76 ± 0.34	a □
CTo	50.62 ± 7.99	A β	507.34 ± 12.28	* A □	64.27 ± 0.13	* C □
CT-Co	46.39 ± 2.27	ab β	418.53 ± 15.84	a □	60.74 ± 0.13	b □
ETo	49.74 ± 0.23	* A β	330.24 ± 2.02	* D □	64.69 ± 0.30	* C □
ET-Co	38.78 ± 0.67	b β	265.16 ± 2.18	c □	58.47 ± 0.57	d □
GTo	46.33 ± 6.67	* A β	416.83 ± 4.08	* B □	65.42 ± 0.15	* B □
GT-Co	41.33 ± 1.18	ab β	337.17 ± 8.56	b □	59.71 ± 0.12	c □

Different letters indicate a statistically significant difference among treated wines according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins addition is expressed with a capital letter on wines Wo, CTo, ETo, and GTo (A, B, C,D). The effect of chitosan addition is expressed with a lowercase letter on wines W-Co, CT-Co, ET-Co, and GT-Co (a, b, c, d). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. The Greek symbol (ə) is used if the value is significantly higher than the non-oxidized sample, and the symbol (β) is used if the value is significantly lower than the non-oxidized sample. All data are expressed as means ± standard deviation.

It is interesting to observe that the additions of C positively impacted the final concentrations of acetaldehyde in all treated wine. The best effect was observed for the combination of GT and C. In a previous study [11], the authors showed that C operates against oxidation by means of a direct radical scavenging mechanism, however, it can also act indirectly, via metal chelation [Chinnici et al. 2014], which would block the generation of radical species following the Fenton reaction [Guibal, 2004; Schreiber et al. 2013; Chinnici et al. 2014]. However, it should also be considered that part of acetaldehyde may have been involved in the formation of ethyl-bridged anthocyanins and flavanols [Timberlake and Bridle, 1976]. Concerning total individual anthocyanins, the presence of C did not influence the final concentration in wines, except for the ET-C and GT-C samples, while the effect on each individual anthocyanins was significant in few cases: delphinidin 3-glucoside in GT-C, petunidin 3-glucoside in W-C, malvidin 3-glucoside in ET-C, and malvidin 3-(6II-acetyl)-glucoside in W-C and ET-C (Table 3.8).

Table 3.8. *Oxidation effect on native anthocyanins.*

	Delphinidin 3-Glucoside (mg/L)		Cyanidin 3-Glucoside (mg/L)	Petunidin 3-Glucoside (mg/L)		Peonidin 3-Glucoside (mg/L)	Malvidin 3-Glucoside (mg/L)		Malvidin 3-(6II-Acetyl)-Glucoside (mg/L)		Malvidin 3-(6II-Coumaroyl)-Glucoside (mg/L)
Wo	5.88 ± 0.92	AB β	ND	6.20 ± 0.91	* A β	ND	30.79 ± 1.08	A β	10.92 ± 1.94	* A β	ND
W-Co	4.76 ± 1.02	ab β	ND	4.13 ± 0.69	a β	ND	26.24 ± 6.66	a β	7.40 ± 0.34	a β	ND
CTo	5.61 ± 0.78	A β	ND	4.45 ± 0.75	A β	ND	26.71 ± 5.41	A β	9.18 ± 1.37	A β	ND
CT-Co	5.40 ± 1.02	a β	ND	4.91 ± 0.83	a β	ND	24.67 ± 2.52	a β	6.91 ± 1.55	ab β	ND
ETo	4.26 ± 0.41	BC β	ND	4.55 ± 0.77	A β	ND	27.30 ± 0.12	* A β	10.66 ± 0.38	* A β	ND
ET-Co	3.16 ± 0.93	b β	ND	3.71 ± 0.67	a β	ND	22.44 ± 0.05	a β	5.48 ± 0.02	b β	ND
GTo	3.64 ± 0.44	* C β	ND	4.41 ± 0.86	A β	ND	26.85 ± 3.27	A β	7.75 ± 1.41	A β	ND
GT-Co	2.99 ± 0.27	b β	ND	3.97 ± 0.17	a β	ND	24.59 ± 0.54	a β	6.78 ± 0.63	ab β	ND

Different letters indicate a statistically significant difference among treated wines according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins addition is expressed with a capital letter on wines Wo, CTo, ETo, and GTo (A, B, C). The effect of chitosan addition is expressed with a lowercase letter on wines W-Co, CT-Co, ET-Co, and GT-Co (a, b). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. The Greek symbol (β) is used if the value is significantly lower than the non-oxidized sample. All data are expressed as means ± standard deviation.

The changes in total anthocyanins and chromatic characteristics after oxidation were as expected: a decrease in total anthocyanins and colour intensity and an increase in tonality (Table 3.9). Significant data points were those on ΔE between wines treated and untreated with C: for all experimental wines after oxidation the effect of C was well visible (ΔE > 3) (Table 3.10) and the trend was: ET > GT > CT and W. The great effect of C on ellagitannins agrees with previous results [Castro and Chinnici, 2020] and it deserves future investigation.

Table 3.9. *Oxidation effect on total anthocyanins, colour intensity, and tonality of treated wines.*

	Total Anthocyanins (mg/L)		Colour Intensity (420 nm + 520 nm + 620 nm)		Tonality (420 nm/520 nm)	
Wo	114.49 ± 2.11	* C β	3.01 ± 0.16	* A β	0.73 ± 0.01	* B □
W-Co	104.63 ± 0.56	b β	2.62 ± 0.13	ab β	0.75 ± 0.00	a □
CTo	123.16 ± 2.04	* A β	2.99 ± 0.21	A β	0.74 ± 0.00	AB □
CT-Co	110.74 ± 1.74	a β	2.64 ± 0.17	a β	0.74 ± 0.00	a □
ETo	119.56 ± 1.28	* AB β	2.83 ± 0.12	* A β	0.73 ± 0.01	* B □
ET-Co	94.26 ± 1.67	c β	2.27 ± 0.20	b β	0.77 ± 0.01	a □
GTo	118.09 ± 0.88	* BC β	2.91 ± 0.11	* A β	0.79 ± 0.06	A □
GT-Co	95.81 ± 2.08	c β	2.64 ± 0.15	a β	0.78 ± 0.00	a □

Different letters indicate a statistically significant difference among treated wines according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins addition is expressed with a capital letter on wines Wo, CTo, ETo, and GTo (A, B, C), and the effect of chitosan addition is expressed with a lowercase letter on wines W-Co, CT-Co, ET-Co, and GT-Co (a, b, c). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. The Greek symbol (ə) is used if the value is significantly higher than the non-oxidized sample, and the symbol (β) is used if the value is significantly lower than the non-oxidized sample. All data are expressed as means ± standard deviation.

Table 3.10. *Oxidation effect on ΔE.*

	ΔE	
Wo-WCo	2.79 ± 0.50	c
CTo-CT Co	3.49 ± 0.38	c
ETo-ET Co	7.64 ± 0.11	a
GTo-GT Co	5.09 ± 0.18	b

3.4.2.3 Impact of C and Oxidative Stress on the Reactivity of Wines towards Saliva

The SPI (saliva precipitation index) was performed to evaluate the reactivity of the wines towards salivary proteins, representing an indirect method to measure wine astringency before and after the treatment with C. According to the ANOVA analysis ($p = 0.005$) that considered the effect of C on SPI, the C highly influenced the precipitation of salivary proteins, resulting in a possible decrease in the astringency in control wine and wine treated with condensed tannins (Figure 3.11).

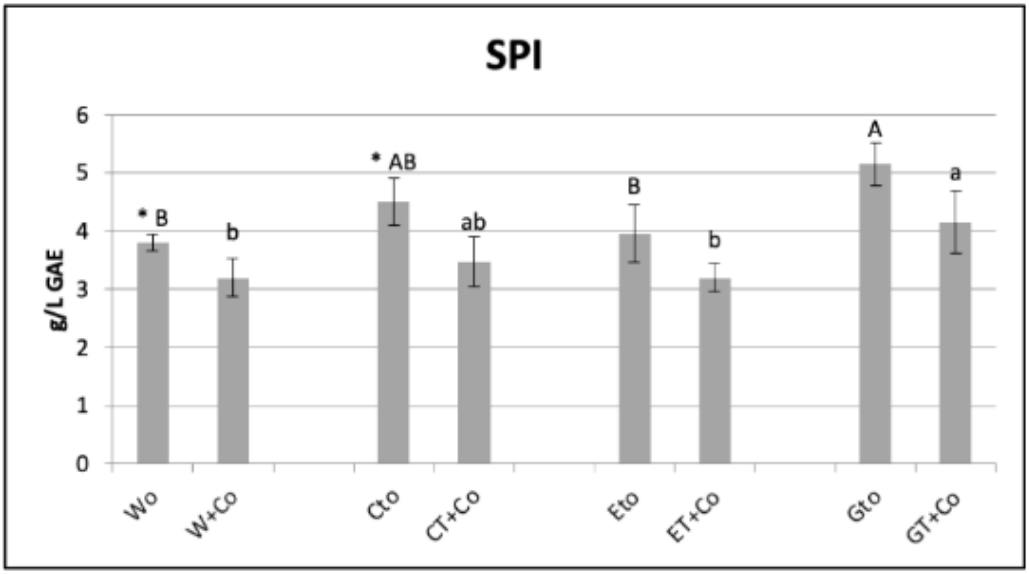

Figure 3.11 Oxidation effect on the SPI (saliva precipitation index) of the treated wines. Different letters indicate a statistically significant difference among treated wines according to Tukey's (HSD) analysis ($p < 0.05$). The effect of tannins addition is expressed with a capital letter on wines Wo, CTo, ETo, and GTo (A, B), and the effect of chitosan addition is expressed with a lowercase letter on wines W-Co, CT-Co, ET-Co, and GT-Co (a, b). The asterisk (*) indicates a statistical difference between each kind of wine before and after the treatment with chitosan. All data are expressed as means ± standard deviation.

This result was also observed in a previous work in which the treatments with C at different phases of winemaking significantly reduced the precipitation of polymeric

tannins reactive to BSA. The most astringent wine was GTo, confirming that the addition of gallotannins can influence the astringency negatively. Even with the addition of CTo tannin (condensed tannin), the wine may result more reactive towards salivary proteins and likely astringent. However, the chitosan treatment decreased the SPI values in a significant way, and the percentage decrease was higher in CT-Co (−23%) than in the other treated wines. The chitosan probably showed a higher affinity for condensed tannins, allowing more precipitation of the formed complexes with salivary proteins [Spagna et al. 1996]. The control wine Wo did not differ from the wine added with ET tannin (ellagitannin), and after the treatment W-Co and ET-Co were less astringent, similar also to CT-Co.

3.4.3 Conclusions

Data obtained in this study showed a significant effect of C on both red wine and tannin-enriched wine. The greatest effect was detected for ET and CT. After oxidation, the treatment with C of wines added with condensed tannins determined the higher formation of polymeric pigments while a great variation in chromatic characteristics was detected for control wines and wines added with CT. For all wines, the treatment with C determined a lower production of acetaldehyde after oxidative stress. The removal of tannins, due to the absorption on C before oxidation, and the lower production of acetaldehyde, due to the antioxidant effect of C, caused a lower formation of polymeric pigments in all wines. This is the first study in which an effect of C on the reactivity towards salivary proteins of wines was detected. As a decrease of SPI was detected, these results suggest a possible use of C to treat wines very rich in tannins that could be not well accepted by consumers for their excessive astringency. Mechanisms involved in the removal of tannins of different origin in red wines should be better elucidated in future studies.

3.4.4 Considerations

The present activity was conducted in collaboration with the entire Enology group of the Department of Agricultural Sciences. The results acquired found editorial placement in an international scientific journal in the form of an original research article entitled "Effect of Chitosan on the Removal of Different Types of Tannins from Red Wines”.

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CHAPTER 4

AGING, ACETALDEHYDE AND POLYPHENOLS

4.1 ACETALDEHYDE

Acetaldehyde is the most common volatile aldehyde in wines. It can result from biological or chemical transformations.

During the initial phase of fermentation, acetaldehyde is formed by yeasts as a byproduct of alcoholic fermentation, even if it is successively degraded by yeasts themselves or by malolactic bacteria. Chemically, acetaldehyde results from the oxidation of ethanol that occurs after fermentation through the Fenton reaction [Waterhouse and Laurie, 2006]. Acetaldehyde is a precursor for the synthesis of other aroma products, thus indirectly contributing to numerous distinctive notes in the aroma profile of wine [Etievant and Schreier 1995]. The production of acetaldehyde has been shown to be influenced by aerobic metabolism, medium composition, the nature of insoluble materials used to clarify the musts, aging procedure, SO_2 content, and aeration.

4.1.1 Microbiological and chemical origin of acetaldehyde in wine

Acetaldehyde is considered a secondary product of the alcoholic fermentation. It is the most important carbonyl compound produced during alcoholic fermentations, with final concentrations ranging from 10 to 200 mg/L. This aldehyde is the terminal electron acceptor in the alcoholic fermentation by *Saccharomyces cerevisiae* [Jackowetz et al. 2011]. It's synthesized from ethanol by *flor* yeasts by means of the enzyme alcohol dehydrogenase in the presence of $NAD^{+}\cdot$. Lactic acid bacteria (LAB) and acetic bacteria can also produce acetaldehyde [Joyeux et al. 1984; Drysdale and Fleet, 1988]. Acetic bacteria oxidize ethanol to acetaldehyde and acetic acid and can produce acetaldehyde contents as high as 250 mg/L. The amount of acetaldehyde formed by LAB varies by species and strain, and is generally less than 30 mg/L [Kneifel et al. 1992]. Acetaldehyde is synthesized through the metabolism of glucose, 2-deoxy-d-ribose-5-phosphate, and threonine [Rysstad et al. 1990; Grozeva et al. 1994]. The increase in the concentration of this compound is one of the more important consequences of microbial activity.

Under the chemical point of view, when wine ages, acetaldehyde, as outlined in the previous chapter is the main by-product of Fenton oxidation of wine (Figure 4.1).

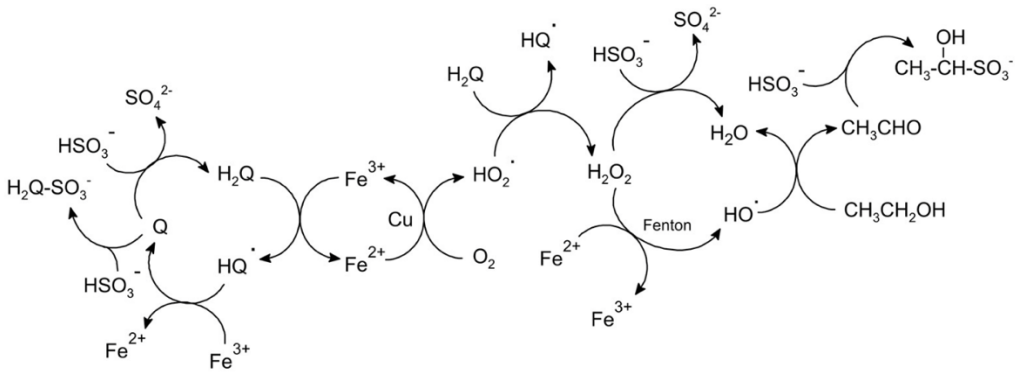

Figure 4.1 Oxidation *mechanism and SO_2 reactions proposed in red wine. Adapted from Danilewicz (2003, 2011, 2014); Danilewicz and Wallbridge (2010), and Waterhouse et al. (2016). Q, quinone; HQ, semiquinone; H_2Q, o-diphenol.*

4.1.2 Behavior of acetaldehyde in wine

Reactions between acetaldehyde and alcohols, as well as between acetaldehyde and various flavonoids (namely flavanols and anthocyanins), SO_2, and glutathione have been identified in wine and are often measured in wines undergoing oxygenation to monitor and manage the effects of such exposure. The condensation of aldehydes with alcohols form a class of compounds called acetals. Depending on the type of hydroxyl compound (alcohol, diols, or polyols) reacting with an aldehyde, three different types of acetals can be formed and have been identified in wine: alkyl heterocyclic acetal alcohols, alkyl heterocyclic acetals, and aromatic alkyl acetals [Es-Safi et al. 2002]. Heterocyclic acetal alcohols formed by the acetalization reaction between glycerol and acetaldehyde have been detected in Madeira, Port, and aged/oxygenated red wines [Camara et al. 2003; da Silva Ferreira et al. 2002; Es-Safi et al. 2002]. Given the relatively high concentration of glycerol in wines 5-20 g/L, they are among the primary secondary products of oxidation, [Cejudo-Bastante et al. 2011; Ribéreau-Gayon 2006]. Four isomers result from this reaction: two five-membered ring structures (*cis*- and *trans*-4-hydroxymethyl-2-methyl-1,3-dioxolanes) and two six-membered ring structures (*cis*- and *trans*-5-hydroxy-2-methyl-1,3-dioxanes). The condensation reaction between 2,3-butanediol and acetaldehyde yields 2,4,5-trimethyl-1,1-dioxolane, an alkyl heterocyclic acetal that has been detected at high levels in white wines undergoing oxidation [Escudero et al. 2000]. Additional acetals were detected in fortified and oxidized wines and derived from reactions of acetaldehyde and other low-level aldehydes with short chain alcohols [Cheynier et al. 2006; Es-Safi et al. 2002]. Diethyl acetal or heterocyclic acetal [da Silva

Ferreira et al. 2002; Moyano et al. 2002; Peterson and Waterhouse, 2016], are reported as potential age markers of Madeira wine [Pereira et al. 2010].

Understanding the formation of aldehydes is further complicated by the number of reversible and irreversible chemical processes in which these compounds are involved. Among the most important reactions of acetaldehyde and other aldehydes in wines there are the ones due to the reversible binding to SO_2 forming α-hydroxyalkylsulfonates (Figures 4.1 and 4.2) [de Azevedo et al. 2007; Grant-Preece et al. 2013]. The α-hydroxyalkylsulfonates of acetaldehyde may be present in unoxidized wines, acting as a reservoir of oxidation-related acetaldehydes that will be slightly released during wine oxidation as the SO_2 is depleted and the equilibria shift [Bueno et al. 2016; Tachtalidou et al., 2022]. These are not the only reactions of acetaldehyde in wine, owing to its electrophilic character it is reactive to other nucleophiles in wine (Figure 4.2). It can also bind reversibly to thiols such as glutathione or cysteine to give α-hydroxysulfides [Lienhard and Jencks, 1966; Sonni et al. 2011; Baert et al. 2015a].

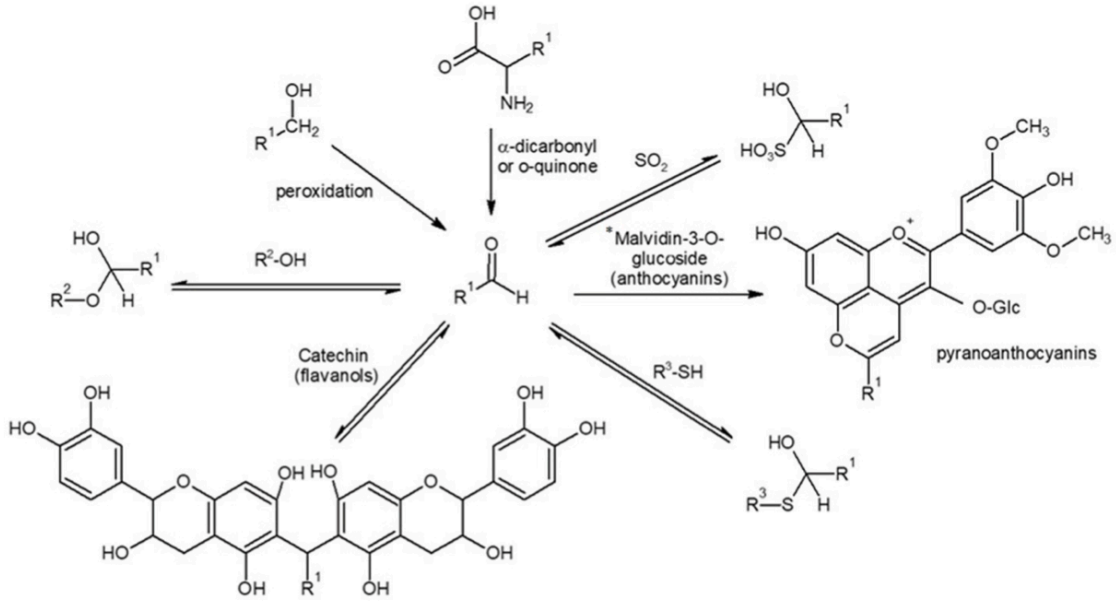

Figure 4.2 *Formation and reaction of aldehydes in wine. Adapted from Grant-Preece et al. (2013) and Waterhouse et al. (2016). *Only aldehydes that can exist in a stable -enol form can produce the pyranoanthocyanins.*

Among the most important reactions of aldehydes in wine there are those with phenolic compounds (Figure 4.2). They different C positions with nucleophilic character that can react with carbonyls such as acetaldehyde, forming a wide range of products, such as

pyranoanthocyanins [Bakker and Timberlake, 1997; Vivar-Quintana et al, 1999; de Freitas and Mateus, 2011; Marquez et al, 2013]. One of the most abundant in red wine is vitisin B that is the cycloaddition product of acetaldehyde and malvidin-3-O-glucoside. The enol tautomer of acetaldehyde attacks the C-4 and C-5 positions of anthocyanin and further dehydrates and oxidizes giving vitisin B to form a pyran ring between C4 and O6 of the anthocyanin [Atanasova et al. 2002; Fulcrand et al. 2006; de Freitas and Mateus, 2011]. Other reaction products between aldehydes and flavonoids are longer dimers or polymers in which the aldehydes act as bridges. In these reactions acetaldehyde gives 8,8-methylmethine bridge, also called an ethyl bridge [Es-Safi et al. 1999, 2002; Escribano-Bailon et al. 2001; Atanasova et al. 2002; Pérez-Magariño et al. 2007; Schmidtke et al. 2011]. The reaction of formation should occur by direct nucleophilic attack on position 8 of flavonoid to introduce a 1-hydroxyethyl group, which dehydrates further and undergoes nucleophilic attack from position 8 (or 6) of the second molecule. These polymeric structures also named acetaldehyde-bridged tannins were suggested to be less astringent than unmodified tannins [Cheynier et al., 2006; Vidal et al., 2004].

4.2 The case study: How acetaldehyde reacts with low molecular weight phenolics in white and red wines

Acetaldehyde is a highly reactive aldehyde featuring both electrophilic and nucleophilic properties and, considering its high volatility, it is a flavor active molecule reminiscent of green grass, bruised apples and nuts with a sensory threshold of about 100 mg/L. Indeed, acetaldehyde can react with other wine compounds including glycerol thus leading to the formation of dioxolane and dioxane acetals responsible for wine oxidative spoilage [Silva et al. 2002]. At higher concentrations, it can be quite pungent with a negative impact on the wine quality. Still, in red, wines acetaldehyde can be even responsible for some beneficial reactions, involving mainly phenolics that could improve wine color stability and astringency over time [Sheridan and Elias, 2015; Drinkine et al. 2007]. Typical adducts formed by the reaction of acetaldehyde with phenolics consist of ethylidene-bridged compounds. Flavan-3-ols, including catechin, epicatechin, proanthocyanidins, and anthocyanins are among the major wine polyphenols reactive to acetaldehyde. Considering that acetaldehyde protonation is the first step of the reaction, pH is a critical parameter to be considered. Low pH values around 2 determine high reaction rates. Consequently, wine pH usually stretching from 3 to 4 causes acetaldehyde to react with

flavonoids more slowly. In this regard, several studies have been conducted in wine model solutions [Dallas et al. 1996; Doco et al. 1996; Es-Safi et al. 1999a; Es-Safi et al. 1999b], while little is currently available in literature about the fate of acetaldehyde in real wines owing to the complexity of the product mixture deriving from the reaction with phenolics. It needs to be underlined that acetaldehyde rapidly reacts with bisulfites that are commonly added to wines as to prevent them from spoilage of either chemical or microbial origin. The product of such reaction is 1-hydroxyethanesulfonate. In addition, when the concentration of sulfur dioxide is twice that of flavanols, reactions of acetaldehyde consuming catechin tend to cease [Sheridan and Elias, 2016]. This is the reason why in this study with the purpose of providing further insights into the acetaldehyde reactivity with phenolics in real wines, we decided to use wines with reduced amounts of bisulfites. When anthocyanins and flavanols simultaneously occur, as in the case of red wines, the reaction of acetaldehyde with anthocyanins is slower than that with flavanols seemingly due to the better nucleophilicity of flavanol's position 8 and, to a lesser extent, of position 6, too. Previous analyses have characterized a number of products of the reaction of acetaldehyde with catechin and malvidin-3-O-glucoside in wine model solution. Specifically, the most abundant product was identified as a catechin-ethyl-malvidin-3-O-glucoside in addition to other ethylidene-bridged oligomers constituted by up to four catechin units or by up to three catechin units containing also malvidin-3-O-glucoside in a terminal chain position. Ethylidene-bridged oligomers containing flavanols and two anthocyanins terminal units have been detected as well [Es-Safi et al. 1999b]. Conversely, unlike flavanols, anthocyanins tend to form preferentially dimers. To make the product mixture even more complex, vinyl flavan-3-ols either as monomers or as subunits included in oligomers can also be formed [Cruz et al. 2009]. Other anthocyanin-derived pigments detected in red wines are pyranoanthocyanins. They are formed by the reactions between anthocyanins and other wine metabolites, such as pyruvic acid and acetaldehyde, or with compounds extracted from grapes, such as cinnamic acids. When malvidin-3-O-glucoside reacts with pyruvic acid and acetaldehyde, vitisin A (1) [Fulcrand et al. 1998] and vitisin B (2) [Oliveira et al. 2009] are, respectively, formed. Pyranoanthocyanins play an important role in wine ageing, as they are more stable in solution as opposed to flavanol–anthocyanin polymers that tend to precipitate over time [Rentzsch et al. 2010]. Also, these pigments with their predominant red–orange hues differ from anthocyanins characterized by a red–purple color. As stated above, in the face of several studies conducted on the reactivity of acetaldehyde with selected wine

phenolics in model solutions, very few are the reports on such reactions in real wines. Although the role of anthocyanins has been extensively investigated, to the best of our knowledge, no studies focused on possible differences between the behavior of white and red wines when added with the same amount of acetaldehyde have been conducted yet. Hence, the present study was designed and carried out with the purpose of gaining preliminary information about the reactivity of acetaldehyde with low molecular weight phenolics in both white and red wines. Additionally, the outcome of such a study was expected to provide further insights into the color evolution of wines.

4.2.1 Materials and Methods

4.2.1.1 *Chemicals and reagents*

Malvidin-3-*O*-glucoside chloride (> 90% HPLC) and (+)-catechin (99% HPLC) standards, formic acid for LC–MS analysis and acetonitrile (hypergrade for LC–MS LiChrosolv®) were purchased from Sigma-Aldrich (Milan, Italy). Aqueous solutions were prepared with Milli-Q water from Millipore (Bedford, MA, USA).

4.2.1.2 Wine samples

Aglianico red wine and Falanghina white wine, both produced in 2018 by the Taburno winery with a standard industry protocol, were added with 190 mg/L of pure acetaldehyde. Aglianico base parameters were: ethanol 13.37%, pH 3.69, titratable acidity 5.4 g/l, volatile acidity 0.54 g/l. Falanghina base parameters were: ethanol 13.12%, pH 3.42, titratable acidity 6.6 g/l, volatile acidity 0.34 g/l. Free and total sulphur dioxide were below detection threshold [Jenkins et al. 2020]. The same Aglianico and Falanghina wines, without the addition of acetaldehyde, were used as control. Treated and untreated wines were stored in 5 L glass flasks hermetically closed in the dark at 20 °C for one year.

4.2.1.3 High-performance liquid chromatography analyses of anthocyanins in red wine samples

The separation of the monomeric anthocyanins was performed according to the OIV method [2020] of analysis using a HPLC Shimadzu LC10 ADVP apparatus (Shimadzu Italy, Milan), consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, an SPD-M 10 AVP detector, and an injection system full Rheodyne model 7725 (Rheodyne, Cotati, CA). A column heating device set at 40 °C was used, with a C18

column, Waters Spherisorb column (250 × 4.6 mm, 4 μm particles diameter) with pre-column (Oiv.int). All the samples were filtered through 0.45 μm filters (Durapore membrane filters, Millipore - Ireland) into glass vials and immediately injected into the HPLC system. A 50 μL loop was used. Elution was carried out using a flow rate of 0.80 mL/min. Eluents were: solvent A consisting of water milli-Q (Sigma-Aldrich)/formic acid (Sigma-Aldrich ≥ 95%)/acetonitrile (Sigma-Aldrich ≥ 99.9%) (87:10:3 v/v/v) and, solvent B consisting of water/formic acid/acetonitrile (40:10:50 v/v/v). The following gradient was used: zero-time conditions 94% A and 6% B; after 15 min the pumps were adjusted to 70% A and 30% B, at 30 min to 50% A and 50% B, at 35 min to 40% A and 60% B; at 42 min through the end of analysis, to 94% A and 6% B. For calibration the external standard method was used: the calibration curve was plotted for the malvidin-3-O-glucoside on the basis of peak area. The concentration of the following monomeric anthocyanins was determined in red wine used as control, after 1 year of incubation, and expressed as mg/L: delphinidin 3-O-glucoside (39.05 ± 1.46); cyanidin 3-O-glucoside (2.82 ± 0.13); petunidin-3-O-glucoside (49.68 ± 1.07); peonidin 3-O-glucoside (22.59 ± 1.01); malvidin-3-O-glucoside (266.77 ± 14.02); peonidin-3-(6II-acetyl)-O-glucoside (8.60 ± 0.40); malvidin 3-(6II-acetyl)-O-glucoside (5.84 ± 0.20); malvidin 3-(6II-coumaroyl)-O-glucoside (34.54 ± 0.43). In red wine added with acetaldehyde, after 1 year of incubation, only malvidin-3-O-glucoside (16.55 ± 0.25 mg/L) was quantified. The remaining anthocyanins were detected only in traces. The concentration was expressed as mg/L of malvidin 3-O-glucoside. All experiments were carried out in duplicate and two analytical replicas were performed.

4.2.1.4 HPLC and LC-HR ESIMS analyses of red wines

Before LC-HR ESIMS analyses, red wines were preliminary subjected to HPLC separation at a semi-preparative scale using the same elution conditions described above for the identification of anthocyanins, except the employed column that was a Luna 10u Column and the flow rate that was set at 1.8 mL/min. From this chromatographic separation, five fractions were collected: A (from 7 to 24 min), B (from 24 to 25 min), C (from 25 to 26 min), D (from 26 to 28 min), and E (from 28 min to end of the run at 42 min). Each collected fraction was successively concentrated to 1 ml final volume under nitrogen, and finally analyzed by LC-HR ESIMS in the positive and negative ion mode using the same parameters reported above for the analyses of white wines except

the elution gradient. Eluents were: solvent A consisting of water milli-Q (Sigma-Aldrich)/formic acid (Sigma-Aldrich ≥ 95%)/acetonitrile (Sigma-Aldrich ≥ 99.9%) (87:10:3) v/v/v and, solvent B consisting of water/formic acid/acetonitrile (40:10:50) v/v/v. The following gradient was used: zero-time conditions 94% A and 6% B; after 20 min the pumps were adjusted to 70% A and 30% B; at 40 min to 50% A and 50% B; at 50 min to 40% A and 60% B; at 51 min through the end of the analysis (65 min) to 94% A and 6% B.

4.2.1.5 CIELAB coordinates and color intensity and hue.

The CIELAB parameters (L*. a*. b*) were determined using the software Panorama (PANORAMA SOFTWARE UPGRADE PATH), following the recommendations of the Commission Internationale de L'Eclariage (CIE). Color differences (ΔE⁄ab) were calculated as the Euclidean distance between two points in the 3D space defined by L*, a*, and b*. Color intensity (Abs 420 nm + Abs 520 nm + Abs 620 nm) and hue (Abs 420 nm/Abs 520 nm) were determined spectrophotometrically using a UV spectrophotometer. All analyses were carried out in duplicate.

4.2.2 Results and Discussion

Each finished red and white wine was subdivided into two aliquots, respectively: one was used as control and the other was treated with an excess of acetaldehyde. Treated wines and control wines were stored under the same conditions for 1 year. This was a reasonable stretch of time usually elapsing for a typical wine from bottling to consumption. The purpose of the addition of an excess of acetaldehyde was to favor the formation of products deriving from the reaction between the aldehyde and wine phenolics at a concentration high enough to be detected and analyzed. These reactions, though only a part of those taking place during wine fermentation and oxidation, are crucial for the stability of the wine color especially during the first years of aging. As for white wines, information on the effect of acetaldehyde on phenolics is still quite scarce. In our study, the comparison between the treated and untreated white wines allowed to shed some light on the evolution of phenolics following the addition of acetaldehyde. This was achieved by means of LC-HR ESIMS. In red wines, the addition of acetaldehyde induced a massive formation of polymeric compounds. This was ascertained when a preliminary HPLC-based analysis was conducted. Unlike the control wine, the occurrence of polymeric pigments in the fortified wine was reflected by a significant drift of the UV/vis

chromatogram baseline recorded at 518 nm. Hence, to get qualitative insights into the complex polymeric fraction of the treated wines, a HPLC-based chromatographic separation at a semi-preparative scale was initially performed, as reported in Experimental. Five fractions were collected (A–E) and each of them was subjected to LC-HR ESIMS.

4.2.2.1 Analysis of white wines

Samples of treated and untreated white wines were analyzed by LC-HR ESIMS after one year of incubation. The comparison of the total ion chromatograms (TIC) of the white wine used as control (sample #1) and the white wine (sample #2) added with the aldehyde brought to light a significant decrease of the relative abundance of some chromatographic peaks along with a concomitant increase of others (Figure 4.3). Discrepancies in terms of peak intensities were ascertained by measuring the areas of the ion peaks evidently differing in the chromatograms of sample #1 and #2. In more detail, in the TIC of sample #2 we observed the increment of peaks eluting at 12.40, 13.32, 14.70, 14.90, and 16.37 min, respectively. Peaks eluting at 12.85, 15.12, 16.20, and 20.00 remained approximately unaffected; whilst, peaks at 9.65, 11.15, 13.51 and 14.50 min, respectively, appeared to be less intense when compared to the TIC of sample #1 (Table 4.1). By comparison with pure standards, the two peaks eluting at 11.15 and 13.51 min (m/z 289) were attributed to catechin and epicatechin (Figure 4.4), respectively. On account of previous reports, the peaks eluting at 9.65 min (m/z 577) and 14.50 (m/z 865) were assigned to a procyanidin dimer and trimer, respectively (Figure 4.4) [Bravo et al. 2006; Frost et al. 2018]. The remaining peaks were reasonably interpreted as the result of the reaction between wine flavanols and acetaldehyde. More specifically, based on their retention times and m/z values (315), the two ion peaks at 14.90 and 16.37 min were assigned to a vinyl-catechin and vinyl-epicatechin derivative (Figure 4.4), respectively. The two ion peaks differed indeed from either catechin or epicatechin by 26 u.m.a., corresponding to a C2H2-moiety [Drinkine et al. 2007]. Such vinyl derivatives are likely the result of the dehydration of ethyl alcohol-flavan-3-ol adducts deriving from the nucleophilic attack of the flavan-3-ol C8 or, to a lesser extent, C6 to protonated acetaldehyde. In the treated wines, we did not detect ethyl alcohol-flavan-3-ol adducts, but peaks at 12.85 and 15.12 min (m/z 359) were likely the result of these latter derivatives with an extra vinyl moiety (Figure 4.4) linked to either 6 or 8 position. Hence, these molecules were identified as ethyl alcohol-vinyl-catechin adducts (12.85 min) and

ethyl alcohol-vinyl-epicatechin adducts (15.12 min). It is reasonable to infer that such derivatives underwent dehydration thus turning into 6,8-divinyl-catechin (16.20 min; m/z 341) and 6,8-divinyl-epicatechin (20.00 min; m/z 341), respectively. Finally, the two ion peaks eluting at 13.32 and 14.70 min both sharing the same m/z value of 603 were assigned to dimeric vinyl-procyanidins. Only the peak at 12.40 remained undetermined. Interestingly, no ion peaks corresponding to possible ethylidene linked flavan-3-ols oligomers were detected.

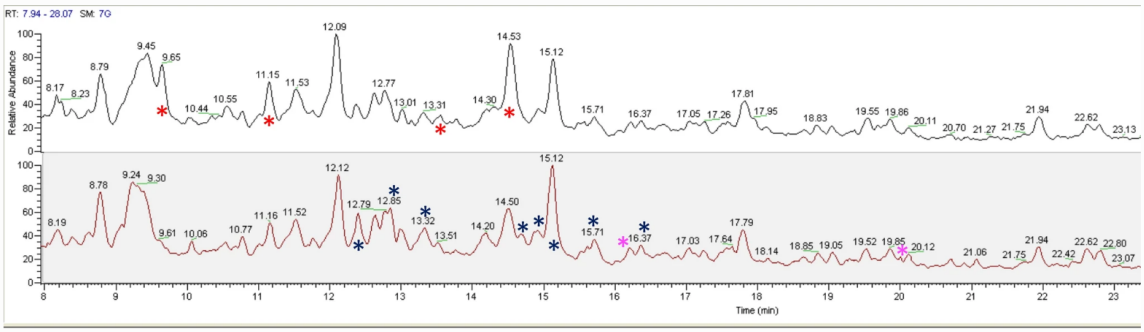

Figure 4.3. *Total ion current chromatograms of a sample of control white wine (above) and of a sample of white wine treated with acetaldehyde. Red and blue asterisks indicate ion peaks that decreased or increased, respectively, after the addition of acetaldehyde (Table 4.1); magenta asterisks indicate peaks that seem to have been not affected by the addition of acetaldehyde.*

Additionally, quantitative analyses were conducted by LC-HR ESIMS as reported in Experimental. Quantitation of catechin and epicatechin was carried out by comparison with pure standards. All of the other identified flavanol derivatives were quantified by assuming they had the same molar response as catechin. On the basis of our results, a significant decrease of over 80% was observed for procyanindin dimers and trimers, confirming previous reports according to which procyanidin B2 in model solution added with acetaldehyde was no longer detectable after 15 days [Dallas et al. 1996]. Catechin and epicatechin appeared to be reduced by approximately 50% in the treated wines (sample #2) in comparison to untreated ones (sample #1). At the same time, though, the relative abundances of ethyl alcohol-vinyl-flavan-3-ols and of divinyl-flavan-3-ols were comparable in the treated and untreated wines, as opposed to vinyl-flavan-3-ols that remarkably increased in sample #2. This datum likely reflects the complex evolution of flavanols mixtures characterized by C–C bond breaking and forming that take place even under the wine mild conditions. It needs to be underlined that several studies have been conducted on the reactivity of flavan-3-ols towards acetaldehyde, in which the major

observed products were oligomers, mainly dimers, consisting of catechin and epicatechin linked by an ethylidene bridge. However, to the best of our knowledge, experimental studies on the reactivity of flavan-3-ols with acetaldehyde have been conducted mainly in wine like solutions, while quite limited are the reports on real wines. In our studies, in white wines treated with an excess of acetaldehyde, we detected the occurrence of a number of newly formed products as reported in Table 4.1, but no ethylidene linked oligomers of flavanols. On the basis of our MS-based analyses, the detected compounds turned out to be all vinyl adducts of flavan-3-ols. According to the proposed mechanism of reaction6, such vinyl adducts can have a double origin. As already discussed above, they can derive from the dehydration of ethyl alcohol-flavan-3-ol adducts initially obtained from the reaction between acetaldehyde and flavan-3-ol at either position 8 or 6, even if this latter position appears to be less reactive due to steric hindrance. Alternatively, the formation of vinyl derivatives can be the result of the depolymerization of ethylidene linked flavan-3-ol oligomers. In this case, we can hypothesize that if such oligomers had been likely formed in the first place, their breakdown occurred as well. It has been in fact proposed that the rate of ethylidene-bridged flavan-3-ol formation is comparable to its rate of breakdown [Drinkine et al. 2007]. Hence, the depolymerized products could rearrange to form vinyl flavan-3-ols. Interestingly, a comparison of the relative abundance of flavanol derivatives between the treated and untreated wines brought to light substantial equal levels of those adducts featuring a double substitution at position 6 and 8 (ethyl alcohol-vinyl-(epi)catechin and divinyl-(epi)-catechin). This would be of some interest and worth to be fully investigated in model solutions to clarify their origin and rate of formation. It should also be taken into account that a possible source of such compounds may be due to yeasts metabolisms and this would pave the way towards microbiological studies, too. Finally, our results under the enological point of view suggest that in white wines flavanols tend to react with acetaldehyde, which in turn will be not initially involved in other reactions leading to the formation of off-flavors including cyclic acetals and sotolon [Pons et al. 2010]. Our results highlight that, regardless of the origin of acetaldehyde in wine, flavanols in white wine are helpful to quench this highly reactive carbonyl by affording more stable compounds. Therefore, on one hand the “catechol” B-ring of flavonoids can be oxidized by quinones through electron transfer reactions leading to flavanoid quinones, precursors of browning products, but on the other they can prevent the negative off-flavors deriving from acetaldehyde produced during oxidation [Ma and Waterhouse, 2018].

Table 4.1. *Compounds whose concentrations was modifed by the addition of acetaldehyde to white wines. Untreated wines (sample #1); treated wines (sample #2)*

Retention time	[M-H]$^-$ *m/z*; Δ (ppm)	Formula	Compound	mg/L in sample #1	mg/L in sample #2	Fragment ions [MS/MS] (*m/z*)
9.65 min	577.1345; Δ = 0.741	$C_{30}H_{25}O_{12}$	Procyanidin dimer	7.10 ± 0.04	1.05 ± 0.02	407, 289, 245
11.15 min	289.0711; Δ = 1.610	$C_{15}H_{13}O_6$	catechin	2.02 ± 0.05	0.92 ± 0.03	245, 203, 159, 151, 137, 125, 109
12.40 min	429.2121	n.a	Undetermined	–	–	–
12.85 min	359.1143; Δ = 1.876	$C_{19}H_{19}O_7$	ethyl alcohol-vinyl-catechin	2.84 ± 0.03	2.93 ± 0.04	341, 313
13.32 min	603.1496; Δ = -0.137	$C_{32}H_{27}O_{12}$	vinyl-Procyanidin dimer	0.12 ± 0.08	1.95 ± 0.1	467, 289
13.51 min	289.0709; Δ = 0.987	$C_{15}H_{13}O_6$	epicatechin	0.73 ± 0.02	0.36 ± 0.05	245, 203, 187, 151, 137, 125, 121, 109
14.50 min	865.1973; Δ = − 1.395	$C_{45}H_{37}O_{18}$	Procyanidin trimer	0.25 ± 0.03	0.04 ± 0.02	577, 289
14.70 min	603. 1496; Δ = − 0.137	$C_{32}H_{27}O_{12}$	vinyl-Procyanidin dimer	0.08 ± 0.04	2.03 ± 0.1	467, 289
14.90 min	315.0866; Δ = 0.874	$C_{17}H_{15}O_6$	vinyl-catechin	0.05 ± 0.01	0.57 ± 0.03	287
15.12 min	359.1143; Δ = 1.876	$C_{19}H_{19}O_7$	ethyl alcohol-vinyl-epicatechin	1.48 ± 0.1	1.69 ± 0.05	341
16.20 min	341.1036; Δ = 1.579	$C_{19}H_{17}O_6$	6,8-divinyl-catechin	1.11 ± 0.08	1.08 ± 0.05	323, 295
16.37 min	315.0866; Δ = 0.874	$C_{17}H_{15}O_6$	vinyl-epicatechin	0.04 ± 0.02	0.59 ± 0.09	287
20.00 min	341.1036; Δ = 1.579	$C_{19}H_{17}O_6$	6,8-divinyl-epicatechin	2.32 ± 0.01	2.12 ± 0.02	295

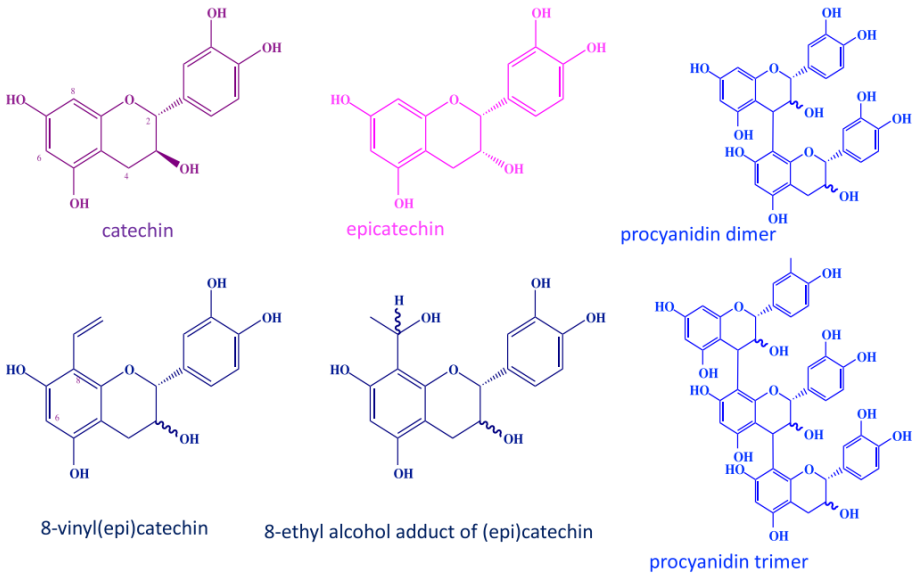

Figure 4.4. *Stereostructures of compounds detected by LC-HR ESIMS in white wines*

4.2.2.2 Analysis of red wines

After 1 year of incubation, the different evolutions of the wine used as control and the one added with acetaldehyde were suggested by their relative HPLC chromatograms registered at l = 518 nm. As mentioned above, unlike the control, the chromatogram of

the treated wine was characterized by a significant drift of the baseline likely due to a massive formation of polymeric pigments. Initially, the official OIV method [2020] was applied to analyzed the two wines. In the untreated wine, a number of anthocyanins was identified and quantified, as reported in Experimental. Conversely, in the wine fortified with acetaldehyde only malvidin-3-O-glucoside could be detected. This datum confirmed that acetaldehyde had caused a dramatic loss of monomeric anthocyanins. In this regard, Dallas and colleagues suggested that, in addition to malvidin-3-O-glucoside, two new colored compounds, obtained from the reaction of peonidin-3-O-glucoside and the procyanidin–acetaldehyde adduct, were simultaneously observed after 1 h, they increased in 2 days and then began to decrease and were no longer detectable after 10 days.

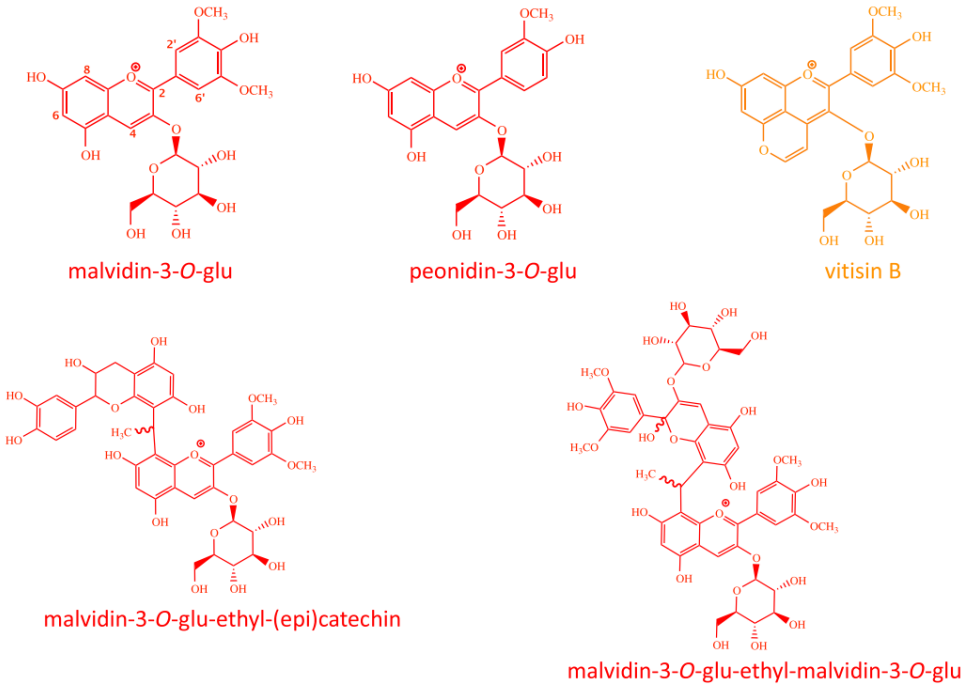

Figure 4.5 *Stereostructures of pigments detected by LC-HR ESIMS in red wines*

It is possible that the same phenomenon occurred in the wines used in our study. To better investigate the wine added with acetaldehyde, a preliminary semi-preparative HPLC separation of this wine was carried out and five fractions (A–E) collected. Each of these fractions was then analyzed by LC-HR ESIMS. By means of LC–MS, in fraction A, we detected an ion peak at m/z 493 attributable to malvidin-3-O-glucoside (Figure 4.5, Table 4.2, SI 1a), while peonidin-3-O-glucoside (m/z 463) was present just in traces (SI 1b).

This was consistent with what initially observed by the HPLC-based analysis performed according to the OIV method.

Table 4.2 *Compounds detected by LC-HR ESIMS (positive ion mode) in fractions A–E obtained from a red wine sample added with acetaldehyde*

Fraction Retention time	$[M]^+$ *m/z*; Δ (ppm)	Formula	Compound	Fragment ions [MS/MS] (*m/z*)
A				
12.47 min	463.1259; Δ = 5.208	$C_{22}H_{23}O_{11}^+C_{23}H_{25}O_{12}^+$	Peonidin-3-*O*-glucoside	301
13.39 min	493.1326; Δ = − 3.818		Malvidin-3-*O*-glucoside	331
B				
15.64 min	561.1227; Δ = − 2.106	$C_{26}H_{25}O_{14}^+$	Vitisin A	399
17.15 min	517.1317; Δ = − 4.491	$C_{25}H_{25}O_{12}^+$	Vitisin B	355
C				
18.17 min	809.2274; Δ = − 1.644	$C_{40}H_{41}O_{18}^+$	Malvidin-3-*O*-glucoside-ethyl- catechin	647, 519, 357
18.97 min			Malvidin-3-*O*-glucoside-ethyl-epicatechin	647, 519, 357
D				
41.53 min	1029.2827; Δ = − 4.220	$C_{48}H_{53}O_{25}^+$	Malvidin-3-*O*-glu-ethyl- malvidin-3-*O*-glu	867, 705
E				
59–61 min	1097.2872; Δ = − 4.492	$C_{55}H_{53}O_{24}^+$	Malvidin-3-*O*-glu-ethyl-dimeric procyanidin	935
62–63 min	1125.3197; Δ = − 4.313	$C_{57}H_{57}O_{24}^+$	Malvidin-3-*O*-glu-ethyl-(epi)catechin-ethyl-(epi)catechin	963, 673

The LC–MS spectrum of fraction B contained two ion peaks at *m/z* 561 and 517 attributed to two pyranomalvidin-3-*O*-glucosides, namely vitisin A and B, respectively (Figure 4.5; SI 2) [Laitila et al. 2019]. Two ion peaks centered at *m/z* 809 in fraction C led us to infer the presence of dimers constituted by a malvidin-3-*O*-glucoside unit linked to a catechin unit (Rt = 18.17 min) and an epicatechin unit (Rt = 18.97 min), by an ethylidene bridge (SI 3). A mixture of diastereoisomers constituted by ethylidene dimers involving an anthocyanin unit and either catechin or epicatechin is not to be ruled out, considering that the ethylidene subunit deriving from acetaldehyde can connect the 8 position of the anthocyanin with either the 8 or 6 position of either flavan-3-ol (Figure 4.5) [Forino et al. 2020]. In the LC–MS spectrum of fraction D, there was an ion peak that was attributed to a dimer constituted by one malvidin -3-*O*-glucoside in its flavylium ion form and one malvidin -3-*O*-glucoside unit in its pseudobase form linked by an ethylidene bridge (*m/z* 1029) (Figure 4.5) [Atanasova et al. 2002]. Eventually, in E, some ion peaks turned out to be associated to a polymeric fraction eluting from 59 to 63 min (SI 4). These peaks were centered at *m/z* 1029 (the same as that contained in fraction D), 1097, and 1125, respectively. In Table 4.2, the above ion peaks are listed along with their molecular formulas and tentative chemical identification based on data available in literature [Es-Safi et al. 1999a; Es-Safi et al. 1999b] (Table 4.3).

***Table 4.3**. Content of native anthocyanins in red wines expressed as mg/L*

	Red wine (ctrl)	Red wine + acetaldehyde
Dp3glc	39.05 ± 1.46	tr
Cy3glc	2.82 ± 0.13	tr
Pt3glc	49.68 ± 1.07	tr
Pn3glc	22.59 ± 1.01	tr
Mv3glc	266.77 ± 14.02	16.55 ± 0.25
Pn3acglc	8.60 ± 0.40	tr
Mv3acglc	5.84 ± 0.20	tr
Mv3cmglc	34.54 ± 0.43	tr

Dp3glc=delphinidin 3-O-glucoside, Cy3glc=cyanidin 3- O-glucoside, Pt3glc=petunidin 3-O-glucoside, Pn3glc=peonidin 3-O-glucoside, Mv3glc=malvidin 3-O-glucoside, Pn3acglc=peonidin 3-(6IIacetyl)-O-glucoside, Mv3acglc=malvidin 3-(6II-acetyl)- O-glucoside, Mv3cmglc=malvidin 3-(6II-coumaroyl)-O-glucoside, tr=trace

Finally, vinyl-(epi)catechin and (epi)catechin-ethyl-(epi)catechin derivatives were identified in the LC-HR ESIMS spectra of fraction A of the treated wines acquired in the negative ion mode. As discussed for white wines, the ion peaks at m/z 315 were attributed to vinyl-catechin (Rt = 14.82 min), and to vinyl-epicatechin (Rt = 16.61 min). Additionally, an ion peak at m/z 605.1651 ($C_{32}H_{29}O_{12}^-$; Δ = − 4.313; Rt = 13.95 min) was indicative of the formation of (epi)catechin-ethyl-(epi)catechin adducts [6]. From a quantitative standpoint, analyses on the identified compounds were not reliable as we were not able to precisely assess the potential loss of analytes following the semi-preparative HPLC separation of the analyzed wines and the successive concentration of the obtained fractions. However, some deductions of some interest for enologists in regards to wine production and aging were inferred. On the basis of our experimental evidence, it was proposed that the anthocyanin moiety is not preferentially attacked by the protonated acetaldehyde, which seems to react first and preferentially with flavanols due to their better nucleophilicity when compared to anthocyanins. Subsequently, after the loss of a water molecule, the acetaldehyde-flavanol adduct attacks either another flavanol or an anthocyanin unit. In our studies conducted on real wines (pH = 3.69), we only detected polymers containing just one anthocyanin unit, even if molecules of

unreacted malvidin-3-O-glucoside were still present in the analyzed samples. On the contrary, flavanol-ethyl-flavanol adducts were detected. Thus, we could suggest that wine pH must play a central role in determining the outcome of the polymerization reaction involving acetaldehyde, anthocyanins and flavanols. It is reasonable to hypothesize that a higher pH renders acetaldehyde a worse electrophile thus causing it to react to a lesser extent with any available nucleophile, including flavanols and anthocyanins. As a consequence, in real wines characterized by milder acidic environments compared to the more acidic model solutions with a pH value usually around 2, just one anthocyanin moiety seems to be involved in the formation of ethylidene-bridged flavanol polymers. This is a crucial piece of information, as it implies the importance of properly modulating the ratio between native pigments and flavanols as to regulate the desired quantity of new pigments responsible for the wine coloration.

4.2.2.3 Chromatic characteristics of wines

The main spectrophotometric data (abs 420 nm, abs 520 nm and Abs 620 nm) and CIELAB color space using the L*a*b* coordinates were determined to understand if changes due to acetaldehyde addition were perceived by human eyes. The color of wine samples was determined as abs units at wavelength usually used to characterize white (abs 420 nm) and red (abs 420, abs 520, abs 620 nm) wines and by determining the CIELAB coordinates in the a*b* color plane, in which color is indicated along the green–red axis (-a* + a*) and blue–yellow axis (-b* + b*). For white wines, the addition of acetaldehyde determined an increase of abs 420 nm and a* and b* coordinates (Table 4.4). Normally, white wines are inside the area defined by the green (negative a* values) and yellow (positive b* values) color. Therefore, the yellow color component was positively influenced by acetaldehyde to a greater degree. The Chroma values, which define chromatic intensity against pure white, were positively correlated with acetaldehyde treatment. Thus, the wines treated with acetaldehyde exhibited higher C* and hue values than the untreated wines. The effect observed on color was also significant in terms of abs 420 nm but the shift was lesser than 0.8 abs units, a value generally considered detrimental for the quality of white wines. The acetaldehyde addition had effects on the chromatic parameters of red wines as well (Table 4.5). The color intensity (abs 420 nm + abs 520 nm + abs 620 nm) increased and hue (abs 420 nm/abs 520 nm) decreased as already observed[9]. A dramatic increase occurred in the chroma values (C^*_{ab}), while the decrease in the lightness (L^*) was up to 50 CIELAB units. The greater

decrease in lightness observed in the case of malvidin-3-*O*-glucoside should, therefore, be interpreted in terms of a greater coloration of wine treated with acetaldehyde. In the wine treated with acetaldehyde, the hue dropped to lower values and wines showed higher bluer hues as expected [Forino et al. 2020].

Table 4.4 *Abs 420 nm and CIELAB coordinates of white wines*

	White wine (ctrl)	White wine + acetaldehyde
Abs_{420nm}	0.15 ± 0.00	0.19 ± 0.00
L*	77.75 ± 0.66	77.58 ± 0.17
a*	1.40 ± 0.00	1.68 ± 0.05
b*	7.10 ± 0.12	9.78 ± 0.10
C*	7.20 ± 0.12	9.95 ± 0.13
H	78.93 ± 0.25	80.25 ± 0.19
ΔE		2.78 ± 0.08
Δhue		1.33 ± 0.39

Table4.5 Color intensity, hue and CIELAB coordinates of red wines

	Red wine (ctrl)	Red wine + acetaldehyde
Abs_{420nm}	2.91 ± 0.00	3.11 ± 0.10
Abs_{520nm}	3.23 ± 0.01	4.92 ± 0.15
Abs_{620nm}	0.64 ± 0.01	1.49 ± 0.05
Color intensity	6.78 ± 0.01	9.52 ± 0.29
Hue	0.90 ± 0.00	0.63 ± 0.00
L*	67.08 ± 0.29	11.15 ± 0.06
a*	17.83 ± 0.10	31.38 ± 0.05
b*	13.05 ± 0.13	17.48 ± 0.05
C*	22.08 ± 0.13	35.95 ± 0.06
H	36.20 ± 0.27	29.10 ± 0.08
ΔE		57.71 ± 0.35
Δhue		−7.10 ± 0.22

4.2.3 Conclusions

Although many studies have been performed on the reactivity of acetaldehyde in wine model solutions, quite poor are the reports on the fate of such molecule in real wines. Wines are complex chemical matrices including several compounds often involved in mutual reactions and equilibria. Thus, investigating the fate of natural metabolites in wines can prove challenging. The study we conducted, by providing insights into the chemical response to acetaldehyde of both white and red wines, constitutes a significant step forward towards the understanding of the chemical bases of the wine stability during the aging. More specifically, in white wines we ascertained the massive formation of vinyl-favan-3-ol derivatives, while in red wines the formation of ethylidene bridged red pigments was predominant. These latter positively enhanced the color properties of red wines and consequently their stability over time; while in white wines favan-3-ols prevented acetaldehyde from inducing undesired browning effects as well as to reactions that ultimately affect the quality of wine by the production of of-favors. The outcome of our research can be of some interest to enologists, since on such knowledge winemakers can hinge the implementation of appropriate technological practices, to opportunely modulate the natural chemical composition of wine metabolites as to guarantee stable high-quality products over time, without having to resort to undesirable external additives. Finally, the improvement of the color observed in red wines after the addition of an excess of acetaldehyde might be of some interest even to the dyeing industry for the production of stable and intense colorants by reusing wine byproducts quite rich in anthocyanins and tannic pigments.

4.2.4 Considerations

The present activity was conducted in collaboration with the entire Enology group of the Department of Agricultural Sciences. The results acquired found editorial placement in an international scientific journal in the form of an original research article entitled "How acetaldehyde reacts with low molecular weight phenolics in white and red wines". The article, to date, has been cited by different groups of Authors for the development of new original research articles.

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CHAPTER 5

AGING, ASTRINGENCY PERCEPTION

Wine mouthfeel and astringency play a vital role in shaping organoleptic quality. As a key factor in wine products, it drives wine quality and consumer preference [Kang et al. 2019]. A balanced level of astringency is the requisite of high-quality red wines; while in excess, it detracts from other sensory precepts. Astringency has long been associated with colloidal interactions between tannins and salivary proteins, and a depletion in the lubricating salivary film. Astringency is a complex sensation that has several contributing sub-qualities that may each have different physicochemical origins. In addition to astringency, the mechanisms of other wine mouthfeel attributes are yet to be fully understood.

Wine mouthfeel is defined as "the group of sensations characterized by a tactile response in the mouth" [DeMiglio et al., 2002]. It is as important as well as wine appearance, aroma, and taste, in shaping the wine organoleptic quality [Laguna et al., 2017]. It includes a broad term of sensations like astringency, body, burning, balance, pricking, warmth, viscosity, etc. [Gawel et al., 2000; Jackson, 2016]. During wine consumption, mouthfeel is affected by not only tannin but also matrix composition including ethanol, acidity, polysaccharide, and their interaction with the oral components [Fontoin et al., 2008; Gawel et al., 2016; Laguna, et al., 2017; Ramos-Pineda et al., 2018; Rinaldi et al., 2012]. Wine mouthfeel encompasses particularly complex and multidimensional sensory experience with over 30 different astringency sub-qualities while more than 20 other mouthfeel terms have been identified and distinguished sensorially and frequently used by wine tasters [Gawel et al., 2000].

Astringency is one of the most investigated wine mouthfeel sensations by far. It also exists and plays a significant role in the sensory experience of a wide range of foods and beverages, including wine, tea, soymilk, coffee, fruits, nuts and legumes [Bajec & Pickering, 2008]. Astringency is typically associated with plant-based polyphenols, which undergo colloidal interactions with salivary proteins during oral consumption [Jöbstl et al., 2004]. The formation of colloidal polyphenol-protein aggregates and loss of a lubricating salivary film have been considered the main mechanism eliciting this sensation [Schöbel et al., 2014; Upadhyay et al., 2016] but, it can still arise without such interactions occurring; that suggests astringency is a much more complex sensation that is not dependent on a single physical or chemical mechanism (oral epithelial cells or

trigeminal innervation may be involved) [Payne et al. 2009; Rossetti et al. 2009; Kurogi et al. 2011; Carpenter, 2013; Schöbel et al., 2014].

5.1 ROLE OF SALIVA

In the oral cavity, saliva is important for speech, chewing, lubrication during swallowing and initiation of digestion; it also represents a protective barrier for teeth and soft tissues [Varga, 2012]. Plays a crucial role in the oral processing of food, contributing to the structural breakdown of the food and its sensory perceptions. Food oral processing has drawn extensive attention in recent years and become a new research frontier of the Food Science discipline [He et al., 2022]. Generally speaking, oral processing of food consists of three major physiological aspects: mastication with teeth, tongue movements, and saliva secretion. Through different enzyme activities expressed by the complex protein pattern, saliva plays a key role not only in the eating process but also in sensory perception due to its instantaneous interaction with food and its components [Mosca et al. 2019; Zhang et al 2020]. The hydrolytic actions on nutrients promoted by salivary enzymes, the dilution effect of some food components, and especially the formation of complexes between food components and salivary proteins are mechanisms that, in addition to bringing changes in the perception of food texture, modulate the perception of flavor. [Van Eck et al. 2021; Zhang 2022].

5.1.1 Saliva Composition

Saliva is normally a colorless, dilute fluid with a density ranging from 1002 to 1012 g/L and with a pH of about 6.64, although it varies depending on the level of CO_2 in the blood [Guyton 1986]. It can be considered a complex biological fluid secreted incessantly in the oral cavity (daily production, in healthy individuals, of up to 1500 ml) and classified as stimulated and unstimulated saliva, with very different compositions and physicochemical properties [Humphrey and Williamson, 2001]. Generally, saliva is composed mainly by water (up to 99.5 percent when stimulated), and by a small but important percentage of other compounds, such as electrolytes, proteins, glycolipids, and carbohydrates [Van Eck et al. 2020]. Among the inorganic constituents, Na^+ and K^+ (and perhaps Ca^{++}) are the main cations of osmotic importance in saliva, while the main osmotically active anions are Cl^- and HCO_3^-. Although the percentage of total protein in saliva is low compared with serum, specific proteins, such as the enzyme α-amylase, are synthesized in salivary glands and may be present in saliva in higher levels than in serum. Other organic components of saliva include maltase, serum albumin, urea, uric acid, creatinine, mucin, ascorbic acid (vitamin C), several amino acids, lactate, and some

hormones such as testosterone and cortisol [Guyton 1986]. In addition, gases such as CO_2, O_2 and N_2 and immunoglobulins such as IgA, IgG and IgM are also present in saliva. Salivary levels of K^+, Ca^{++}, urea, uric acid and aldosterone are highly correlated with plasma levels. As for the physiological significance of other saliva constituents, such as trace minerals, epithelial growth factor, neural growth factor, several enzymes and some proteins (kallikreins and calmodulin), remains unknown [Chicharro et al. 1998]. In addition to the electrolytes mentioned above, these components also include phosphate, SCN^-, Mg^{++}, F^-, urea, ureic acid or maltase, enzymes, minerals and gases (CO_2, O_2 and N_2), and proteins [Roblegg et al. 2019]. All these substances determine the functional properties of saliva by highlighting its difference from water. More specifically, saliva is a complex mixture of water, mucins, electrolytes, proteins, lipids, urea, ammonia, and food residues [Humphrey and Williamson, 2001]. By virtue of both its compositional and physicochemical characteristics, saliva can be considered an aqueous barrier to the more viscous mucus barriers of gastrointestinal tisuues and has a mucin concentration of about 200 μg/mL [Rayment et al. 2000].

5.1.1.1 Saliva proteins

Salivary proteins, even if represent a small fraction of the composition of saliva, are extremely complex in their composition [Mosca and Chen, 2017], a study of the salivary proteome identified more than 2,500 different proteins [Sanguansermsri et al. 2018]. Moreover, the complexity of saliva is enriched by individual variations in both composition and properties and sensitivity to many influencing factors, such as health status, lifestyle, and dietary habits [Louro et al 2021]. The percentage of total proteins in saliva is low in comparison with serum, specific proteins, such as the enzyme α-amylase, are synthesized in the salivary glands and may be present in saliva in levels exceeding that of serum. The proteome of whole saliva, in contrast to that of serum, is highly susceptible to a variety of physiological and biochemical processes. First, salivary protein secretion is under neurologic control, with protein output being dependent on the stimulus. Second, extensive salivary protein modifications occur in the oral environment, where a plethora of host- and bacteria-derived enzymes act on proteins emanating from the glandular ducts. Salivary protein biosynthesis starts with the transcription and translation of salivary protein genes in the glands, followed by post-translational processing involving protein glycosylation, phosphorylation, and proteolysis. This gives rise to salivary proteins that are grouped in families, consisting of structurally closely

related family members. Once glandular secretions enter the non-sterile oral environment, proteins are subjected to additional and continuous protein modifications, leading to extensive proteolytic cleavage, partial deglycosylation, and protein-protein complex formation. All these protein modifications occur in a dynamic environment dictated by the continuous supply of newly synthesized proteins and removal by swallowing.

Saliva contains a wide variety of secreted proteins, including a-amylase, an enzyme involved in the digestion of starch; lysozyme, peroxidase, immunoglobulins (IgA), many additional proteins that have antibacterial and/or antiviral properties and mucins, which are multifunctional glycoproteins involved in mechanical protection and prevention of dehydration of the oral epithelia, as well as in lubrication for solid food and trapping of microorganisms. Most of the proteins in saliva are secreted by the acinar cells. Salivary proteins exhibit vectorial transport from the rough endoplasmic reticulum, where they are synthesized, through a succession of membrane-bounded compartments including the Golgi complex, condensing vacuoles, and secretory. The secretory granules migrate to locations within the cell close to the apical membrane prior to the release of their contents into the acinar lumen.

Protein compositions are highly variable between subjects and depend on many factors, such as age, diet, medication and/or pathologies and sensory stimulation during food intake [Vandenberghe-Descamps et al 2016]. In any case, apart from water, saliva is composed mainly of proteins. In fact, it contains thousands of different proteins and peptides. These are either secreted by the salivary glands or originate from the crevicular fluid (an exudate of plasma reaching the oral cavity through the gingival crevices), epithelial cells, oral microbiota, or food remnants. The estimation of the proportions of the different proteins suggests that the most abundant proteins are those secreted by salivary glands, such as mucins, α-amylase, cystatins, histatins, statherin, and Proline Rich Proteins -PRPs-, together with immunoglobulins and albumin [Messana et al. 2008]. PRPs are of special interest for this thesis because of their high affinity for tannins. They represent up to 70% of proteins secreted by the parotid glands and 20−30% of proteins in whole saliva. Fragments of PRPs also represent a large part of the adult saliva peptidome and most oligopeptides identified in human infant saliva [Vitorino et al. 2009].

Alpha-amylase from human saliva

Salivary alpha-amylase is a major component of human saliva and is one of the most abundant and important components in saliva, it has been extensively investigated for its

functional roles in eating and sensory perception [Zhang et al. 2022]. It not only plays a role in the initial digestion of starch but is also involved in the colonization of bacteria involved in early dental plaque formation. Human salivary α-amylase (Figure 5.1a) is composed of 496 amino acid residues, one calcium ion (Figure 5.1b), one chloride ion (Figure 5.1c) and 170 water molecules [Ramasubbu et al. 1996].

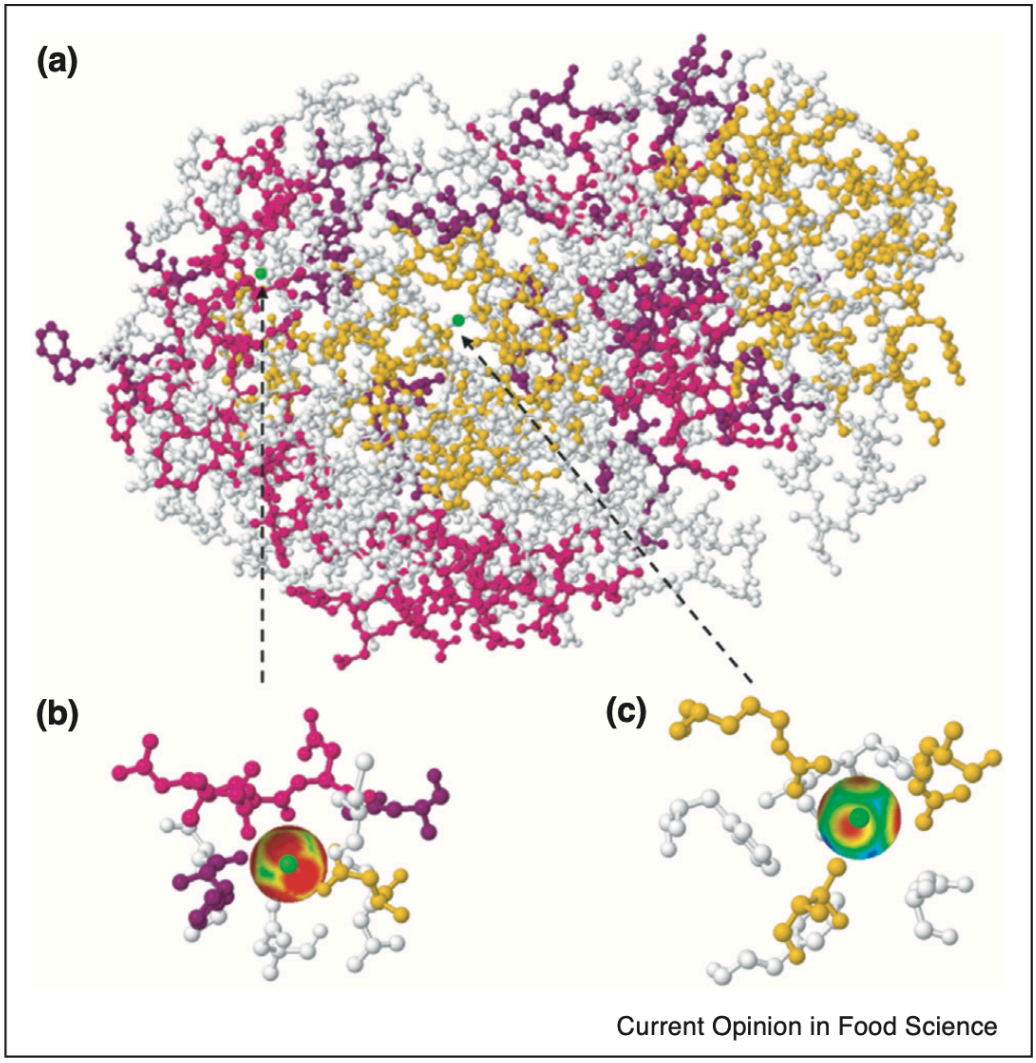

Figure 5.1 *a-c Three dimensions (3D) structure of human salivary amylase (a), the calcium ion (b), the chloride ion (c). All the 3D views were exported from the worldwide Protein Data Bank (PDB) Nature Structural Biology with PDB ID of 1SMD. The 3D views were presented by choosing Style in Ball and stick, Color scheme in Structure.*

Salivary amylase is a monomeric calcium-binding protein with a single polypeptide chain. Its 3D-structure consists of three domains, A, B and C. Domain A has a (β/α)8

barrel structure, domain B has no definite topology, and domain C has a Greek-key barrel structure. The calcium ion is bound to Asn100, Arg158, Asp167, His201 and three water molecules. The chloride ion is bound to Arg195, Asn298 and Arg337 and one water molecule. A highly mobile glycine-rich loop 304-310 acts as a gateway for substrate binding and is involved in a 'trap-release' mechanism in the hydrolysis of substrates. The active site is located at the C-terminal end of the central β-barrel, and the residue Trp58 has been shown to be critical for enzyme activity. The C domain is loosely linked to the rest of the molecule (Ramasubbu et al 1996).

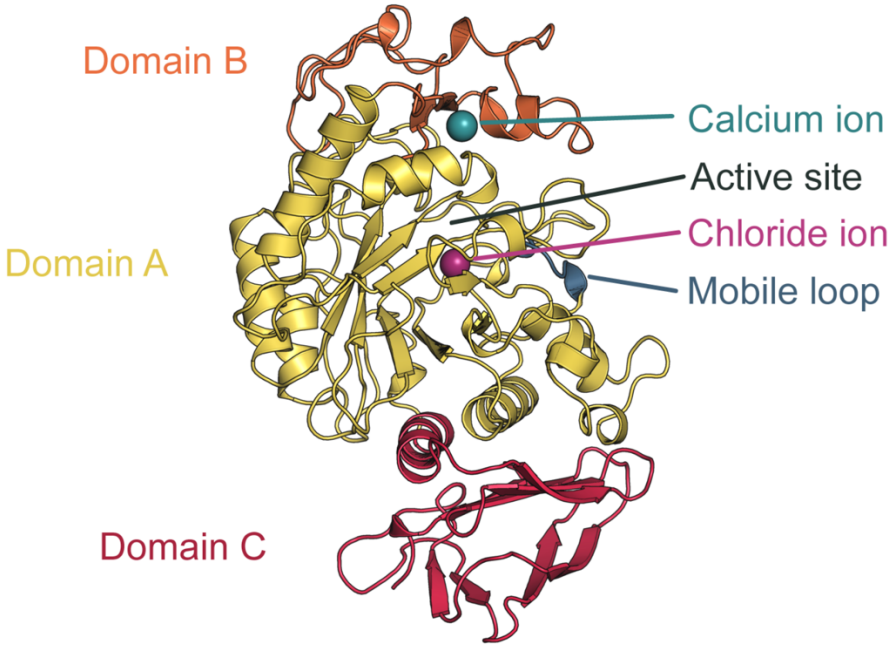

Figure 5.2 *Human salivary amylase (PDB 1SMD). Colours are as follows: domain A (yellow), domain B (orange), domain C (red), calcium ion (green), chloride ion (magenta), mobile glycine-rich loop (dark blue)*

How such a structure and its variation influence its functions remains a very interesting question to biological and sensory studies. Various techniques, including circular dichroism, synchronous fluorescence, fluorescence spectroscopy, ultraviolet-visible spectroscopy assay, and so on, have been proved for such purposes.

Salivary α-amylase (SA) performs two functions during food oral processing that both have a significant impact on sensory perceptions. SA hydrolyzes starch components and leads to the structural breakdown of food during oral processing [Laguna et al. 2021]. SA has a retention effect on taste and aroma compounds by providing binding sites for small molecules [Munoz-Gonzalez et al. 2021]. For the hydrolysis function, SA has the greatest effect on starch-based foods when compared to its effects on other kinds of foods. In the

aspect of retention effect, SA tends to interact with hydrophobic flavor molecules within foods [Zhang et al. 2022].

Salivary α-amylase (SA) can also provide numerous binding sites for small molecules, which makes it an effective modulator for aroma compounds release. SA binds with hydrophobic aroma compounds and decreases their release rates, which results in prolonging the retention time and influencing the aroma perception. Previous studies have confirmed that the release rate of beer aroma compounds such as ethyl hexanoate and linalool decreases in the presence of SA, which results in a decreased fruity perception in beer [Ramsey et al. 2020]. The interaction between SA and aroma compounds highly depends on hydrophobicity. A lower release rate has been observed for aroma compounds with higher log P ($2 < \log P < 5$) [Piombino et al. 2019]. However, the combinations between SA and polyphenols within wine can increase the release rate of aroma compounds [Pittari et al. 2021]. It was also found that the release of ethyl hexanoate volatility increased above 60% compared to that of the control with no presence of polyphenols [Perez Jimenezet et al. 2020]. However, the interaction mechanisms of SA with aroma compounds are still largely debatable. In terms of binding behavior, the interactions between SA and aroma compounds are not only influenced by the SA concentration, but also by the food and wine matrix.

5.1.2 Kinetics of saliva proteins tannins interactions

Two families of salivary proteins, proline-rich proteins (PRPs) and histatins, are generally recognized as tannin-binding salivary proteins (TBSPs). However, there is evidence for the presence of other, different types of proteins. These are characterized by their high affinity to tannins, but they differ greatly in terms of molecular size, amino acid composition, structure, and taxonomic distribution in mammals. PRPs were first detected in human saliva [Mandel et al., 1965]. They have also been found in the saliva or salivary glands of several laboratory animals and wildlife (Table 1). In these mammals, PRPs are thought to be the most prevalent group of proteins in the saliva; PRPs account for 70% of all salivary proteins in humans [Mehansho et al., 1987]. TBSPs in mammalian saliva suggest that these proteins participate in the maintenance of oral homeostasis, counteract the effect of tannins, and/or trigger the sensation of astringency, which signals a high tannin concentration in food. Regarding the impact of the phenolic structure on tannin–protein interactions, the affinity of proanthocyanidins for proteins increases with the number of constitutive units and their level of galloylation [Ricardo-da-Silva et al. 1991].

The presence of a third hydroxyl group on the B-ring has also been reported to increase the affinity of tannins for proteins [Siebert et al. 1999]. Indeed, the number of hydroxyl groups increases the stability of the tannin-protein interaction via the establishment of hydrogen bonds, their positions are also important [Bacon et al. 1998]. The establishment of interactions also seems to depend on the polyphenol spatial configuration [McManus et al 1985]. For example, molecules with flavan-3-ol monomers linked via C4–C6 linkages have a higher affinity for proteins than their isomers with C4–C8 linkages [Laurent et al. 2007]. Moreover, *cis*-2,3-flavan-3-ols have a slightly higher affinity for proteins than *trans*-2,3-flavan-3-ols [Morzel et al. 2002]. PRPs have a higher capacity to protect proteins from aggregation by tannins than other salivary proteins. This capacity has been attributed to their intrinsically disordered conformation and to their tandem repeat sequences composed of proline clusters surrounded by flexible amino acids. The rigid proline clusters provide stable binding sites to initiate the interaction with tannins, while the flexibility of surrounding amino acids allows structural rearrangement, stabilizing the interaction through the establishment of additional hydrogen bonds between the peptide chain and tannins [Morzel et al. 2022]. This structural rearrangement may explain the higher affinity of long chain PRPs compared to proline-rich peptides. The several tandem repeat sequences also provide several binding sites, allowing several stoichiometries of interaction. Once soluble noncovalent complexes are formed, bound tannins, which are described as multidentate ligands, may bridge tannin·PRP complexes, leading to the formation of aggregates. At least three tannins per protein is required to form aggregates. PRP−tannin aggregates grow with tannin concentration up to their precipitation [Ferrer-Gallego et al. 2015; Soares et al. 2018]. Beside PRPs, histatins have been reported to precipitate tannin more effectively than acidic PRPs (aPRPs) at pH 7.4, while acidic PRPs show a greater ability at pH 3.0 [Soares et al. 2018]. This difference may result from different mechanisms of interactions. Nevertheless, both histatins and aPRPs efficiently protected salivary amylase from tannin inhibition. Interaction of tannin with histatins has been reported to involved π−π stacking between the ring of aromatic amino acids and the phenolic ring of tannins. The same authors [Soares et al. 2018] evidenced that acid PRP, histatins, and statherins are more prone to be precipitated by tannins than basic PRP (bPRP) and glycosylated PRP (gPRP). For bPRP, this effect could be due to Coulombic repulsion between the bPRP−tannin soluble complexes decreasing the formation of aggregates, as bPRPs are highly charged at the acidic pH used in this study. Regarding glycosylated PRP (gPRP), the presence of glycosylation has been

reported to increase the solubility of the gPRP−tannin supramolecular structures compared to nonglycosylated PRPs [Morzel et al. 2022].

5.2 DOCKING AS TOOL TO UNDERSTAND SALIVA PROTEINS TANNINS INTERACTIONS

Molecular docking [Irwin et al. 2002] is a key tool in drug discovery and molecular modeling applications. The goal of ligand–protein docking is to explore the predominant binding mode(s) of a ligand when it binds to a protein with a known three-dimensional structure. In molecular docking, based on the protein structures, tens of thousands of possible poses of ligand are generated; the binding poses are evaluated by a scoring function (SF) [Jhoti and Leach, 2007], which guides and determines ligand poses. As the most important component of molecular docking, scoring functions have three major functions: the first is to determine the binding mode and site of a ligand binding to a protein [Hermann et al. 2007]; the second is to predict the absolute binding affinity between the protein and ligand in lead optimization; the third is virtual screening, which can identify the potential drug leads for a given protein target by searching a large ligand database [Seifert et al. 2007; Schneider, 2010]. Previous research has classified the scoring functions into three major classes: force field, empirical and knowledge-based SFs [Wang et al. 2013]. However, since current research has greatly improved the studies for scoring functions [Ain et al. 2015; Khamis et al. 2015], especially in protein–ligand interactions, it is necessary for us to review the recent scoring functions for protein–ligand interactions with new classification scheme [Liu and Wang 2015], which classifies the scoring functions into physics-based, empirical, knowledge-based and machine learning-based SFs.

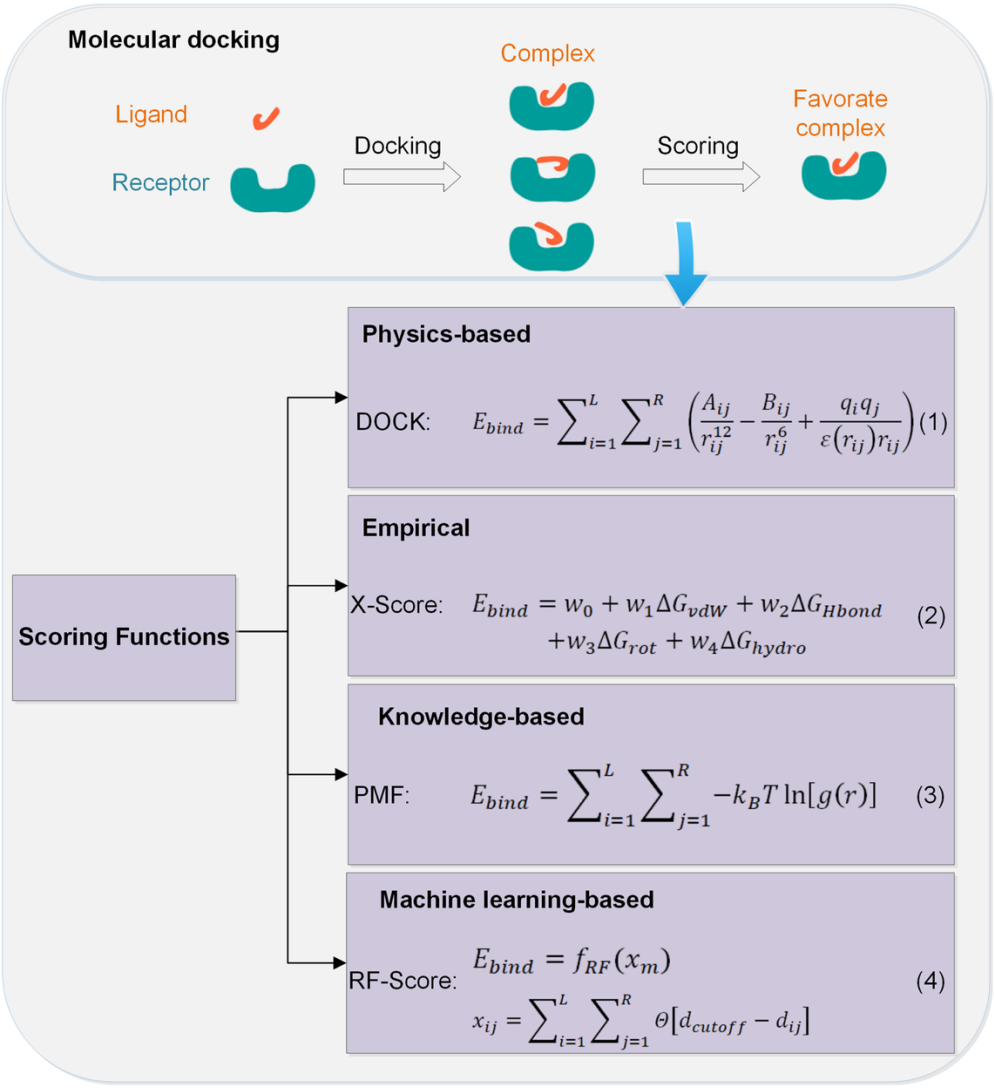

Figure 5.3 *Four categories of the scoring functions for protein–ligand docking [Li et al. 2019]*

The first three classical scoring functions are classified according to the types of feature items, and they mainly use the linear regression method. The fourth type of scoring function incorporates the nonlinear regression machine-learning method. The four types of soring functions are described in Figure 5.3 Here, SFs are introduced, and the scope of their applications discuss is discussed.

Figure 5.4 describes the physics-based SFs including the scoring functions based on force field, solvation models and quantum mechanics methods [Maurus et al. 2008].

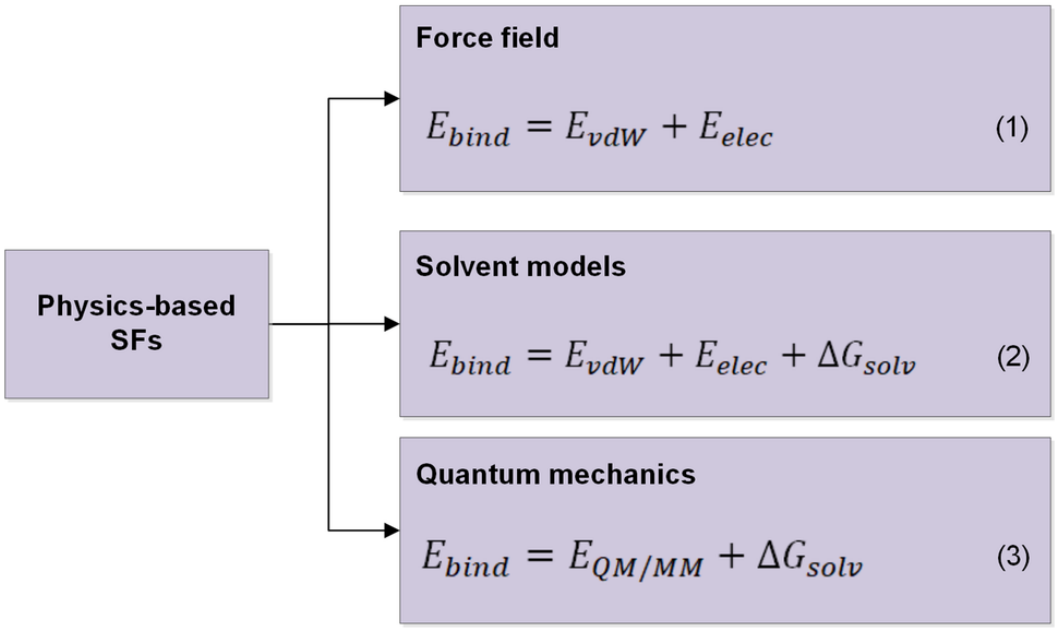

Figure 5.4 *The computational processing of knowledge-based SFs. Here, ρij(r)ρij(r) is the number density of the protein–ligand atom pair i − j at distance rr. ρ∗ijρij∗ is the pair density in a reference state and g(r)g(r) is the relative number density of atom pairwise i − j at distance rr. kBkB is the Boltzmann constant and TT is the absolute temperature [Li et al. 2019]*

The classical force field-based SF computes the binding energy by accumulating the van der Waals and electrostatic interaction between the protein–ligand atom pairs Equation 1 of Figure 5.4), which considers the contribution of enthalpy to energy [Meng et al. 1992]. Since it neglects entropy and solvent effect, the performance of the force field-based SF is not good enough [Huang et al. 2010]. Thus, the force field-based SF is improved by incorporating the torsion entropy [Kramer et al. 1999] of ligands and the solvation/desolvation effect described by explicit [Uehara and Tanaka, 2016; Kumar and Zhang, 2013] and implicit solvent models [Sun et al. 2014; Chen et al. 2016] (Equation 2 of Figure 5.4). However, the predictive accuracy for the binding energy is significantly subjected to the functional form of the potential energy and related parameters that are hard to locate because this type of scoring function is based on the force field.

Therefore, recent studies have developed the SF based on quantum mechanics (QM) to address the challenges of covalent interactions, polarization, and charge transfer in docking [Kulik 2018; Chaskar et al. 2017]. However, the QM-based SF has greater accuracy and computational cost than the force field-based SF. For this reason, a hybrid quantum mechanical/molecular mechanics (QM/MM) approach (Equation 3 in Figure 5.4) was developed to compromise the computational cost and predictive accuracy [Senn

and Thiel, 2009]. How to speedup QM/MM computing has become a hot research area. Though there are still many existing technical difficulties, QM has significant potential to replace the force field when evaluating the direct interactions between proteins and ligands, which represents the current novel research trend.

In general, physics-based SFs can directly compute the interactions between the atoms of protein and ligand. Physics-based SFs are appropriate to compute binding free energy between proteins and ligands with relatively greater predictive accuracy than other types of SFs due to the consideration of the enthalpy, solvation, and entropy. However, MM or QM models of physics-based SFs are computationally expensive.

Empirical Scoring Functions [Friesner et al. 2006; Zheng and Merz, 2011] estimate the binding affinity of a complex by summing up the important energetic factors for protein–ligand binding, such as hydrogen bonds, hydrophobic effects, steric clashes, etc. We usually employ a training set with known binding affinities to optimize the weights of the energetic factors for empirical SFs by linear regression analysis. An example of empirical SFs: X-score can be written as Equation 2 in Figure 5.3.

Empirical SFs are comprised of two research directions. One direction is how to employ a large and high-quality training data set to optimize the protein–ligand structures; the other direction is how to choose appropriate energy terms by stepwise variables and systematic selection regarding the target protein [Catana and Novel, 2007; Sotriffer et al. 2008]. Currently, empirical SFs are commonly employed by protein–ligand docking programs [Jain, 2003, Trott and Olson, 2010].

Although the empirical SFs decompose protein–ligand binding affinities into several individual energy terms, similar to physic-based SFs, they usually employ a flexible and intuitive functional form other than using the well-established models that physics-based SFs use. Because of their simple energy terms, these SFs are good at predicting binding affinity, ligand pose, and virtual screening with low computing cost [Li et al. 2014], but they are poorly suited for describing the relationship between binding affinity and the crystal structures and they encounter double-counting problems.

Knowledge-based SFs [Gohlke et al. 2000] derive the desired pairwise potentials from three-dimensional structures of a large set of protein–ligand complexes based on the inverse Boltzmann statistic principle. It is assumed that the frequency of different atom pairs in different distances is related to the interaction of two atoms and converts the frequency into the distance-dependent potential of mean force. Figure 5.5 describes the computational flow for knowledge-based SF.

The greatest advantage for knowledge-based SFs is compromising the computing cost and predictive accuracy compared with the physics-based and empirical SFs. However, it is difficult for knowledge-based SFs to locate the reference state. Currently, there are two classical strategies used to determine the reference state. One is approximating the reference state by the random distribution of atomic pairs in the training set [Neudert and Klebe, 2011]; the other is introducing the corrections item based on the first strategy to improve the accuracy of the knowledge-based SF, such as the volume factor correction method, physics-based iterative method [Huang and Zou, 2014; Forli and Olson, 2012], and so on. Currently, most researchers focus on extending the pairwise potentials to many-body potentials by introducing several new parameters [Lu et al. 2008; Xu et al. 2017;], which will significantly increase the predictive accuracy while we have difficulty to locate these newly introduced parameters.

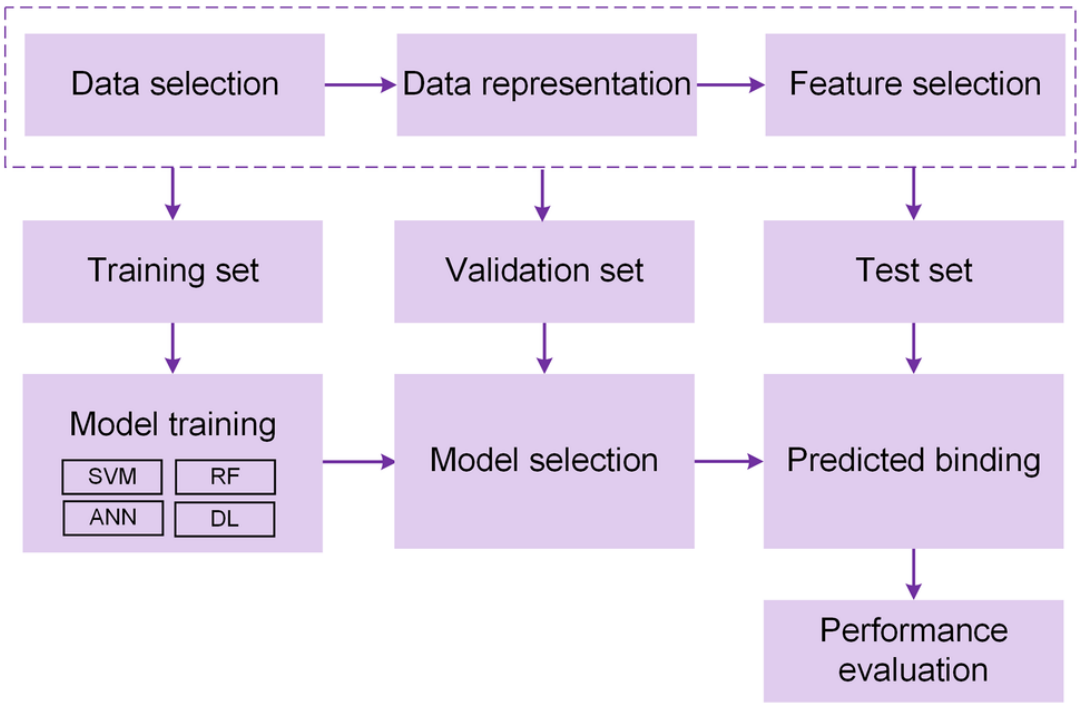

Figure 5.5 *Workflow of training a machine-learning-based SFs [Li et al. 2019]*

Since the training sets for these potentials only consist of structural information and are independent of the experimental binding affinity data, they can avoid possible binding affinity ambiguities caused by experimental conditions, indicating that knowledge-based SFs are suitable for binding poses prediction rather than the binding affinities.

Physics-based, empirical, and knowledge-based SFs are commonly used in previous studies [Gohlke et al. 2000; Meng et al. 1992]. These SFs have been successfully

incorporated into popular docking software, such as DOCK [Meng and Kuntz, 1992], SYBYL [Jones et al. 1997], Discovery Studio [Krammer et al. 2005], Schrodinger [Friesner et al. 2004], Autodock [Morris et al. 2015], Autodock vina [Trott and Olson, 2010], and so on. However, since each SF has obvious shortcomings, the docking software cannot perform well in every aspect.

Nevertheless, since it is time-consuming, how to speed up the physics-based SFs has already become a major research field [Steinmann et al. 2018; Chaskar et al. 2014]. Empirical SFs can obtain fast computing capacity, but their simple function form and the linear regression method (Equation 2 in Figure 5.4) may mask the relationship between binding affinity and crystal structure. Knowledge-based SFs can compromise the demand between speed and accuracy; theoretically extending the pairwise potentials to many-body potentials will help with improving the accuracy, but it is difficult for us to locate too many introduced parameters [Xu et al. 2017; Li et al. 2014; Park and Saitou, 2014]. Given that classical scoring function has its advantages and limitations, combining different scoring functions (hybrid SFs) is generally used to improve the accuracy of SFs. Examples of the hybrid SFs are MultiScore [Terp et al. 2001], GFscore [Betzi et al. 2006], SeleX-CS [Bar-Haim et al. 2009], VoteDock [Plewczynski et al. 2001] and so on. The hybrid SFs combine different individual scores by consensus strategies, such as vote-by-number, number-by-number, rank-by-number, average rank, linear combination, etc. Hybrid SFs are prone to perform better than standalone scoring functions. The reason may be that combining dissimilar types of scoring functions can compensate for the weaknesses of each. However, such scoring functions are obviously more time-consuming. For this reason, machine learning-based scoring function research [Ma et al. 2013; Cheng et al. 2012] has become a current hot research area. Although the descriptors for this type of SF are difficult to explain, machine learning-based SFs can outperform conventional SFs in practice. More importantly, we can increase the predictive accuracy for machine-learning-based SFs by enlarging the training data set.

Since increasing structural and interaction data will be accumulated from academic and industrial fields, it was considered that machine-learning SFs have the potential to dominate future SFs. However, there is no such universal SF that can work well for every molecular docking computation; thus, the scoring functions for a specific aim were developed [Zhang et al. 2017; Poli et al. 2018] by integrating different type of SFs in the distant future [Baek et al. 2017; Debroise et al. 2017]. Moreover, new types of features (intermolecular features, ligand-only and protein-only features) can be employed to

improve the performance of the SFs, and it is also very important to generate these SFs as open software for more researchers in this field to use.

5.3 THE CASE STUDY: COMPUTATIONAL EXPLORATION OF THE INTERACTIONS BETWEEN CATECHIN, MALVIDIN OR MALVIDIN-3-GLUCOSIDE-ETHYL-CATECHIN COMPLEX AND HUMAN SALIVARY *A*-AMYLASE

Anthocyanins are pigments that are chemically reactive to the pH of wine and therefore can easily react with a range of electrophiles and nucleophiles present in red wine during aging. One of the most important reactions involves the interaction between anthocyanins and flavan-3-ols (e.g., catechins and procyanidins), resulting in the formation of new, more stable complexes with different physicochemical characteristics [Dueñas, et al. 2006; Escribano-Bailón et al. 2001; Salas et al., 2004]. As highlighted in the past decades, the coloration of newly formed polymer pigments is less dependent on pH changes than their anthocyanin precursors [Bakker and Timberlake, 1997]. In addition, the new compounds are also involved in modulating astringency [Vidal et al., 2004].

The first reaction described in red wines was the condensation reaction between anthocyanins and flavan-3-ols mediated by acetaldehyde with the formation of purple pigments [Noniera et al. 2011].

However, if and how these reactions can affect astringency is not yet fully understood, also because of the connection or contribution of the various proposed mechanisms explaining wine astringency is still unknown [Canon et al., 2018, Huang and Xu, 2021]. The most accepted mechanism is based on the precipitation of salivary proteins by polyphenols. To date, the relationship with prolin rich protein PRPs, an important group of proteins subdivided into acidic, basic, and glycosylated PRPs, has been most investigated. PRPs associated with astringency are mostly basic and acidic [Canon et al., 2013]. In addition to PRPs, other relevant salivary protein families that have been linked to astringency include α-amylase [Guerreiro et al. 2012; Silva et al., 2017]. Although α-amylase is the most abundant enzyme in saliva, the interaction of novel formation complexes and single molecules with α-amylase appears to be less investigated than with other proteins.

Based on these considerations, the final phase of the doctoral activity was directed toward the study of computational tools [Yuriev et al. 2013] capable of investigating the interactions of anthocyanins and their polymeric pigments against salivary α-amylase; thus, an in silico molecular docking study was performed that could predict the preferred orientation of a receptor-bound ligand to form a stable complex. This computational

investigation provides several insights into the mechanisms of interaction of salivary α-amylase with anthocyanins and their polymeric pigments that may be useful in enriching knowledge regarding the mechanisms involved in salivary protein precipitation and thus initiate the development of new wine aging strategies.

5.3.1 Material and methods

5.3.1.1 Ligands

Catechin, malvidin- 3-glucoside, and their polymeric complex, as malvidin-3-glucoside-ethyl-catechin were used as ligands. The three-dimensional structures of ligands were obtained from the online data-bank "zinc database".

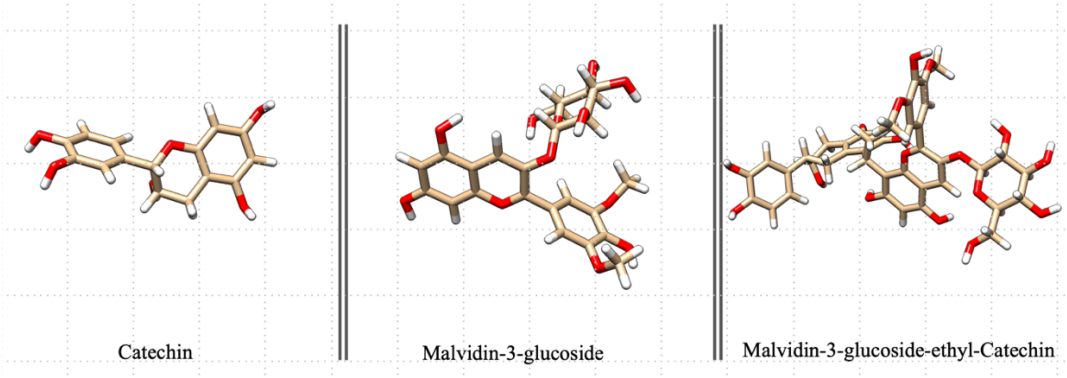

Figura 5.6 *Three-dimensional structures of Catechin, Malvidin-3-glucoside and their complex*

5.3.1.2 Protein target

Human salivary amylase (HSA) was used as receptors (Figure 5.2). The three-dimensional structure of HSA (PDB ID: 1SMD) was obtained from the online Protein Data Bank (PDB). Water molecule, solvent molecules, ions and crystallized ligand were removed from the crystal structure of HSA followed by addition of hydrogen atoms using Discovery Studio Visualizer.

5.3.1.3 Docking

Rigid-flexible molecular docking was performed by AutoDock Vina 4.2 . Preliminary blind docking studies were conducted that included the entire protein and showed a clear preference for docking at the hydrophobic core. 1SMD was then docked within a 9410696 Å box, using a grid spacing set at 0.182 Å, which contained the hydrophobic cleft. Nine binding modes were generated for the most favorable bindings. Maestro 2014 and swiss

model was used to plot their interactions. The standard protonation of amino acids at neutral pH was considered.

5.3.2 Results and Discussion

The docking studies showed that hydrogen bond (H-bond) interaction, played an important role in binding. The results indicated that hydrophobic interaction and electrostatic interaction were also vital in binding between ligands and both HPA and HSA, and the involved residues were given in Figure 5.7 – 5.9.

Nine predicted conformations of protein-ligand and binding energy as well as RMSD (Å) were obtained through AutoDock Vina. According to the principle of energy minimization [Guilbert et al. 2008], in other word, a lower binding energy meant that the ligand was easier to bind with the protein, mode 1 was considered as the best conformation of each ligand. The affinities of molecular binding between ligands and HSA were in the order catechin > malvidin-3-glucoside-ethyl-catechin > malvidin which indicated that polymeric pigments exhibited more binding capacities, stabilization and probably inhibition activity than their malvidin precursors. As to the interaction between ten ligands and 1SMD, catechin had the lowest binding energy followed by polymeric complex and malvidin.

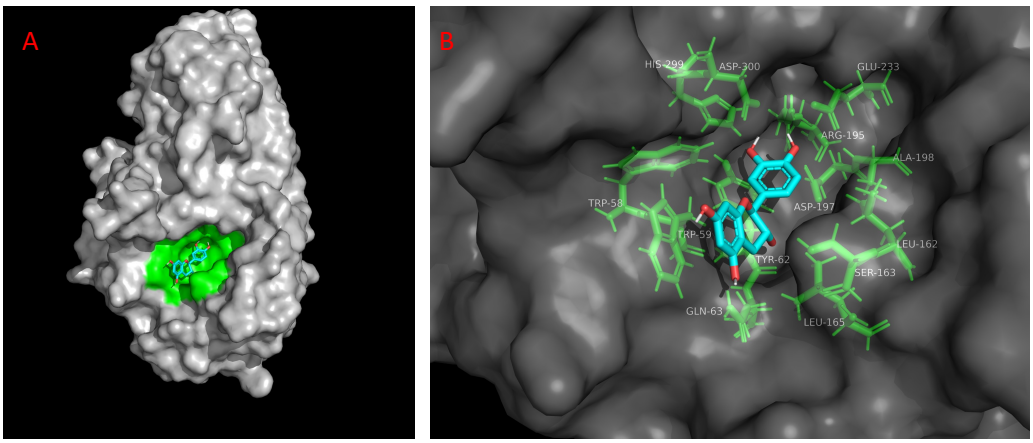

Figure 5.7 *(A) Surface representation of salivary alpha-amylase (PDB ID:1SMD) showing the ligand* ***(catechin)*** *at the predicted binding site (green) and (B) the detail of the predicted binding site showing the spatial arrangement of the catechin (cyan stick) and the alpha amylase residues (green sticks) with which the interaction is created.*
Binding Energy Score -8.6 kcal/mol
Residues: TRP58 TRP59 TYR62 GLN63 HIS101 LEU162 SER163 LEU165 ARG195 ASP197 ALA198 GLU233 HIS299 ASP300

Of the three ligands considered, catechin, in fact, is the one that establishes with the 1SMD protein an interaction characterized by the highest binding energy (Score -8.7). In detail, as reported in Figure 5.7, catechin interacts with the chain A of the human salivary 1SMD protein by establishing hydrogen bonds with 14 amino acid residues (TRP58 TRP59 TYR62 GLN63 HIS101 LEU162 SER163 LEU165 ARG195 ASP197 ALA198 GLU233 HIS299 ASP300).

On the other hand, the least performing binding energy was recorded between malvidin and human salivary protein 1MSD. In this case, an interaction characterized by an energy score of -8.0 kcal/mol with the involvement of 21 amino acid residues was recorded (Figure 5.8)

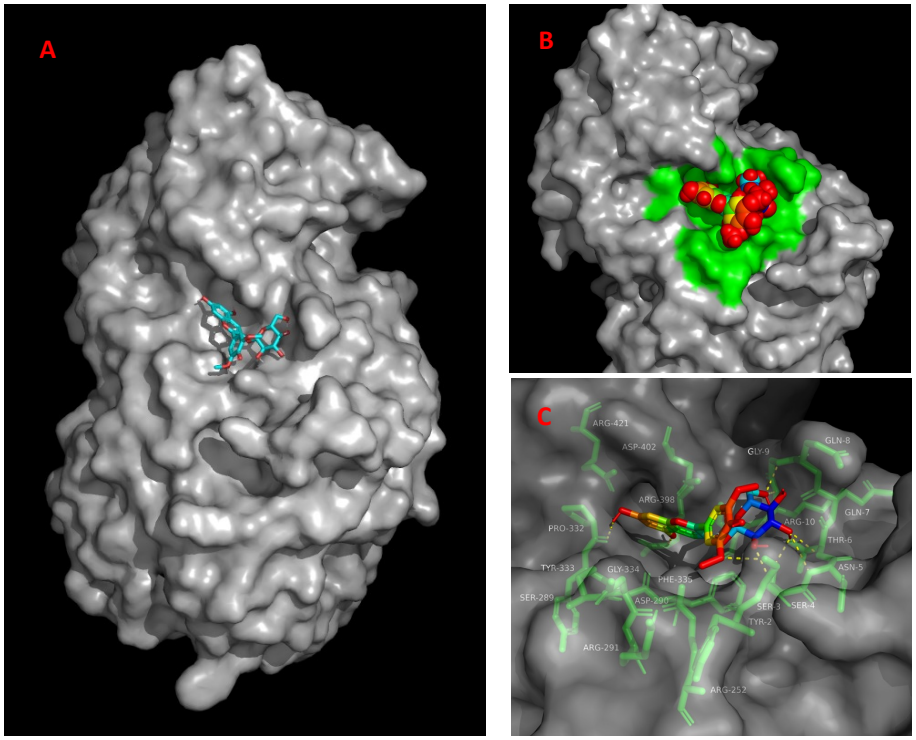

Figure 5.8 *(A) Surface representation of salivary alpha-amylase (PDB ID: 1SMD) showing the ligand (**malvidin**) at the predicted binding site. (C) Detail of the surface representation of the predicted binding site (green) and the ligand (spheres). (B) Spatial arrangement of the ligand (coloured sticks) and the alpha amylase residues (green sticks) at the predicted binding site. Hydrogen bounds are reported with yellow dotted lines.*
Binding Energy Score -7.8 kcal/mol
Chain A*: TYR2 SER3 SER4 ASN5 THR6 GLN7 GLN8 GLY9 ARG10 THR11 ARG252 SER289 ASP290 ARG291 PRO332 TYR333 GLY334 PHE335 ARG398 ASP402 ARG421*

In an intermediate position between catechin and malvidin is the malvidin-3-glucoside-ethyl-catechin complex. This compound establishes interactions with human salivary

protein always in the A chain involving 22 amino acid residues. Computational analysis recorded a binding energy characterized by a score of -8.3.

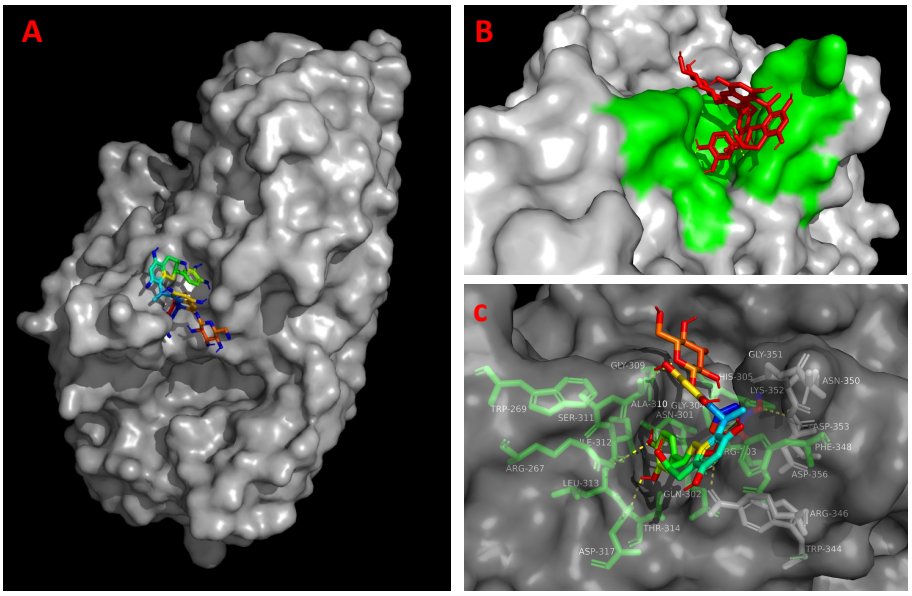

Figure 5.9 *(A) Surface representation of salivary alpha-amylase (PDB ID: 1SMD) showing the ligand (**malvidin-3-glucoside-ethyl-catechin**) at the predicted binding site. (B) Detail of the surface representation of the predicted binding site (green) and the ligand (red stick). (C) Spatial arrangement of the ligand (coloured stick) and the alpha amylase residues (green sticks) at the predicted binding site. Hydrogen bounds are reported with yellow dotted lines.*
Binding Energy Score -8.3 kcal/mol
Chain A*: ARG267 TRP269 ASN301 GLN302 ARG303 GLY304 HIS305 GLY309 ALA310 SER311 ILE312 LEU313 THR314 ASP317 TRP344 ARG346 PHE348 ASN350 GLY351 LYS352 ASP353 ASP356*

Docking results indicated that both the two monomers as well as their complex showed excellent abilities to actively interact with the 1SMD protein by influencing its conformation. However, the binding energy between the three ligands and the enzyme is significantly different exerting different influence on astringency. The results confirm that catechin has a greater effect on astringency than malvidin. In addition, the astringency produced by malvidin-3-glucoside-ethyl-catechin is greater than malvidin but lower than catechin.

5.3.3 Conclusions

The results showed that docking tools can offer useful results regarding the interactions between polyphenolic compounds and salivary proteins. The kinetics and evolution of polyphenolic compounds during wine aging combined with computational study can offer extraordinary information to help the development of appropriate aging strategies.

5.3.4 Considerations

The present activity was conducted in collaboration with the Randall Centre for Cell & Molecular Biophysics, within the Faculty of Life Sciences & Medicine at King's College London.

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CONCLUSIONS

The acquired results allow to enrich knowledge around the role of acetaldehyde and polyphenols in the wine aging, also considering the cornerstones around which modern consumer choices revolve, such as quality of health interest and sustainability. As far as the health aspect is concerned, adequately aged wines rich in phenolic compounds positively influence the expression of a family of proteins, the *sirtuins*, which promote actions of health interest. Noteworthy are the results that allowed the development of a new and rapid protocol to predict the evolution of phenolic compounds. A tool, this last, that was then adopted to evaluate the effect of a protective and sustainable agent, such as chitosan, on the oxidative evolution of the polyphenolic component. The understanding of such reactions between acetaldehyde and polyphenols has offered the most significant information regarding the oxidation process and has been useful for subsequent studies relating to the study of interactions between salivary proteins and newly formed polymeric pigments. A study of molecular docking between the malvidin-ethyl-catechin, malvidin and catechin and salivary alpha-amylase allowed to understand the impact of these reactions of acetaldehyde on the precipitation of salivary proteins. Data pointed out that the binding energy between the three ligands with salivary alpha-amylase is significantly different thus exerting different influence on astringency.

Therefore, the thesis work shows that docking tools can offer useful results regarding the interactions between polyphenolic compounds and salivary proteins. The study of the evolution of phenolic compounds during wine ageing, combined with a computational study, can offer remarkable insights for the development of appropriate ageing strategies.