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Innovative strategies to control oxidation in wine

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Esame finale anno 2016

Declaration

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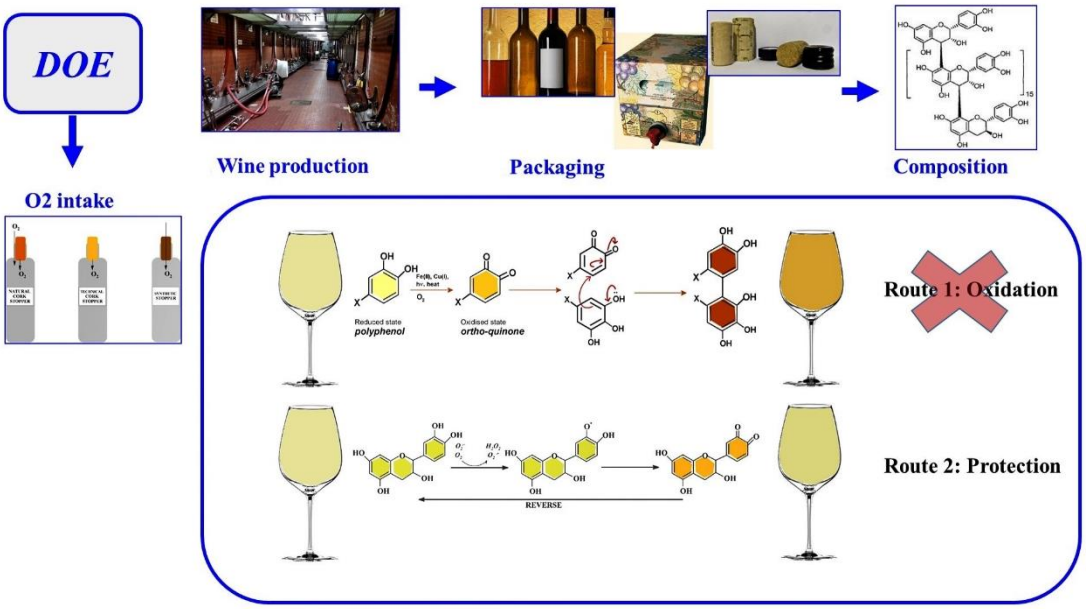
Summary

The topic of wine oxidation and the need of innovative strategies to prevent its extent were the subject of this PhD thesis.
The complexity of the oxidative chemical reactions occurring in wine during its conservation were highlighted, and multiple analytical approaches were used to provide a more comprehensive understanding of wine oxidation and to plan tailored strategies to avoid its occurrence.
The complexity of wine oxidation could be in a simplified manner attributed to the following main factors: wine composition, storage conditions, and oxygen exposure. An integrated theoretical and experimental approach was used, including study of chemical, physical and technological variables involved in wine production and storage.
Standard protocols currently used to analyse the wine composition were implemented, if needed, and the lab scale trials were coupled with monitoring real case study along the supply chain. In particular, the effectiveness of plant extracts (tannins) commonly used in oenology was also evaluate in order to better understand their antioxidant properties and to encourage an harmonized regulation of their use in winemaking.
The information provided and the scientific approach proposed in this thesis work may be useful in future work aimed to study practical implications and effective strategies to control oxidation in wine.



This PhD dissertation is dedicated to Valerio, Renata, Cecilia, the family that I was glad to have, and Francesco, the family that I was proud to choose.

This PhD dissertation is also dedicated to people who supported me improving my professional and human capacities, and several more people with whom I had the good fortune to come in contact during my life.

To conclude, this PhD dissertation is dedicated to Enea, the most beautiful flower of 2016, and to his wonderful parents.

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PREFACE

This PhD dissertation is organized in eight chapters. A preliminary literature review is presented, then research results are introduced separately and written according to the Food Chemistry (Elsevier) Journal style. The overview and research results are followed by a conclusive section including a general discussion on the major findings, limitations and future perspectives, as follows:

Chapter I GENERAL INTRODUCTION AND PROJECT AIMS

Chapter II LITERATURE REVIEW

A review of wine oxidation including causes, mechanisms and the role of oxygen management

Chapter III RESEARCH RESULTS

Monitoring the oxygen intake along the supply chain

Chapter IV RESEARCH RESULTS

Shelf life experiments.

Case study (A): Kinetic and thermodynamic approaches to the browning onset after bottling of selected Italian white wine varietals bearing different packaging;

Case study (B): the influence of selected closures in the evolution of oenological parameters of Muller Thurgau Italian white wine.

Chapter V RESEARCH RESULTS

Antioxidant effect of wine polyphenols in international red and white wines.

Chapter VI RESEARCH RESULTS

The antioxidant capacity of natural extract (tannins) in oenological conditions.

Chapter VII GENERAL DISCUSSION AND CONCLUSIONS

CONTENTS

7.1 BRIEF OVERVIEW

7.2 GENERAL DISCUSSION OF FINDINGS ACCORDING TO ORIGINAL OBJECTIVES

7.2.1 Objective I: Monitoring of the oxygen intake along the supply chain.

7.2.2 Objective II: Evaluate the impact of different packaging and closures in the shelf-life of wines.

7.2.3 Objective III: Determine the structure-activity relationship between polyphenols occurring in wine and their antioxidant capacity. Objective IV: Proposal of innovative analytical strategies to determine TPC and AA parameters in wine and oenological products.

7.2.4 Objective V: Highlight the potentiality of oenological additives (tannin) to assist the sulphur dioxide action thanks to their expected antioxidant properties.

7.3 MAJOR FINDINGS: LIMITATIONS AND NOVELTY VALUE – IMPLICATIONS. FUTURE PERSPECTIVES.

7.4 FINAL REMARKS

List of abbreviations

TAC	Total Antioxidant Capacity
AA	Antioxidant Activity
ROS	Reactive Oxygen Species
DPPH	2,2-diphenyl picryl hydrazil radical
GSH	Glutathione
ROH	Alcohols
OD	Optical Density
GAE	Gallic Acid Equivalent
TO	Total Oxygen
MW	Molecular Weight
DOE	Design Of Experiment
PET	PolyEthylene Terephthalate
PETA	Active PolyEthylene Terephthalate
EVOH	Ethylene-Vinyl alcohol
BIB	Bag in Box

Chapter 1

Introduction and Project Aims

Chapter 1: Introduction and project aims

1.1 Introduction

Commercial food and beverage could undergo severe and hazardous alterations during their shelf life, due to incorrect processes or microbial attacks; but also a slight variation of the properties of the product, although not harmful to health, may reduce consumers' confidence and address their preferences (Cleveland, Rojas-Méndez, Laroche, & Papadopoulos, 2016; D. Oliveira, Machín, Deliza, Rosenthal, Walter, Giménez, et al., 2016; Saxby, 1996). For this reason, strong emphasis is given to a proper conservation but also in exalting the distinctive benefit properties of food products. Wine is emblematic in this sense, being a product that belongs to an ancient tradition and becoming synonym of cultural identity and quality in more recent times (Lindh, Olsson, & Williams, 2016; Mora, 2016a, 2016b; Silva, Jager, van Bommel, van Zyl, Voss, Hogg, et al., 2016). The main concern in wine storage and trading regards its susceptibility to oxidation, induced by complex mechanisms mainly involving oxygen permeation/migration during processing and storage, and specific catalysers. These mechanisms affect sensory properties of red and especially white wines inducing the loss in varietal aroma, the development of off-flavours like farm-feed, woody-like and kerosene (Jackson, 2009; Paul A Kilmartin, 2009), and the colour tuning from red or pale yellow to brownish hues: this phenomenon is commonly referred as *browning* (Li, Guo, & Wang, 2008; Sioumis, Kallithraka, Makris, & Kefalas, 2006). There are several factors affecting wine oxidation; some of them, referred as external factors, are related to processing, packaging and storage conditions: in 2009 AWRI estimated that 48% of the wines that were rated as faulty in wine competitions exhibited off-flavours that were developed as a result of the erroneous management of oxygen (Ugliano, Kwiatkowski, Travis, Francis, Waters, Herderich, et al., 2009). Another crucial issue to explain wine susceptibility to oxidation is related to the intrinsic properties of wine, including the content in natural antioxidant and catalysts (pro-oxidants), its alcohol content and pH value (Lingua, Fabani, Wunderlin, & Baroni, 2016). Several reviews focused to both the chemistry of wine oxidation (Li, Guo, & Wang, 2008; Waterhouse & Laurie, 2006) and the impact of storage conditions (Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011; Ghidossi, Poupot, Thibon, Pons, Darriet, Riquier, et al., 2012; Karbowiak,

Gougeon, Alinc, Brachais, Debeaufort, Voilley, et al., 2009). It follows that the ability of effectively measure the antioxidant capacity of wine and the impact of processing and storage conditions are critical issues in oenological chemistry. A critical approach to the problem of oxidation provides two different moments, (i) the first includes the identification of critical points for the intake of oxygen during the production process and the management of the oxygen intake following bottling, which can be summarised as shelf-life durability; (ii) the second provides the measurement of the total antioxidant capacity (TAC) of wine. Several analytical approaches have been developed to measure the attitude of wine to prevent or limit oxidative damages; the main mechanism to be monitored is provided by the formation and scavenge of reactive free radicals (C. M. Oliveira, Ferreira, De Freitas, & Silva, 2011). Nevertheless, due to the complexity of the matrix wine and to the difficulty in reproducing processes involving radicals on a laboratory scale, there are several approaches that are followed in the current practice, each offering specific advantages and some limitations (Fogliano, Verde, Randazzo, & Ritieni, 1999; Paul A. Kilmartin, Zou, & Waterhouse, 2001; Versari, Parpinello, Scazzina, & Rio, 2010; Yilmaz & Toledo, 2006).

1.2 Project aim

The goal of this PhD study titled "Innovative strategies to control oxidation in wine" was to elucidate the main factors affecting the oxidation in wine, as well as to propose alternative and innovative strategies to monitor its potential occurrence, and to limit the detrimental effects in commercial products. An accurate understanding of the problem associated with a good practice of the cellar and with the availability of fast and accurate tools for supply chain monitoring cannot take effect in each individual case, but can reduce the impact of oxidative degradation in the global wine market. A multiple analytical approach allowed to measure oxygen intake, catalysed chemical processes, occurrence of wine antioxidants and their activity, which are the main factors affecting wine oxidation. In order to achieve this aim, several experiments were designed in order to reach five specific **objectives**:

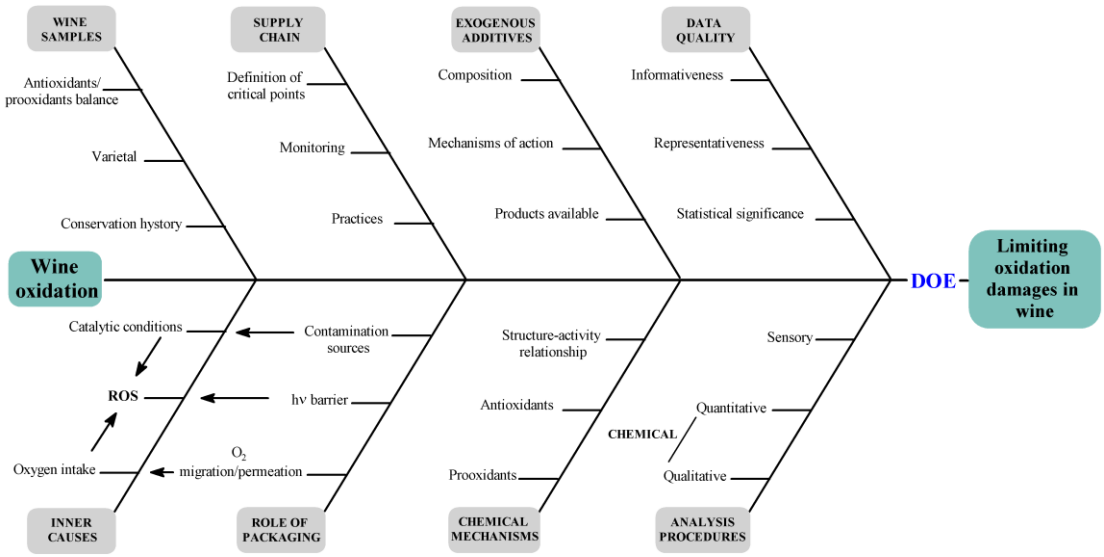
I. To identify best practice approach in order to optimize oxygen management during winemaking and storage;
II. To enhance the shelf-life of wines by using different packaging and closures;

III. To improve the antioxidant activity assay in wine;

IV. To elucidate the structure-activity relationship between main polyphenols of wine and their antioxidant activity;

V. To disclosure the antioxidant properties of oenological commercial tannins.

To conclude, the use of a representative sampling and multivariate models would enable to implement the information of this thesis for the creation of a more statistically representative database in the future.



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Chapter 2

A Review of Wine Oxidation

Causes, chemical species involved, role of gas permeation/migration, packaging

Chapter 2: A Review of Wine Oxidation: Causes, chemical species involved, role of gas permeation/migration, packaging

2.1 Introduction

A thorough understanding of the problem of wine oxidation is the key approach for the identification of appropriate strategies to prevent or limit its occurrence. Prediction of chemical oxidation following vinification is a complex issue due to the simultaneous contribution of different physical, chemical and technological factors.

One challenge for the systematic approach to the problem of oxidative stress in wine is to take into account that different chemical compounds and external factors may produce additive effects or result in a balance of antioxidant and pro-oxidant mechanisms (Mark P. Bradshaw, Cheynier, Scollary, & Prenzler, 2003; Hötzer, Henriquez, Pino, Miranda-Rottmann, Aspillaga, Leighton, et al., 2005). In this view, the scientific literature available on this topic could be ideally divided in two groups, the first one devoted to the elucidation of chemical reactions leading to oxidation by-products and wine alteration (Danilewicz, 2007; Andrew L. Waterhouse & V. Felipe Laurie, 2006; Wildenradt & Singleton, 1974), the second focused on the impact of the technological practices, mainly the gas permeation through selected packaging, on the occurrence of oxidation (Karbowiak, Gougeon, Alinc, Brachais, Debeaufort, Voilley, et al., 2009; Silva, Julien, Jourdes, & Teissedre, 2011; Tao, García, & Sun, 2014; Waters, Peng, Pocock, & Williams, 1996).

We assume that monitoring the evolution of wine during shelf-life provides information on the quality of winemaking process and on the impact of different packaging solution actually available. Although this approach is informative, we focus instead on the time occurring between vinification and bottling as the most significant in the prevention of the chemical oxidation. In this phase the monitoring has a deterrence function, enabling producers to adopt the most suitable strategies to protect their wines.

Chapter 2 is an updated and critical description of factors inducing oxidation in wine, based on the scientific literature available.

2.2 The chemical oxidation of wine and the role of oxygen

Figure 2.1 after Andrew L. Waterhouse represents the distribution of chemical classes occurring in wine (Waterhouse, 2012), with many compounds involved in wine oxidation at different levels. In a general perspective, we can consider a two-stage mechanism for wine oxidation. At the first step, the oxygen exert a selective action against wine minor components: the polyphenolic compounds act as pro-oxidant due to their low redox potential, and the simultaneous presence of dioxygen and solvated Fe(II) ions leads to the formation of aromatic quinones and hydrogen peroxide as by-products (**figure 2.2**). Oxidation by-products have different fates in the wine environment: quinones, bearing increasing stability due to the high electron delocalisation over the aromatic structure, can further stabilise their structure combining with thiols, or undergoing reverse processes mediated by SO_2 to rebuild the original hydroxylated form. Conversely, the hydrogen peroxide is involved in the Fenton mechanism, initiating the radical chain in the second oxidation stage: the oxygen molecule is activated by the progressive formation of species with increasing reactivity, bearing lower selectivity, which indiscriminately oxidise major constituents of wines (alcohols, acids) and produce aldehyde, ketones, adducts and brown pigments; the latter compounds are responsible for significant variation in the sensory properties of wine. In the next two sections the second stage of wine oxidation is analysed in detail according with formation of radical species, and subsequent alteration of the oenological substrate.

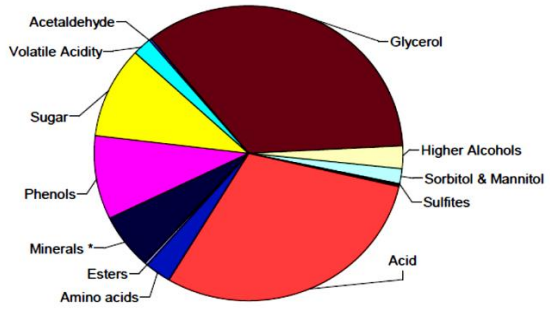

Figure 2.1. Schematic representation of wine constituents involved in oxidation after A.L.Waterhouse (2012).

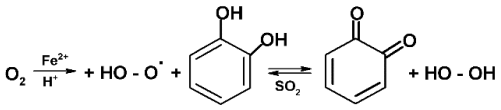

Figure 2.2. Formation of an aromatic quinone following the oxidation of an *ortho*-polyphenol (catechol).

2.2.1. Reactive oxygen species: ROS

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; this mechanism has been extensively described (Singleton, 1987; Danilewicz, 2003; Waterhouse & Laurie, 2006), and it is schematically summarised in **figure 2.3**. On a chemical point of view, the oxygen molecule exhibits a low reactivity with respect to organic substrates, being paramagnetic in its ground state (**figure 2.4**); there is a need of an additional energy supply for the oxygen in its triplet electronic state ($^3O_2$) to be converted in its singlet electronic state ($^1O_2$), which is highly reactive and evolves into chemical species bearing increasing reactivity; this energy supply is equal to the distance between the ground state and the $^1\Delta$ excited state of molecular oxygen, 22.4 kcal (Bradley & Min, 1992). The reactive oxygen species, commonly referred as ROS, includes oxygen radicals and non-radical derivatives characterised by high reactivity, like singlet oxygen, hydrogen peroxide (H_2O_2) and ozone (O_3) (Choe & Min, 2006; Singleton, 1987). The formation of singlet oxygen lowers the activation barrier for the reaction of oxygen with organic substrates, and initiates the formation of ROS; on a kinetic point of view, this process is highly competitive.

To exemplify the order of magnitude of the energies involving reactions between oxygen radicals and other reactive species, we can cite some studies about ROS reactivity on isolated systems. Christensen et al. (Christensen, Sehested, & Corfitzen, 1982) have reported an Arrhenius activation energy of 3.4 kcal mol^{-1} for the reaction of hydroxyl radicals with hydrogen peroxide, that are also the main oxidising agents following the progression of the radical chain wine; although the experiment was performed at pH 7.8, the authors noticed that the activation barrier was even lowered when moving toward lowest pH values. Again, the rate constants for the abstraction of an hydrogen atom by peroxy radicals was estimated by Ingold (Ingold, 1969) as $5x10^3$ mol^{-1} sec^{-1} when the hydrogen belong to a simple phenol, and $20x10^3$ mol^{-1} sec^{-1} when the hydrogen is abstracted from a methylated phenolic compound (2, 6-dimethylphenol).

ROS are unstable chemical species, bearing very short and vastly different lifetimes, which reflects their different reactivity. The superoxide anion ($O2^{\bullet -}$) exhibits a moderately long lifetime, ranging between 10^3-10^4 s, and also peroxyl radical (ROO•)

have a half-life in the time-scale of seconds (7 s); these species are highly selective and are able to diffuse in their medium to reach targeted molecules (Akaike, Ijiri, Sato, & Maeda, 1995; Sies & Stahl, 1995). On the opposite, the lifetime of other ROS is drastically reduced: the singlet oxygen ($^1O_2$) is characterised by a lifetime of 10^{-5} s, followed by the alkyl radical (•OR), 10^{-6} s. The hydroxyl radical (HO•) is the more unstable and reactive species of the radical chain produced by oxygen, with a lifetime in the scale of 10^{-9} s; it has been estimated in 1.97±0.26 ns using a picosecond fluorescence experiment (Schwarzwald, Monkhouse, & Wolfrum, 1987). The oxidative reaction of HO• with organic molecules in solution is diffusion-limited, taking place at the site of generation with high rate constants and any selectivity (Sies & Stahl, 1995; Steenken, 1987).

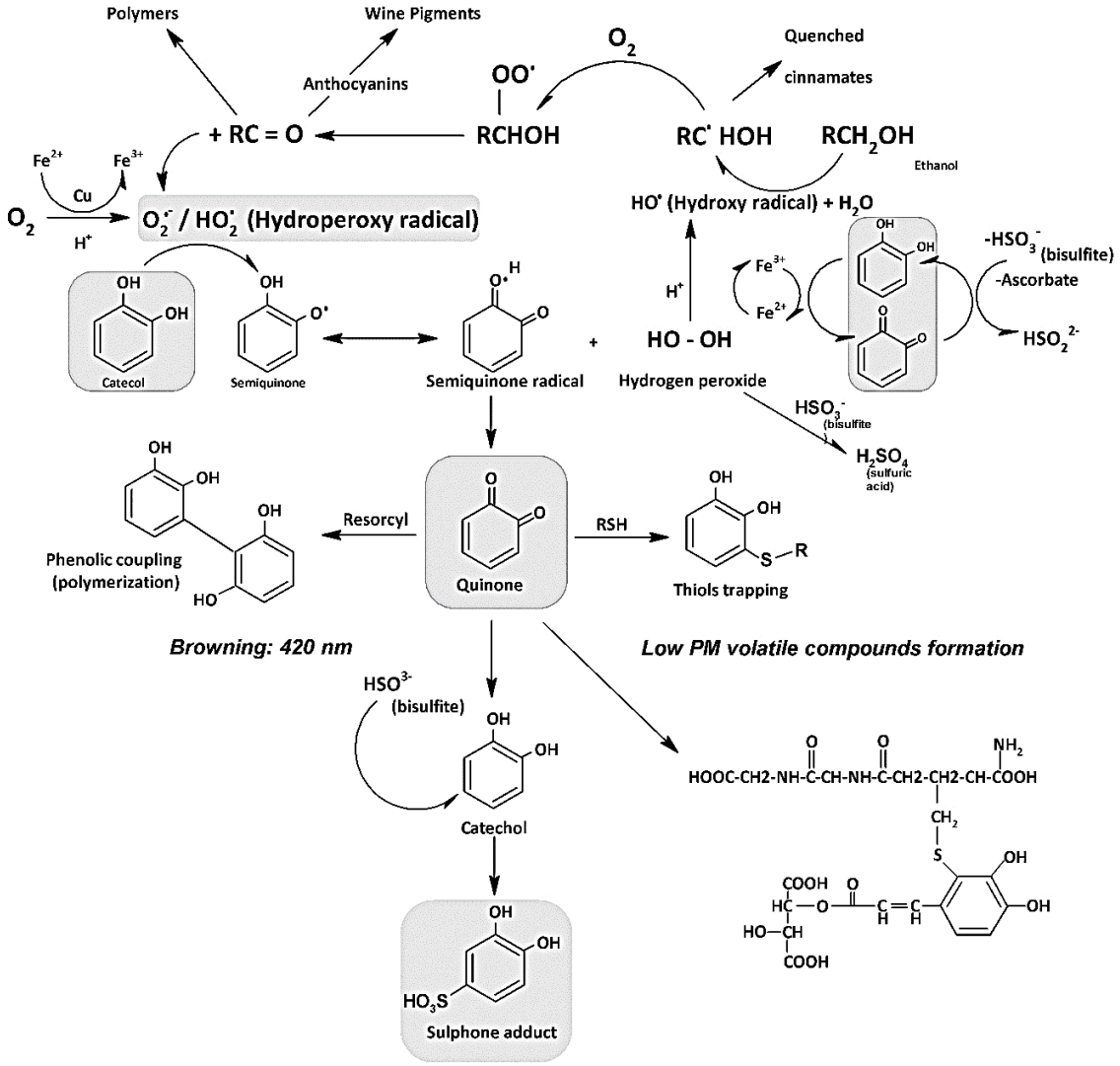

Figure 2.3. Simplified schematic representation of the oxidation mechanism and related by-products in wine.

Due to their high lability, the thermodynamic parameter of ROS, like the enthalpy of formation of reactive radicals ($\Delta H(R\bullet)$) and the molecular bond dissociation (D_o) of their precursors are routinely investigated in isolated systems. **Table 2.1** lists some of these values reviewed from the literature, when available, to provide comparative orders of magnitude for thermodynamic parameters of ROS and polyphenol species involved in wine oxidation. Data are extrapolated from different experiments, their direct comparison with related parameters in wine is made difficult by the influence of matrix effects occurring in real conditions.

Compounds	**Enthalpy of radical formation ΔH_{f0} (kcal mol^{-1})**	**Homolytic O-H dissociation energy D_0 (kcal mol^{-1})**	**Bibliographic references**
Hydroxyl radical R-OH > RO$^{\cdot}$ - H	8.85±0.07	101.76±0.07	(Ruscic al., 2002)
Alkil radical CH3COOH > CH3COO$^{\cdot}$ - H	-	112±3	(Wojciechowski & Ortiz de Montellano, 2007)
Hydrogen peroxide H2O2 > HOO$^{\cdot}$ - H	-	90±3	(Benson, 1965)
Peroxyl radical ROOH > RO$_2^{\cdot}$ - H	5	≈70 (range 86-88)	(Benson, 1965; Foti & Daquino, 2006)
o-benzoquinone	-23.1±4.1	(range 78-86)	(Fattahi et al., 2005; Foti & Daquino, 2006)
m-benzoquinone	6.8±4.1	(range 78-86)	(Fattahi et al., 2005; Foti & Daquino, 2006)
p-benzoquinone	-27.7±3.0	(range 78-86)	(Fattahi et al., 2005; Foti & Daquino, 2006)
catechol	-	70.9 (range 78-86)	(Foti & Daquino, 2006; Ordoudi et al., 2006)
protocatechuic acid	-	72.9 (range 78-86)	(Foti & Daquino, 2006; Ordoudi et al., 2006)
caffeic acid	-	69.6 (range 78-86)	(Foti & Daquino, 2006; Ordoudi et al., 2006)
vanillic acid	-	73.7 (range 78-86)	(Foti & Daquino, 2006; Ordoudi et al., 2006)
ferulic acid	-	72.4 (range 78-86)	(Foti & Daquino, 2006; Ordoudi et al., 2006)
2,2-diphenil pycryl hydrazyl	-	78.9 (N-H)	(Foti & Daquino, 2006)

Table 2.1. Thermodynamic parameters for ROS calculated under different analytical conditions (see references in the right column). (-) No literature values available.

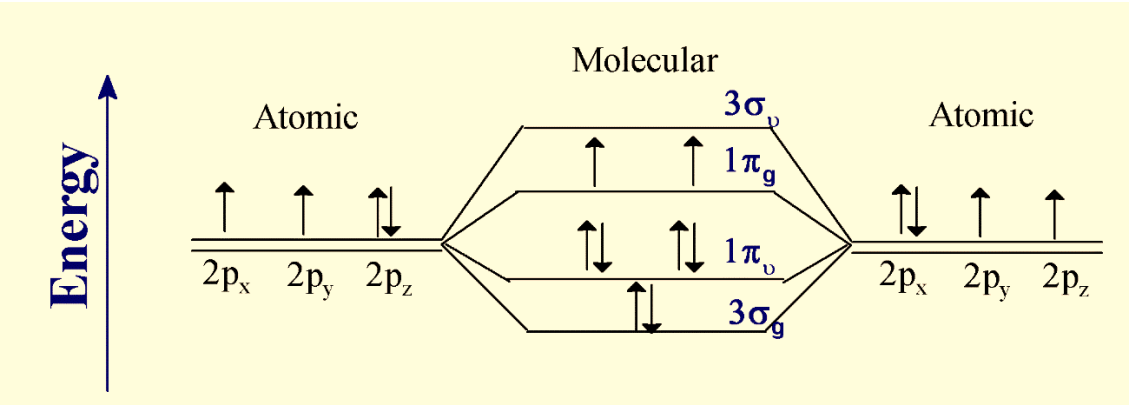

Figure 2.4. Schematic representation of the outer molecular orbitals of the paramagnetic $^3O_2$ molecule.

2.2.2. Oxidation of oenological substrates

The chemical oxidation, also called non-enzymic to distinguish from the enzymatic degradation occurring in grape and musts, happens in wine as a result of the interaction of chemical compounds and dissolved oxygen with specific catalysers, transition metals to cite the main ones, or in the presence of catalytic conditions, i.e. light exposure, high temperatures and pressures.

The first analytical evidence of an ongoing oxidative process is a colour tuning from purple-red or pale yellow to brownish hues, the so-called browning effect; while oxidative phenomena and colour variation are largely associated to a refinement of the product during the storage of red wine, in the case of white wines the occurrence of browning usually accompanies the development of undesired olfactory and taste sensations, among which the loss in varietal aroma and the development of off-flavours like farm-feed, woody-like, boiled potato, onion and kerosene (Jackson, 2009; Kilmartin, 2009). Polyphenols are the most susceptible to oxidation, and they possess the dual nature of antioxidants and pro-oxidants due to their redox properties (**figure 2.5**); although they are able to protect wine limiting the propagation of ROS, they are also considered the main starters of oxidative processes (Waterhouse, 2012). The flavonoid and phenolic acids bearing *ortho*-diphenol moieties, mainly caffeic acid and its esters, flavan-3-ols, anthocyanins and related polymers, and gallic acid, are considered to be the most susceptible to oxidation in the chemical browning process, and the levels of flavan-3-ols mostly affect the development of browning in white wines; among the possible oxidation paths promoted by these polyphenols there is the formation of quinone structures, which are

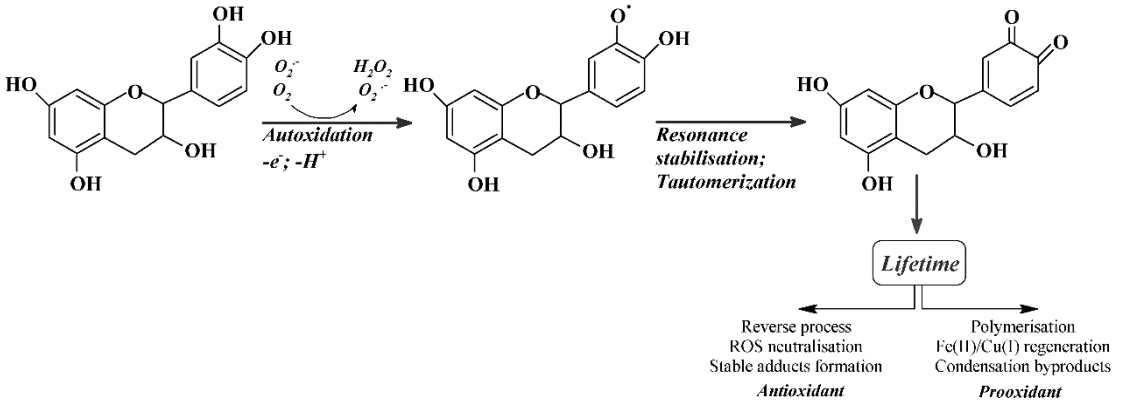

Figure 2.5. Formation of phenolic quinones and their possible reaction routes in oenological conditions.

pretty unstable and tend to stabilise through the formation of molecular adducts or through polymerising condensation processes (Danilewicz, 2011; Oliveira, Ferreira, De Freitas, & Silva, 2011; Waterhouse & Laurie, 2006). Other routes involve condensation between polyphenols acetaldehyde or glyoxylic acid (derived from the oxidation of tartaric acid), or the oxidation of ethanol produced by coupled oxidation of polyphenols or catalysed by transition metals which produce some acetaldehyde having an high impact in the olfactory perceptions of wine (Escudero, Asensio, Cacho, & Ferreira, 2002; Nykänen, 1986; Wildenradt & Singleton, 1974).

As illustrated in **figure 2.2**, the hydrogen peroxide is released during a preliminary oxidation of wine phenolics in the first stage of the oxidation onset; in this regard, the antioxidant function of SO_2 to its reaction with H_2O_2, which subtract the latter to further oxidative stages has been previously described (Singleton, 1987; Danilewicz, 2007). According to Danilewicz (2007), when the hydrogen peroxide is not bonded to sulphite ions added in wine, the presence of transition metals, mainly iron and copper, play a key role in wine oxidation, because H_2O_2 in association with ferrous ion (Fe^{2+}) tends to generate ROS following the Fenton or Harber-Weiss like reactions (Fenton, 1894; Fenton & Jackson, 1899; Koppenol, 2001), a well-known chemical mechanisms involving a stepwise formation of radicals with increasing oxidative capacity. Both mechanisms schematically shown in **figure 2.6** terminate with the production of hydroxyl radical ($HO^{\cdot}$), a powerful antioxidant non-selective with high reactivity (see section 2.2.1.), able to oxidise almost all the components of the wine (Danilewicz, 2007; Li, Guo, & Wang, 2008).

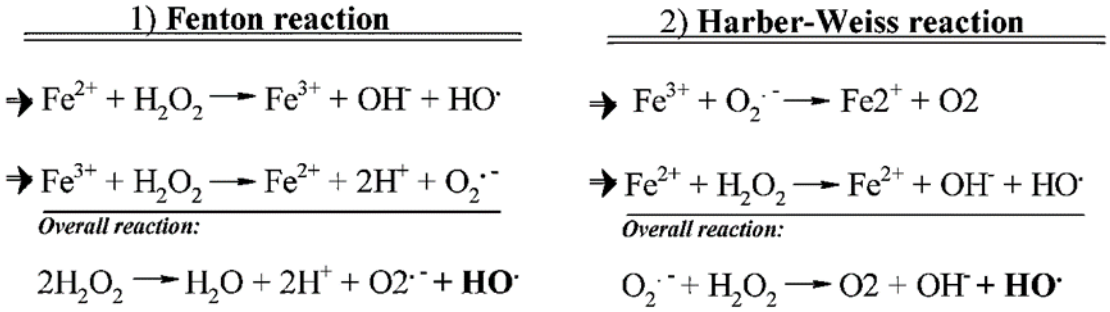

Figure 2.6. Fenton and Harber-Weiss reactions responsible for the initiation of the radical chain in wine.

The radical chain producing ROS requires the addition of four electrons to O_2:

(I). $O_2 + e^-, H^+ \rightarrow HOO\bullet\ (+H_2O) + e-$

(II). $HOO\bullet + e^-, H^+ \rightarrow H_2O_2$

(III). $H_2O_2 + e^-, H^+ \rightarrow \bullet OH$

(IV). $\bullet OH + H^+ \rightarrow 2H_2O$.

This radical chain also provides the formation of the superoxide radical ($O2\bullet-$) and peroxide (O_2^{2-}) intermediates, which may be directly reduced by phenolic molecules and are better oxidants than O_2.

Wine oxidizable substrate, with a special emphasis on alcohols (ROH), play a key role in the modification of the chemical composition and sensory properties. The most important modification occurring in red wine colours are driven by the presence of acetaldehyde produced by oxidation of ethanol in solution, which allow the development of colour and flavour changes related to wine varietals: the acetaldehyde mediate the condensation between anthocyanins and flavanols to produce pigments, but also drives the cross-linking of catechins and produce methylmethine-linked flavanol adducts; it also mediate the self-condensation of anthocyanins and reacts directly with malvidin-3-*O*-glucoside to produce visitin pigment (Mateus, Silva, Vercauteren, & de Freitas, 2001; Timberlake & Bridle, 1976; Vivar-Quintana, Santos-Buelga, & Rivas-Gonzalo, 2002).

The production of acetaldehyde from ethanol and ketones from organic acids are not the only reaction expected in wine; emblematic in this sense is the presence of glycerol, a major constituent of wine (5 to 20 g L^{-1}), that provides several oxidation products (da Silva Ferreira, Barbe, & Bertrand, 2002; Ribéreau-Gayon, Glories, Maujean, &

Dubourdieu, 2006). Oxidation by-products of glycerol have not been extensively studied, and there is a lack of systematic characterisation of the resulting aldehyde and ketone-like structures (Waterhouse & Laurie, 2006). Other wine components evolves into electrophilic products during the oxidation route: sugars are converted in further ketones, malic acid is converted into pyruvic while tartaric forms aldehydes compounds (Oliveira, Ferreira, De Freitas, & Silva, 2011).

According with volatile compounds, responsible for wine aromas, previous studies have demonstrated how volatile thiols decrease during wine ageing and after bottling when oxidative processes occur (Blanchard, Darriet, & Dubourdieu, 2004; Brajkovich, Tibbits, Peron, Lund, Dykes, Kilmartin, et al., 2005). Thiols are unstable species which easy undergo modifications when subjected to the presence of catalysers at the oenological conditions, and the react through nucleophilic, acid-catalysed, substitution reactions with procyanidins, which also explain the disappearance of GSH in wines aged in contact with ellagitannins released from oak barrels (Jocelyn, 1972; Labarbe, Cheynier, Brossaud, Souquet, & Moutounet, 1999; Lavigne, Pons, & Dubourdieu, 2007; Matthews, Mila, Scalbert, Pollet, Lapierre, Hervé du Penhoat, et al., 1997). Quinones formed following polyphenols oxidation are electrophiles and they react directly with thiols, being nucleophilic compounds, by a Michael addition reaction (Cheynier, Trousdale, Singleton, Salgues, & Wylde, 1986), and they form low MW volatile compounds (**figure 2.3**).

Figure 2.7 after Nikolantonaki et al. (Nikolantonaki, Chichuc, Teissedre, & Darriet, 2010) summarizes the possible reaction pathways leading to the degradation of volatile thiols (RSH) in wine.

This is a simplified overview on the complexity of chemical structures which potentially affect the properties of wine, thus reflecting the complexity of its composition.

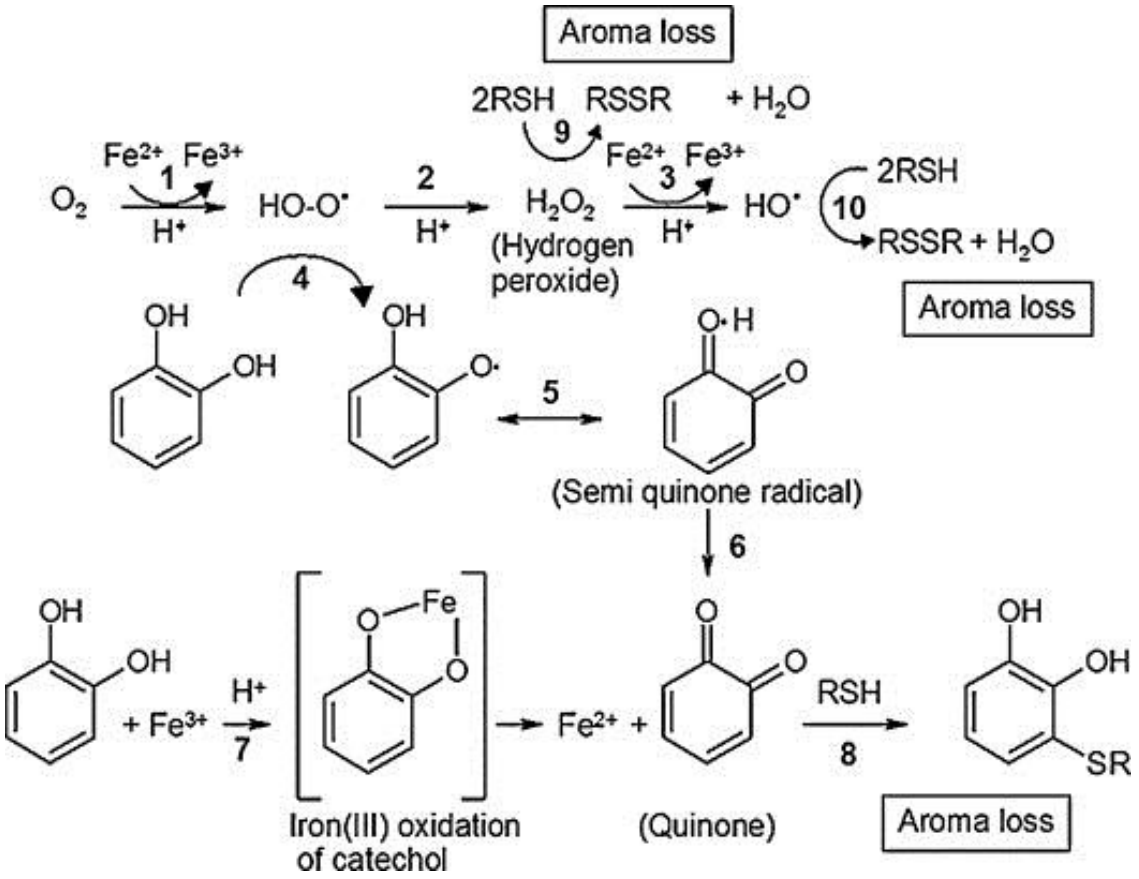

Figure 2.7. Schematic representation of the loss in varietal aroma due to the decomposition of volatile thiolic compounds (RSH) (Nikolantonaki et al. 2010; the figure is presented with the kind permission of prof. Darriet and with copyright permission by Elsevier Limited).

2.3. The role of polyphenols as antioxidants in wine

The effectiveness of phenolic compounds in avoiding oxidation could be attributed to the synergistic effects of their mixtures naturally occurring in wine, since every compound of the chemical class bears a specific molecular structure with a specific activity (Bors & Michel, 2002; Heim, Tagliaferro, & Bobilya, 2002; López-Vélez, Martínez-Martínez, & Valle-Ribes, 2003; Rice-Evans, Miller, & Paganga, 1996). Phenolics are easily oxidizable substrates, recognised to be stronger hydrogen bonding acids toward oxy radicals (Mahoney & DaRooge, 1975), and the intermediate that is formed following the polyphenols oxidation is generally a quinone radical, which exhibit an increased stability due to delocalization of the unpaired electron around the π- system of the aromatic ring. The most active polyphenols are usually the flavonoid compounds, which preferentially interact with metal ions, and ROS. Polyphenols are efficient chelating agents, which are able to coordinate iron and copper, the main

catalysers of wine oxidations, at different valences; the formation of coordination complexes reduce the solvated ions available from the catalysed oxidative reactions. Catechol groups (*o*-dihydroxy substituted aromatic rings) are considered the main responsible for antioxidant properties of polyphenols. In a simple catechol, the active site consists indiscriminately of one of the –OH group, where the homolytic O-H cleavage occurs; the resulting polyphenolic radical is highly stable due to resonance. In catechol derivatives, as well as in different phenolic compounds, the –OH positions are no longer equivalents due to the different substitutions at the aromatic ring, with rearrangement of the electronic density. For this reason, electrons – hydrogen atoms are available in different extent, depending on the field inductive and resonant effects induced by substituents. Furthermore, flavonoid compounds (catechins) are able to recycle themselves, converting the quinone to the original hydroxylated form. The aromatic *o*-substitution provides an additional resonant stabilisation, higher than in *meta*- and *para*- substitutions; furthermore the electron affinity of *o*-benzoquinone was computed to be 0.09 (0.06) eV larger than that for *p*-benzoquinone (Fattahi, Kass, Liebman, Matos, Miranda, & Morais, 2005).

The interaction with free radicals provides the scavenging of ROS through the formation of oxidised intermediates (PhO•) that could be reduced and possibly regenerated by other phenolic compounds, which are less active and undergo further chemical interactions.

Ph(A) + ROO• – Ph(A)O• + ROOH

Ph(A)O• + Ph(B) - Ph(A) + OxPh(B)

This chain-break effect allow to create an alternative reaction path neutralising highly reactive ROS and releasing in solution more stable structures. The radical centres formed at the polyphenol following oxidation are stabilised through a mechanism which delocalize the odd electron along the π-acceptor groups of the unsaturated system (aromatic ring) delocalize the unpaired electron, and also the heterocycle commonly referred as C (pyran ring) in flavonoid compounds provides an additional stabilisation through the lone-pair-donor group, involving a three-electron interaction between a lone pair on the heteroatom and the unpaired electron at the radical centre. The hindered phenolic compounds are able to scavenge radicals mainly through the donation of an electron followed by deprotonation; but also the stabilisation of the resulting antioxidant radical of the polyphenolic molecules involved (gallic acid and gallic acid esters, phenolic acids, flavonoid compounds, oligomeric and polymeric tannins) play a

key role in the targeted antioxidant action, as well as the mechanism by which the stabilization occurs: singlet electron resonant stabilisation, molecular adducts formation, intramolecular hydrogen bonding (Frankel & Meyer, 2000; Guo, Zhao, Shen, Hou, Hu, & Xin, 1999; McPhail, Hartley, Gardner, & Duthie, 2003; Andrew L. Waterhouse & V. Felipe Laurie, 2006). Furthermore, in more complex structures like flavonoids, the donation of non-phenolic hydrogens have been demonstrated to contribute to the antiradical mechanisms of catechins, due to their favourable dissociation and stabilization enthalpies (Kondo, Kurihara, Miyata, Suzuki, & Toyoda, 1999a, 1999b).

Due to the high variability and complexity of reactions following the oxidation of polyphenolic compounds, it is quite difficult to provide a unique explanation and a unified mechanism of reaction for all antioxidants in wine. Furthermore, the study of the effects of chemical reactions involved in the formation and scavenging of radicals in wine requires analytical techniques with high temporal resolution (Maillard, Ingold, & Scaiano, 1983; Schwarzwald, Monkhouse, & Wolfrum, 1987; Zhao & Zhang, 2015), which are usually not readily available in laboratories working on food quality control; for these reason most of the antiradical assays reproduce in the laboratory scale the formation and subsequent removal of reactive species bearing longer lifetimes, to enable the monitoring of their evolution when in contact with wine antioxidants.

2.4. Exogenous additives to protect wines against oxidation

As it has been described so far, the protection of wine is a critical issue for producers, which requires a tailored protection strategy during the winemaking process and before bottling. One of the most common practices in winemaking is the addition of exogenous substances which limit or delay the occurrence of oxidation in time. Sulphur dioxide and ascorbic acid have been the most implemented additives for this purpose, which are described in detail in the following sections; furthermore, alternative options for SO_2 and ascorbic acid have been assessed for the protection during wine storage, including the addition of caffeic acid, gallic acid and glutathione (El Hosry, Auezova, Sakr, & Hajj-Moussa, 2009; Roussis, Lambropoulos, & Tzimas, 2007). Among them section *2.4.3.* provides a description of botanical extracts, commonly referred as tannins, used

as antioxidants in the oenological practices: particular attention has been devoted to the effectiveness of this compounds and experimental results are reported in **chapter 6**.

2.4.1. Sulphur dioxide (SO_2)

Sulphur dioxide is widely added during the winemaking process from grape pressing to bottling in form of potassium bisulphite salt ($KHSO_3$), to provide protection to musts and wines against oxidation and harmful microbial species, like yeasts and bacteria. Considering the European legal limits, maximum levels of SO_2 in conventional wines are 150 mg L^{-1} for reds and 200 mg L^{-1} for whites and rosés, whereas in organic wines will be 100 mg L^{-1} for red wines and 150 mg L^{-1} for whites and rosés (*Council Regulation (EC) No 479/2008 of 29 April 2008 on the common organisation of the market in wine; Commission Implementing Regulation (EU) No 203/2012*); by comparison, the maximum legal level of added sulfites in the United States is 350 mg L^{-1}. Bisulphite ion HSO_3^- formed in solution at oenological pH values is able to limit the oxidation by reaction with H_2O_2, and oxygen radicals. SO_2 inhibits aldehyde formation by competing for hydrogen peroxide reduction (Elias & Waterhouse, 2010); furthermore, it enhances the rate of reverse conversion of quinones derived from polyphenols to their hydroxylated form (Danilewicz, Seccombe, & Whelan, 2008). Danilewicz has calculated a molar reaction ratio 1:2 between O_2 and SO_2, consistently with the reaction of one mole equivalent of SO_2 with H_2O_2 and the second with one mole equivalent of quinone; this ratio is decreased in the case of red wine to 1:1.7, due to the presence of nucleophilic substances competing to quinone (Danilewicz, Seccombe, & Whelan, 2008).

SO_2 also promotes addition reactions with carbonyl compounds to form non-volatile bisulphite adducts; this stage is very important to avoid the development of undesirable aroma generated by sulphur compounds. In wine solution the sulphite also bind with acetaldehyde, anthocyanins, pyruvic acid, glutaric acid, glucose or phenolic compounds, particularly, caffeic acid and *p*-coumaric acid. The formation of molecular adducts improve stabilisation of unstable species in wine, for this reason there is a specific distinction between free sulfur dioxide, referred to HSO_3^- and SO_2, the active form, and the binded sulfur dioxide involved in the formation of adducts (bound SO_2) (Nikolantonaki, Chichuc, Teissedre, & Darriet, 2010).

The antioxidant capacity of sulphur dioxide in wine has been extensively studied, and several mechanisms have been proposed (Barril, Clark, & Scollary, 2012; Danilewicz, Seccombe, & Whelan, 2008; Danilewicz & Wallbridge, 2010), including indirect evidences on the catalytic action of transition metals to promote oxidation routes in wine (Danilewicz, 2007). In particular, in a wine model solution including SO_2 and 4-methylcatechol (4-MeC) as an oxidizable substrate, any significant SO_2 oxidation is observed, while the consumption of SO_2 is enhanced when adding both iron and copper ions, showing a synergistic effect between the two ionic species. Copper was proposed as an intermediate, which by interacting with oxygen, facilitates redox cycling of iron. Furthermore, the consumption rate is dependent on the concentration of 4-MeC (Danilewicz, 2007).

Unlike the multiple protective properties of sulphur dioxide in wine, its use has been significantly reconsidered in recent years due to its potential impact on the health of consumers, since it has been recognized as an allergen: parental, oral and topical exposures to sulphites have been reported to induce clinical effects in sensitive individuals, like dermatitis, urticaria, flushing, hypotension, abdominal pain, diarrhoea and asthmatic reactions (Rolland, Apostolou, Deckert, de Leon, Douglass, Glaspole, et al., 2006; Vally, Misso, & Madan, 2009).

2.4.2. Ascorbic acid

Ascorbic acid is a powerful antioxidant naturally present in grapes, being a strong reducing agent: its reduction potential is ~210 mV, as measured in cyclic voltammetry (*vs* Ag/AgCl) at pH 3.6 (Paul A. Kilmartin, Zou, & Waterhouse, 2001). It is usually rapidly consumed during crushing, when it plays a key role on the protection of polyphenols from the enzymatic oxidation. In recent times the use of ascorbic acid as an additive at various stages of the winemaking process has been evaluated to provide increasing protection against the oxidation of white wines; the typical dosage recommended ranges from 50 to 150 mg L^{-1} (Barril, Clark, Prenzler, Karuso, & Scollary, 2009), with 250 mg L^{-1} being the legal limit for its addition in wine (OIV 2006). Unfortunately, there is an important drawback to the exclusive use of ascorbic acid as an antioxidant in wine, as in oenological conditions exhibits a pro-oxidant secondary effect (Mark P. Bradshaw, Cheynier, Scollary, & Prenzler, 2003; Mark P. Bradshaw, Prenzler, & Scollary, 2001). When reducing the quinones and reactive

species, the ascorbic acid is converted into dehydroascorbic acid and hydrogen peroxide, and further degradation routes produce carboxylic acids, ketones, and aldehydes, (Marc P. Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011), as schematically represented in **figure 2.8**. Furthermore, ascorbic acid has been demonstrated to increase the production of phenolic pigments in a model wine solution containing (+)-catechin, through the formation of coloured xanthylium cations (Mark P. Bradshaw, Prenzler, & Scollary, 2001).

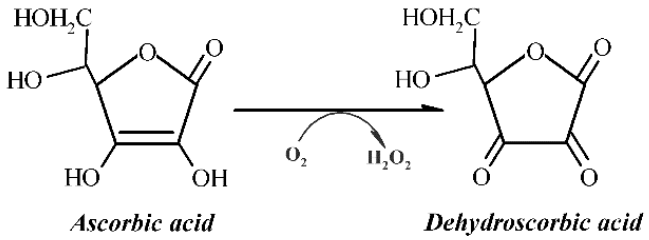

Figure 2.8. Oxidation route of ascorbic acid in oenological conditions.

The only option in using ascorbic acid as an antioxidant in wine is the combined use of a supporting antioxidant, which effectively scavenge the oxidation by-products; SO_2 and GSH have been tested for this purpose (Mark P. Bradshaw, Scollary, & Prenzler, 2004; Roussis & Sergianitis, 2008).

In conclusion, experiments provided so far clearly stated that ascorbic acid can support but not replace the use of traditional antioxidant products in wine.

2.4.2. Oenological tannins

Grape naturally contains polymerised flavan-3-ol compounds, namely procyanidins, which contribute to mouthfeel and colour stabilisation in wine (Harbertson, Parpinello, Heymann, & Downey, 2012; Somers, 2003; S. Vidal, Francis, Noble, Kwiatkowski, Cheynier, & Waters, 2004); these compounds belong to the class of tannin compounds. Tannins are secondary metabolites of plant produced by several botanical species with protective purposes, which accumulate in specific plant components: wood and woody parts, leaves, fruits (Laghi, Parpinello, Rio, Calani, Mattioli, & Versari, 2010); the main sources of tannins in grape are skin and seeds, for this reason wines made avoiding skin contact, like white and sparkling wines, result in a low concentration of these compounds. Tannin extracts are mainly constituted by polyphenolic compounds bearing variable degree of polymerisation and often linked with sugars; Grape seed and skins

tannins are condensation products composed of oligomers and polymers including catechin, epicatechin, epicatechin-gallate and epigallocatechin subunits (Haslam, 2007).

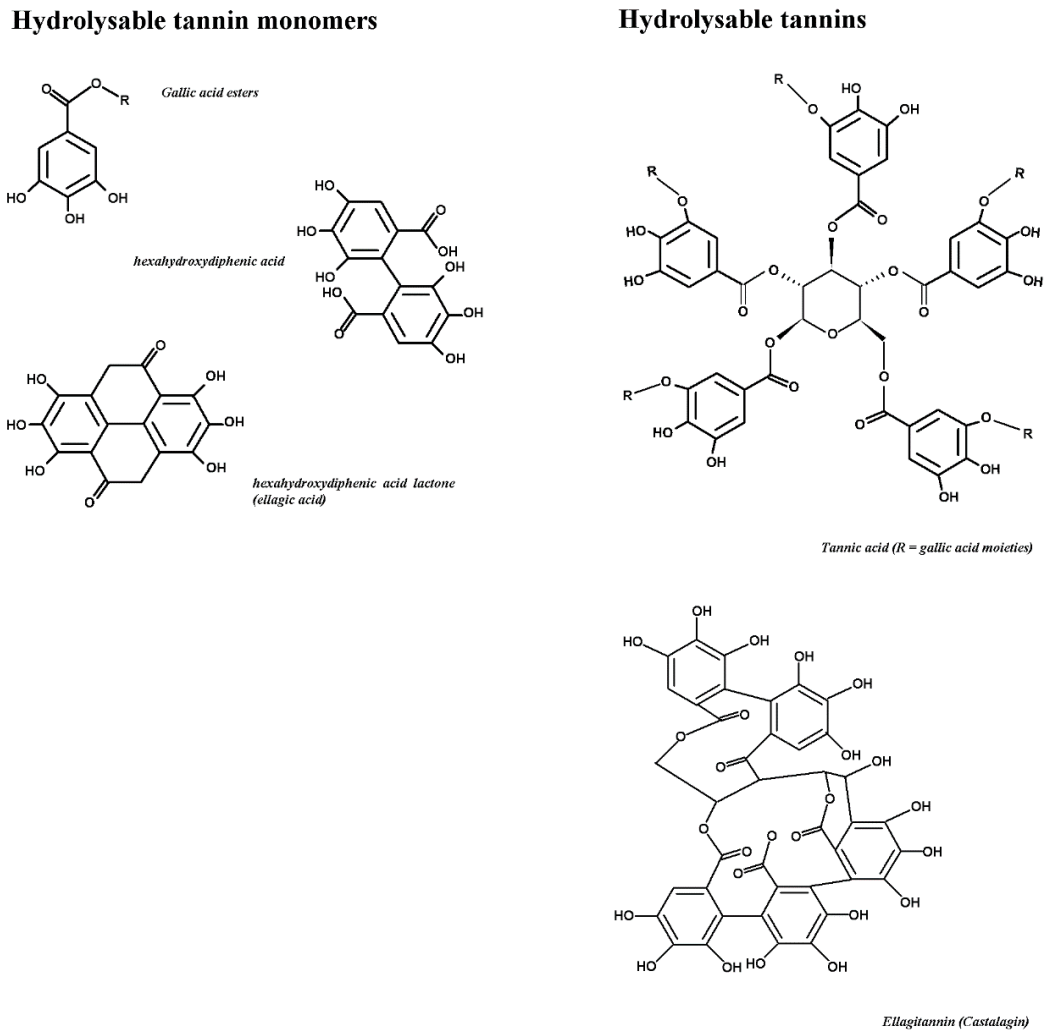

Figure 2.9. Hydrolysable tannins: monomeric precursors and polymerized compounds.

Endogenous tannin compounds derived from grape play a key role in the development of pigmented polymers in red wines and in sensory attributes like bitterness and astringency (Harbertson, Parpinello, Heymann, & Downey, 2012). Exogenous tannins are derived from different botanical sources: oak, chestnut, grape, green tea, quebracho to cite some, they can be both hydrolysable or condensed, and their use is currently allowed in winemaking as clarifying agents, (*OENOLOGICAL TANNINS, INS N°: 181, Oeno 12/2002 modified by Oeno 5/2008, 6/2008 and OIV-Oeno 352-2009*), due to their ability to complex organic molecules like protein and sugar and precipitate the resulting adducts, and as flavourings for food and beverage production (EC No 1334/2008, EU Regulation No. 872/12). Tannins are commercially available as lyophilized powders or water suspension stabilised by adding sugars (i.e. arabitol), and they can be a unique

extract or provided as a blend, with different degrees of purity (Versari, du Toit, & Parpinello, 2013). Suppliers provide general information on the source of the extract and indications for the specific use of each product: most of the technical reports suggest their use as "antioxidant". The antioxidant activity of tannins is due to similar mechanisms of parent monomers: they are effective reducing agents and radical scavengers, and they act as bidentate chelators, complexing solvated ions of transition metals which are responsible for the Fenton and Harber-Weiss reactions.

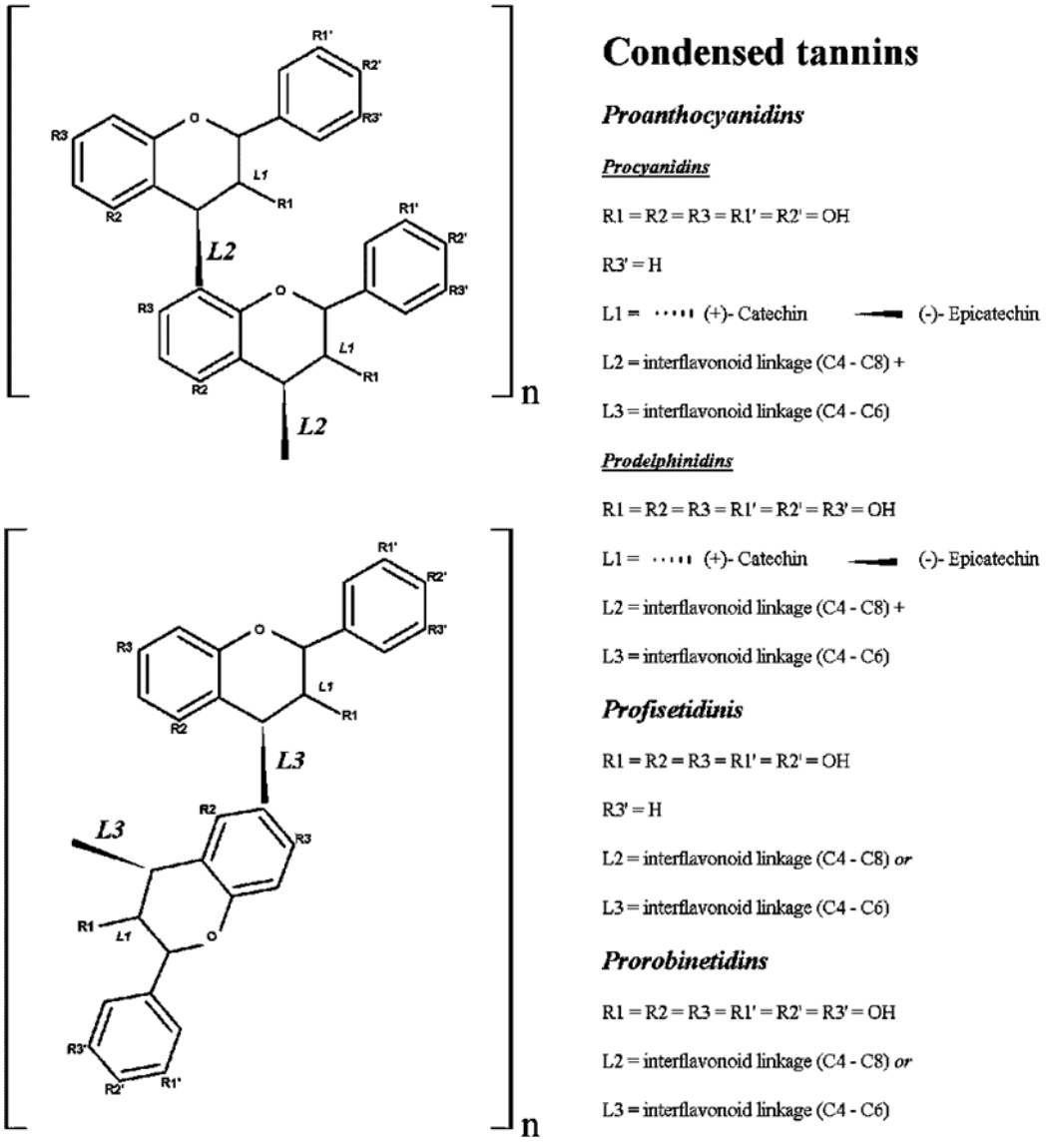

Figure 2.10. Schematic description of the most common condensation structures of procyanidins.

The potentiality of their use is limited by their impact in the bitterness, astringency and colour perceptions, and there is a need to further investigate their dose-effect response

and their sensory threshold when added in wine (Ricci, Lagel, Parpinello, Pizzi, Kilmartin, & Versari). In this thesis they have been investigate according with their performances as antioxidant in model-wine system, and the satisfactory preliminary findings will be discussed in detail in **chapter 6**; results highlighted the need to further implement the design of experiment (DOE) with trials in real system, in order to monitor their antioxidant activity and sensory impact during long-term conservation of wine.

2.5. The influence of packaging on the shelf-life of bottled wines

This thesis work has focused on the impact of selected packaging in wine neglecting their technological performances, whose study provide an approach based primarily on the study of the properties of materials. However, a brief descriptive more than technical review on the types of packaging commercially available is presented in this section, to improve understanding of the importance of a proper oxygen management during shelf-life.

Packaging has a fundamental role in ensuring safe delivery of wine throughout supply chains to the end consumer; processing constraints, customer expectations, the cost of materials, environmental impact, chemical and microbiological inertia and technical characteristics are all parameters that are considered when choosing the most appropriate solution (Winder, Ridgway, Nelson, & Baldwin, 2002).

Packaging should also protect wine against oxidation which is driven by the control of gas exchanges, mediated by the porosity in the case of the closures and the permeability of composite materials (plastics and cardboard) alternative to traditional glass bottles.

In recent years, many materials have been introduced in the market with the aim of providing increasingly specific solutions to the different products. This variety arises from the awareness that there is not a global strategy, comprising the needs of different wines and different foreseen shelf lives: it is not possible to define the ultimate best packaging, but only the most suitable to the actual product specification.

For this reason, it is common practice in wine sciences to monitor the evolution of oenological parameters of bottled wines (Fu, Lim, & McNicholas, 2009; Moio, Ugliano, Genovese, Gambuti, Pessina, & Piombino, 2004; Robertson, 2009). In addition, when having the same wine varietal, and packaging, storage conditions,

including positioning of the bottles, temperature, exposure to light, influence wine durability, and this is particularly important for worldwide exportation and shipping.
A more detailed overview on packaging and closures is provided in the following two sections.

2.5.1. Wine packaging

Glass containers are traditionally preferred for bottled wines, but in recent times there has been an intensive business with Bag-in-Box©, plastic bottles, and Tetra pack© facing the growing demand for low-cost and environmental friendly solutions for wine bottling (Charters & Pettigrew, 2007). The choice of packaging has a critical impact in the conservation of wine, as it affects the oxidative stress regulating the transfer of gases through the packaging material, allowing a careful management of the oxygen intake during wine production and storage (Ghidossi, Poupot, Thibon, Pons, Darriet, Riquier, et al., 2012; J. C. Vidal & Moutounet, 2006).
Alternative wine preservation technologies provide the use of PET, a polyester synthetic polymer composed by ethylene glycol and terephthalic acid, which form a polymer chain.
PET is processed through extrusion, cooling, and transformation into small pellets, and the final product is characterised by transparency, low cost, performant mechanical properties and strength; it is suitable for the processing on mold and the production of thin films (Girija, Sailaja, & Madras, 2005).
It is used for the wine storage as composite material, which provides a multilayer structure to improve the gas barrier (Van Bree, De Meulenaer, Samapundo, Vermeulen, Ragaert, Maes, et al., 2010). Typical examples of a gas barrier resin include ethylene-vinyl alcohol copolymers (EVOH) and MXD6 nylon. Furthermore, an improved PET polymer doped with a radical scavenger (PETA) has been recently developed and tested in view of its use in the wine industry (Giovanelli & Brenna, 2006).
Bag in Box (BIB) is an environmental friendly alternative to plastic bottles and glass, and it is formed by a cardboard box containing a resistant bladder (or plastic bag), bearing the same PET composite structure described above several layers in the same way as PET multi-layer bottles (Rapp, 2005). In Tetra pack technology the multilayer plastic film is directly applied inside a carton brik; in contrast to BIB, Tetra pack technology is also suitable for the storage of small volumes of wine, up to 250 ml.

The impact of these materials on wine oxidation is related to their barrier properties against oxygen. Several studies relating to different aspects of packaging materials have been carried out. Some studies highlighted the similar performances between PET multi-layer (OxSc-PET) and glass bottles during 7 months storage of white and red Apulia table wines, suggesting that OxSc-PET containers are efficient to slow down certain detrimental phenomena responsible for wine quality decay during storage similarly to glass bottles (Mentana, Pati, La Notte, & del Nobile, 2009). A more recent study have compared the evolution of aromatic profile of a rosè wine stored in glass, PET, recycled PET; in this case, an appreciable differentiation by a targeted number of compounds occurred after 5 months storage, with a pronounced decrease of oxygen sensitive compounds like methionol when using PET (Dombre, Rigou, Wirth, & Chalier, 2015).

The preservation of sensitive volatile compounds like methionol has been improved introducing PETA bottles doped with an oxygen scavenger at different concentration levels (1%; 3%). When comparing the same wine in glass and PETA, only the decrease of trans-1,3-dioxolane in 3%-PETA allowed to discriminate samples; methionol, an oxygen sensitive aroma compound, was preserved in both glass and 3o%-PETA bottles (Dombre, Rigou, & Chalier, 2015).

Considering the effectiveness of the doped films in limiting the loss of varietal aroma of wine, an innovative composite material including ethyl cellulose and grape tannins extract (GT) has been designed and tested for its mechanical, antioxidants and antimicrobial properties, as a part of the PhD project and in view of its potential use in wine packaging (Olejar, Ray, Ricci, & Kilmartin, 2014). The preliminary results of this study, which is still in progress, are presented in **Addendum A** in the form of the research paper originally published by Cellulose journal (Springer).

2.5.2. Closures

Wine undergoes several exposures to oxygen when moving along the supply chain, and it is stabilised at the end of the process by adding the oenological treatment just before bottling. After bottling, oxygen intake depends on the sealing effect of closures, bearing different barrier effect (Godden, Lattey, Francis, Gishen, Cowey, Holdstock, et al., 2005). Natural and synthetic cork, derived by *Quercus suber L.* vegetable coating, are the traditional closures. The use of cork as a closure is very ancient, having its

maximum development in the second half of the XVII century, in France (**figure 2.11**); only in very recent times its use was accompanied by the introduction of synthetic caps and screw caps.

Figure 2.11. The oldest bottled Champagne so far, which according to experts date back to 1800, was found in the summer of 2010 kept in a wreck at 50 meters deep off the Finnish archipelago. The properties of cork have been preserved due to environmental conditions (high pressure, low oxygen levels, dark).
Source: Web (http://www.iprosecco.it/allasta-lo-champagne-dei-record-per-oltre-200-anni-in-fondo-al-mar-baltico/).

Generally the synthetic stoppers exhibit a high oxygen ingress rate, which is reduced in the case of technical corks and screw-caps (Lopes, Saucier, Teissedre, & Glories, 2006; Squarzoni, Limbo, & Piergiovanni, 2004). Due to the variability of the natural source of monolithic cork, the monitoring requires a high number of samplings to obtain statistically significant results on the performances of corked wine; however, it has been evidenced that natural cork stoppers tends to diffuse oxygen inside the bottle at a relatively low rate (Lopes, Saucier, Teissedre, & Glories, 2007).

Figure 2.12 inspired by a recent work after Lopes et al. (2007) report a schematic representation of the oxygen inlet channels in natural cork, technical cork, and synthetic closure, respectively. It is evidenced how the natural cork allows the gas permeation at the interface with the glass, in addition an aliquot of oxygen is released from the pores of the compressed cork following insertion in the bottle neck; the same mechanism of release occurs in the technical cork, whereas the entry of oxygen at the interface with the glass is inhibited by surface treatments with resins. Synthetic caps, which are designed to have specific permeability, allow the oxygen inlet through controlled porosity produced during the polymer extrusion process (Lopes, Saucier, Teissedre, & Glories, 2007).

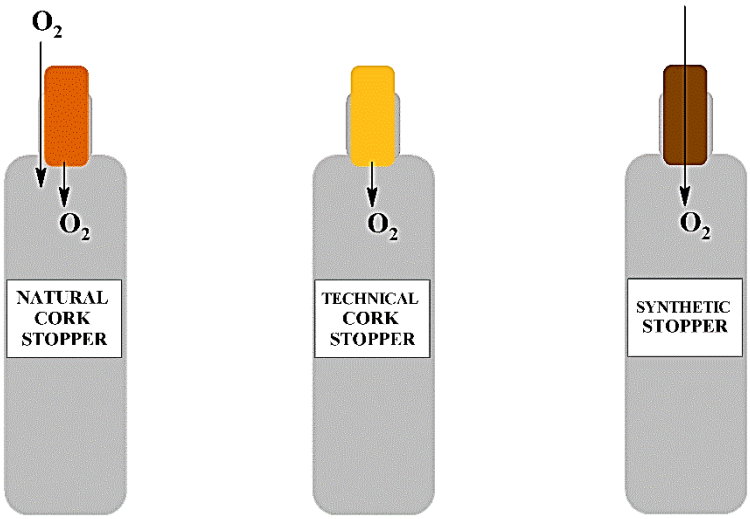

Figure 2.12. Schematic representation of mechanisms of oxygen permeation in wines after bottling, when using different closures; the scheme is inspired by previous studies on permeation of wine closures to oxygen (Lopes et al., 2007).

The screw-cap are generally relatively impermeable to oxygen, even though shelf-life trials have demonstrated performances that are similar to the natural cork in long-term conservation. A Sauvignon Blanc has been sealed under screwcap and cork, and monitored for two years after bottling. Wines under cork and screwcap showed the same SO_2 consumption and CO_2 retention amount, and retained similar levels of volatile compounds, with a slight loss in corked wine (-18÷23%) due to possible absorption of thiols in the cap. Levels of polyphenols and browning development were also similar. And the sensory panel didn't find appreciable differences between the wines (Brajkovich, et al., 2005).

Regardless of the individual cases, the permeability of the packaging to oxygen is a critical issue since "appropriate" oxygen intake can't be ultimately defined; even a low oxygen supply can be detrimental to the wines, generating reduced hints and affecting the fining of aging wines, especially red.

2.5.2. Oxygen meters

The crucial role of oxygen in sensory and chemical attributes of wine is well summarized in a famous sentence of the physicist Louis Pasteur. In 1873 he wrote: "*l'oxigène est le pire ennemi du vin" (the oxygen is the enemy of wine), but he also added: c'est l'oxygène qui fait le vin, c'est par son influence qu'il vieillit*" (oxygen make the wine, it is through its influence that it develops), (Pasteur, 1873). Oxygen and

its complex chemistry is strictly related to the shelf - life of wine, leading to oxidative spoilage but also to the development of characteristic chemical compounds and aromas from precursors; for this reason, a specific attention was paid on the study of diffusion rate of oxygen through closures and on its dissolution in wine; several technologies for the monitoring and control of oxygen content and consumption during shelf - life have been developed so far (Godden, Lattey, Francis, Gishen, Cowey, Holdstock, et al., 2005; Lopes, Saucier, Teissedre, & Glories, 2006; Lopes, Silva, Pons, Tominaga, Lavigne, Saucier, et al., 2009; Pristouri, Badeka, & Kontominas, 2010).

Several approaches to the measurement of dissolved oxygen in bottled wine have been developed to monitor the oxygen ingress rates through closures and to build up the curve of oxygen consumption in bottle during shelf-life (Nevares & del Álamo, 2008; Nevares, Del Alamo, Cárcel, Crespo, Martin, & Gallego, 2008; Salmon, Fornairon-Bonnefond, & Mazauric, 2002).

The Mocon method is based on a coulometric sensor, which is electrochemically activated by contact with oxygen (Poças, Ferreira, Pereira, & Hogg, 2010). Recent studies based in this methodology have allowed to discriminate the different closures on the basis of their permeability; however, the Mocon method only measures oxygen ingress in empty packaging, while it is not able to provide information on oxygen dissolved in the liquid

Recently, a non-destructive colorimetric method was developed for the measurement of OTR (Oxygen Transfer Rate) in the wine bottle. The NomaSense oxygen meter (Nomacorc, Limbourg-Dolhain, France) portable device equipped with fluorescent dots and optical fibres (**figures 2.12-13**) allows a single bottle to be analysed without compromising the closure seal, following the insertion of the dot in the bulk of the bottle (dissolved oxygen) and in the head space (Ugliano, Dieval, Dimkou, Wirth, Cheynier, et al., 2013). This method measures the oxygen ingress through different closures under similar conditions of packaging, since the contribution of the glass of the bottle is corrected for the fluorescence emission of interest.

Another option commercially available provides the use of a sampling chamber filled with nitrogen, and equipped with a probe to perforate the cap of the bottle and sample the wine thus excluding atmospheric oxygen interferences (**figure 2.14**); the aliquot of wine is transferred to an electrochemical cell where the dissolved oxygen content is measured and corrected for the temperature values (Anton Paar, Graz, Austria).

Figure 2.12. The measurement of dissolved oxygen with the colorimetric method after Nomacorc (Limbourg-Dolhain, France).

Figure 2.13. The measurement of head space oxygen with the colorimetric after Nomacorc.

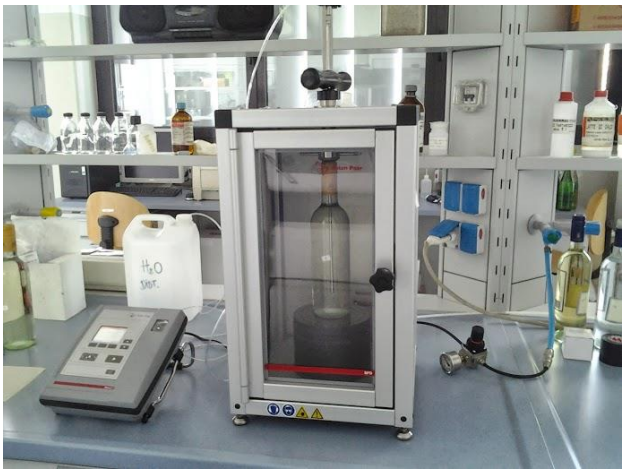

Figure 2.14. The sampling chamber to measure dissolved oxygen in bottled wines after AntonPaar (Graz, Austria).

A further description of oxygen level measurements performed in wine along the supply chain and after bottling are provided in **Chapters 3** and **4**.

2.6. Conclusions

The confidence of wine consumers is strictly related to the preservation of distinctive properties of wines after bottling. The detrimental effect of oxidation is considered one of the main causes affecting the shelf-life of wine, for this reason, and due to the complexity of the mechanisms and factors involved, there is a need for the definition of a protocol to monitor and protect wine against oxidative stress.

During storage, different internal, external or environmental factors may be taken into account; furthermore, with ageing, each bottled wine accumulates a "physiological history" of different events. The internal factor are related with the balance of possible pro-oxidants (mainly transition metals) which promotes and accelerates the oxidative routes, and antioxidants (mainly polyphenols) which allow to limit them; the external factor are related to the addition of additives (sulphur dioxide, ascorbic, oenological tannins, to cite some) and to the use of selected packaging. The environmental factors may include, amongst others, ambient temperature, humidity, and light exposure.

A multitude of review on the mechanisms producing oxidation in wine has been published in order to provide a clear explanation of factor affecting wine conservation;

Considering, however, the complex interaction of the internal, external and environmental factors and their synergistic effect in wine spoilage, some selected parameters have been investigated in detail, and in this work of thesis priority is given to fast and reliable monitoring of significant parameters to build up DOE which can be successfully applied along the supply chain by technicians working in winemaking.

2.7. Literature cited

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ADDENDUM A

Cellulose
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ORIGINAL PAPER

Superior antioxidant polymer films created through the incorporation of grape tannins in ethyl cellulose

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Abstract Agro-wastes represent an abundant and economical source of antioxidant compounds. Extraction and incorporation of antioxidants from these compounds into ethyl cellulose films provides the basis for an active packaging material. Grape tannin extract (GT) incorporation into ethyl cellulose results in hydrogen bonding between polyphenols and ethyl cellulose strands, which allows for the polyphenols to remain active and to be securely incorporated. Incorporation of 0.5 % GT in ethyl cellulose produced a significant increase ($p < 0.01$) in antioxidant activity while not altering physical or mechanical properties. A higher loading of GT at 3.0 % into ethyl cellulose resulted in further improvement in antioxidant activity (12-fold), while a slight decrease in the tensile properties was noted due to the plasticizing effect of GT as a consequence of disruption of the intermolecular hydrogen bonding.

Keywords Antioxidant · Grape tannin · Ethyl cellulose · Packaging · ABTS

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Introduction

Over the last few decades, polymeric materials have emerged as a cost effective, flexible solution for numerous applications in materials and chemical processing industries, as well as commodity goods. According to a recent market analysis, 288 million tons of plastics were produced worldwide in 2012 (PlasticsEurope 2013). One of the major application areas of these materials is in the food-packaging sector and the demand for plastics in this area is expected to increase. However, the consumer is influencing the production of plastics by requiring them to be "green" with regards to environmental impact, and in the case of packaging, to improve the shelf-life and safety of the products contained. As a result of these influences, the plastics industry has turned to the development of active polymers that incorporates natural components.

Active polymers as packaging materials can protect foodstuffs through several mechanisms, including the provision of a general barrier from the environment, but can also impart antioxidant activity. The antioxidant barrier can protect the contents from reactive radicals in the environment or those that may be generated in the foods themselves. Reactive species, such as reactive forms of oxygen, can increase the rate of food spoilage as well as alter flavors and appearance, thus shortening shelf-life (Bradley and Min 1992).

The grape and wine industry produces a readily available agro-waste, which is high in antioxidant

compounds (Aliakbarian et al. 2012; Arvanitoyannis et al. 2006; Fontana et al. 2013). The antioxidant activity associated with grape extracts is linked to the polyphenolic compounds contained therein, including benzoic acids, hydroxycinnamic acids, flavan-3-ols, and flavonols. The individual phenolic compounds extracted and isolated from this waste stream have been shown to be high in antioxidant activity (de Campos et al. 2008; Lafka et al. 2007). Grape tannins involve higher molecular weight polyphenol compounds found in the skins and seeds of grapes. As grape tannins are extracted from a naturally occurring food source, they are generally regarded as safe, which allows for their use in contact with foods.

Natural antioxidants have taken an important role as additives within polymers. The stability, oxidative induction time, thermal degradation, antioxidant capacity and plasticizing affects have been considered for grape extracts, green tea extracts, tomato waste, seaweeds, fish proteins, murta leaves, essential oils of herbs, and other natural extracts. These have been included in a variety of materials; polyolefins, such as polypropylene (Ambrogi et al. 2011; Cerruti et al. 2009; López de Dicastillo et al. 2013; Nerín et al. 2006), ultra-high molecular weight polyethylene (Peltzer et al. 2007); natural polymers, such as chitosan (Moradi et al. 2012; Rubilar et al. 2013; Siripatrawan and Noipha 2012), cellulose (Krizova and Wiener 2013; Quilaqueo Gutiérrez et al. 2012), and others (Blanco-Pascual et al. 2014; Bourtoom et al. 2006; Cerruti et al. 2011; Li et al. 2014). These studies have shown the diversity of natural components that can be incorporated or made into films to produce materials suitable for various applications. Current studies examine the role of multiple isolated components (Silva-Weiss et al. 2013), but research on antioxidant activity has mainly centered on individual compounds isolated from natural sources and incorporated into films (Arrua et al. 2010, 2012; Cerruti et al. 2011; Helal et al. 2012; Iñiguez-Franco et al. 2012; Pastor et al. 2013; Spizzirri et al. 2009).

Cellulose polymers have many applications and are capable of meeting the natural and "green" demands of consumers. Integration of natural extracts has been shown to enhance the properties of these polymers (Krizova and Wiener 2013; Pastor et al. 2013; Quilaqueo Gutiérrez et al. 2012; Wu et al. 2009). Continued research into maximizing the desired effects, optimization of the materials utilized, and processing methods is required. This will ultimately reduce costs and provide a material that is acceptable to industry by meeting regulatory requirements and making use of current processing technologies.

In the current study, grape tannin (GT) has been incorporated into ethyl cellulose (EC), and the antioxidant properties and important physical properties of the active polymer have been assessed. GT was explored as it contains a range of polyphenol antioxidant compounds that are mainly dimeric, oligomeric and polymeric in structure, and are naturally found in an agro-industry waste product. The resulting active polymer utilizes minimal materials and contains compounds that are both natural and are generally regarded as safe for use.

Materials and methods

Potassium persulfate, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), ethyl cellulose (22 cps, 48–49.5 % ethoxy), and ethanol were purchased from Sigma (St. Louis, MO). 18-Ω water was produced on a Barnstead water purification system (Waltham, MA). GrapeEX, grape tannin (GT) derived from grape seeds was obtained from Tarac Technologies Pty Ltd. (Nuriootpa, SA, Australia). The manufacturer established the GT to be 58 % phenolic content expressed as gallic acid equivalents.

Preparation of EC–GT blends

GT was dissolved in 70 mL ethanol to provide various proportions: 0.25, 0.5, 1.0, 2.0 and 3.0 % GT (*w*/*w*). The solution was shielded from light at room temperature and to these solutions 6 g EC was added slowly. The solutions were allowed to mix protected from light at room temperature for 90 min to ensure thorough blending of the compounds. The solutions were subsequently poured onto a 40 cm × 60 cm Teflon™ sheet and allowed to dry overnight at room temperature in the dark producing a film of approximate dimensions of 25 cm × 30 cm.

Free radical scavenging

Triplicate samples of 10 cm^2 total surface area of polymer were cut, weighed, rinsed with 20 mL of 18-Ω water and placed into 20 mL sealable vials. The

ABTS radical scavenging assay was performed using a modified method based upon Re et al. (1999). Briefly, a 7 mM solution of ABTS in water was reacted with a 2.45 mM solution of potassium persulfate to generate the ABTS radical following incubation at room temperature in the dark for 16 h. The stock ABTS radical solution was then diluted to an absorbance of 0.70 ± 0.02 or 1.40 ± 0.10 at 734 nm. 20 mL of the diluted solution was added to the vials containing the polymers and placed in a water bath at 30 °C for 60 min and shielded from the light. After 60 min the absorbance was measured.

The moles of the ABTS radical that had reacted was calculated and used for determination of % scavenged using the following equation:

$$\text{mol scavenged} = (I - F) \times (\text{mol ABTS}\cdot) \tag{1}$$

where $I = A_{734}$ of the ABTS · at $t = 0$ and $F = A_{734}$ of the ABTS · at $t = 60$.

$$\%\ \text{Scavenging} = 100 - \left[\frac{\text{mol ABTS}\cdot - \text{molscavenged}}{\text{mol ABTS}\cdot}\right] \times 100 \tag{2}$$

Leachability studies

Leachability studies were performed by the protocol outlined in *The United States pharmacopeia* (USP), monograph 661 (The United States pharmacopeia 2011). Briefly, 120 cm^2 surface area of control EC and blended EC films were placed into 20 mL of deionized water separately following two rinses with 20 mL of deionized water to remove any dust from the films and flasks. The flasks were placed in a 70 °C oven for 24 h. Each flask was swirled and 20 mL of liquid was extracted and placed into a clean, dry and weighed petri dish. The petri dishes were then placed in a 100 °C oven to evaporate the liquid extract and allowed to cool before reweighing.

Spectroscopic analysis

The ATR spectra were registered using a diamond ATR Smart Orbit™ accessory (from Thermo Optec) with a bouncing refractive infrared beam at 45° angle of incidence, using a DTGS detector with a KBr window and operating in the Mid-IR. ATR spectra of pristine polymeric film, GT extract and polymeric film functionalized with GT (conc. 0–3 %) were recorded placing the powder of tannic extract and small film fragments on the macro ATR crystal, and applying a pressure to optimize the contact. The spectra were obtained in the range of 4,000–650 cm^{-1} with resolution 4 cm^{-1} and averaged over 64 scans. Standard software (Omnic ESP, version 7.2) was used for data acquisition and analysis.

Tristimulus color determination

Color was determined for the EC films with and without the addition of GT utilizing a white background. Color values were determined making triplicate measurements with a Minolta colorimeter CR-300 (Minolta Camera Co., Ltd., Chou-Ku, Osaka, Japan) and recorded in the CIELab color system. The color system consists of a luminance component (L*), a green to red component (a*), and a blue to yellow component (b*), along with chroma (C*), a derivative value that represents the colorfulness in relation to the brightness of white.

Mechanical properties

Mechanical properties of the film samples were determined using an Instron 5567 universal testing machine (Norwood, MA) according to the ASTM D882 standard (ASTM standards [electronic resource] 2000). The gauge length was set to 80.0 mm at a crosshead speed of 5 mm min^{-1}. The test specimens were punched out of the films as 7 mm × 150 mm (width × length) strips and tested utilizing a 50 N load cell. The film thickness was measured at 3 locations along the strip and the measurements averaged. All measurements were undertaken with 10 replicates and the values averaged, giving an experimental uncertainty of ± 5 %.

Statistical analysis

All values reported are from replicate determinations with basic mathematical calculations and analysis being performed using Microsoft Excel 2011 for Mac. Further statistical analysis, one-way ANOVA with Tukey-HSD post hoc testing was performed with JMP 11 for Mac (SAS Institute Inc., Cary, NC).

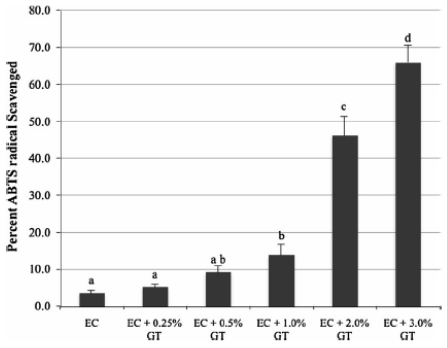

Fig. 1 Antioxidant activity of polymers expressed as percent ABTS radical scavenging for ethyl cellulose (*EC*) films with different additions of grape tannin (*GT*). *Bars* not connected by the *same letter* are significantly different ($p < 0.01$)

Results and discussion

Free radical scavenging of GT incorporated into ethyl cellulose

The incorporation of GT into EC introduces a radical scavenging functionality in this biodegradable polymer, Fig. 1. Compared to pristine EC, effective free radical scavenging activity by incorporating GT at a concentration as low as 0.5 % in EC was clearly seen. However, pristine EC did produce a small antioxidant response, which can be attributed to hydroxyl moieties being oxidizable. Using a two-way ANOVA for analysis, a significant increase ($p < 0.05$) of radical scavenging activity over control EC was obtained for GT concentration of 0.5 % and higher. The scavenging activity was concentration dependent with the range of activity observed in EC films containing GT at the various levels had a minimum of 5.3 ± 0.6 % for 0.25 % GT, and a maximum of 65.9 ± 4.6 % for 3.0 % GT, giving a 12-fold increase in activity resulting from a 2.75 % GT increase in the polymer mix.

Compared to recent studies on introducing antioxidant activity into polymeric materials by various antioxidants from natural sources, GT showed superior effectiveness in combination with EC. Arrua et al. (2012) found that incorporation of gallic acid or caffeic acid in poly(glycidylmethacrylate-*co*-trimethylolpropane trimethacrylate) and poly(*N*-acrylol-tris(hydroxymethyl)aminomethane-*co*-glycidylmethacrylate-*co*-*N*,*N*′-methylenebisacrylamide) polymers can introduce antioxidant activity of approximately 20 %, which could be further enhanced to approximately 50 %, as assayed by ABTS radical scavenging, when using a macro porous polymer. The porosity in the host polymer increased the exposed surface area, and as a result the antioxidant materials could readily interact with the oxidizing agent and thereby produce the expected higher antioxidant activity. However, porous materials have limited applications due to their low mechanical properties. Hence, in the present study the porosity of the matrix polymer was not utilized to enhance the antioxidant activity.

In another recent report by López de Dicastillo et al. (2013), a green tea extract, consisting of seven catechins and gallic acid, introduced free radical scavenging activity into polypropylene to 13 %, and this activity was greater than that of the individual phenolic compounds incorporated into polypropylene on their own. In both the Arrua and López de Dicatillo studies, the concentration of phenolic compounds added in the polymer matrices was approximately 16 %, which greatly exceeds the maximum of 3 % GT used in this study, yet both of the prior studies resulted in a lower scavenging ability than seen in the current study. The higher scavenging ability demonstrated with the GT could be a result of additive and synergistic affects associated with the phenolic compounds found in the GT as well as the diversity of phenolics providing antioxidant activity (Sivarooban et al. 2008; Wang et al. 2012). Additionally, an increased surface concentration of the incorporated phenolics can result in increased scavenging ability.

In a study of ethyl cellulose and polyaniline (PANI) Hsu and Kilmartin (2012) reported a significant antioxidant activity increase, using the oxygen radical absorbance capacity (ORAC) assay, from 5 to 9 % PANI with a more moderate increase from 9 to 17 % PANI. The trend observed by Hsu is also observed in the current study with an exponential increase in antioxidant activity up to 2 % GT followed by a more moderate increase from 2 to 3 % GT. This trend suggests that the surface concentration of ethyl cellulose can reach a maximum of reactive sites.

Physical attributes

Following the USP661 standard protocol, the amount of GT that leached from the 120 cm^2 total surface area

Table 1 Mechanical testing of EC–GT polymer blends

Sample	Yield strength (MPa)	Tensile strain at break (standard) (%)	Ultimate tensile strength (MPa)
EC	34.4 ± 2.4 a	1.8 ± 0.1 a	34.4 ± 2.4 a
EC + 0.25 % GT	34.9 ± 1.0 a	1.9 ± 0.1 a	34.9 ± 1.0 a
EC + 0.5 % GT	32.2 ± 2.7 a	2.1 ± 0.5 a	32.2 ± 2.7 a
EC + 1.0 % GT	31.4 ± 0.5 a, b	2.0 ± 0.3 a	31.4 ± 0.5 a, b
EC + 2.0 % GT	31.9 ± 2.5 a, b	2.1 ± 0.4 a	32.5 ± 2.3 a, b
EC + 3.0 % GT	28.0 ± 1.1 b	1.5 ± 0.1 a	28.0 ± 1.1 b

Values not connected by the same letter within a column are significantly different ($p < 0.05$). EC is ethyl cellulose and GT is grape tannin

of the EC blends was 4 mg or less for all concentrations, and well below the 10 mg limit (The United States pharmacopeia 2011). Thus, the majority of the active material remained within the EC carrier matrix and hence the observed free radical scavenging activity of these blended films is associated with bound GT on the surface of the polymer. It is worth mentioning that GT is a food grade antioxidant and would be safe even if it leached out into solution. However, for prolonged activity it is desirable to retain this active ingredient in the carrier matrix.

A recent report by Colon and Nerin (2012) investigating green tea extracts in polyethylene terephthalate examined the antioxidant activity and the polyphenol incorporation. From the study it was determined that the incorporation of the extracts into the films did not alter the composition of the phenolics. As a result, the phenolics retained their scavenging ability even when attached to the film, supporting the finding of the current study that the GT will remain incorporated and active within the blended films.

The mechanical properties of the films are shown in Table 1. The tensile strain at break was not significantly different over the range of added GT. At the 0.5 % GT concentration the antioxidant activity increased significantly over the control films and the mechanical properties showed no statistical significant difference. Compared to EC, until 1 % GT loading there was a marginal change in mechanical properties in the EC–GT blends, however, at 3 % loading the plasticization effect of GT was noted.

The intermolecular hydrogen bonding of the ethyl cellulose strands provides rigidity to the polymer films and as a result provides tensile strength. This slight decrease in tensile strength at 3 % GT loading is consistent with Cerruti et al. (2011) in a study of GT and a starch–copolyester blend, which showed a decreased tensile strength in the polymers containing 4 % GT. GT is a known plasticizer and its effects result in a decreased tensile strength and decreased yield strength. The GT molecules supposedly alter the hydrogen bonding of ethyl cellulose allowing for an increase in plasticity.

An evaluation of the transparency of the blends is shown in Fig. 2 and the color determination in Table 2. CIELab tristimulus color determination of the film samples show a decrease in the luminosity with greater GT inclusion, when measured on a white background. The hue of the polymers increased in red (larger a*) as the concentration of the GT increased, similarly it also changed from blue to yellow (larger b*). Chroma (C*), the strength of an objects color, was statistically similar in the 0.5 % films compared to the control films. At the same time, good transparency was evident (Fig. 2), which allows for utilization in multiple applications, including as a packaging material where contact clarity is required (Kunte et al. 1997).

Interpretation of molecular interactions between GT and EC

The superior antioxidant activity and the plasticization effect of EC films containing GT could be due to molecular interactions between these two materials. FTIR spectroscopic studies were performed to investigate this possibility. The FTIR spectrum of the GT (Fig. 3) was characterized on the basis of markers related to the moieties that could be involved on the formation of hydrogen bonds with ethyl cellulose; mainly the –H and –OH substituents of the B aromatic rings, and the etherocyclic pyrylium 6-member ring (ring C) of the monomeric flavan-3-ol unit.

Fig. 2 Transparency and coloration of the polymer films. *Each square* (**a**–**f**) contains a 4 × 4 cm^2 of film placed onto a *white background* with *black printing*, to display the coloration of films as well as the transparency, as depicted by the readability of text through the polymer. The *squares* are: **a** pristine EC; **b** EC + 0.25 % GT; **c** EC + 0.5 % GT; **d** EC + 1.0 % GT; **e** EC + 2.0 % GT; **f** EC + 3.0 % GT

Table 2 CIELab color coordinates for the ET–GT polymer blends

Sample	L*	a*	b*	C*
EC	92.02 ± 0.07 a	0.90 ± 0.06 a	−3.70 ± 0.08 a	3.80 ± 0.08 ab
EC + 0.25 % GT	91.09 ± 0.14 a	1.02 ± 0.04 ab	−1.85 ± 0.20 b	2.11 ± 0.15 a
EC + 0.5 % GT	89.19 ± 0.32 b	1.48 ± 0.03 b	1.76 ± 0.08 c	2.30 ± 0.04 a
EC + 1.0 % GT	87.78 ± 0.50 bc	2.16 ± 0.22 c	4.64 ± 0.96 d	5.11 ± 0.96 b
EC + 2.0 % GT	87.09 ± 1.02 c	2.53 ± 0.33 c	6.69 ± 1.20 e	7.14 ± 1.23 c
EC + 3.0 % GT	87.68 ± 0.55 c	2.48 ± 0.14 c	6.53 ± 0.23 e	6.98 ± 0.26 c

Values not connected by the same letter within a column are significantly different ($p < 0.05$). L* is luminosity where 0 is black and 100 is white, a* negative values are green and positive values are red, b* negative values are blue and positive values are yellow, EC is ethyl cellulose and GT is grape tannin

The spectral region 1,617–1,450 cm^{-1} includes the typical vibrational frequencies of the –C=C– bonds in the aromatic and heterocyclic rings (Ping et al. 2012). The strong 1,522 cm^{-1} band suggests a non-gallate procyanidin, due to the absence of the typical doublet occurring for flavanol gallate compounds; the band is related to skeletal stretching mode of the *o*-disubstituted aromatic ring, in particular the B ring of a flavanol or flavan-3-ol based compound (Foo 1981; Socrates 2001). The strong band at 1,609 cm^{-1} is related to the –C=C–O deformation of the heterocyclic C ring in the dominant planar *trans*-form, as the *cis*-form is less sterically constrained and produces an absorption band shifted toward higher frequencies values (1,640 cm^{-1} typically) (Foo 1981). The strong 1,440 cm^{-1} band is related to the in-plane vibration of pyrylium compounds, as for the 1,104 cm^{-1} band, attributed to the asymmetric C–O–C stretching in cyclic ethers (Socrates 2001). The broad band centered at 1,373 cm^{-1}, with a shoulder around 1,320 cm^{-1}, is attributed to the structure of the *o*-disubstituted phenolic ring; the shoulder, combined with the presence of a strong doublet (low resolution) around 1,057–1,040 cm^{-1}, is related to symmetric and asymmetric deformation of the C–O–H group, as well as the weak and broad peak at 770 cm^{-1} (Laghi et al. 2010; Nuopponen et al. 2006; Ping et al. 2012).

The spectral region 1,210–1,100 cm^{-1} is typically characterized by combination bands related to –OH moieties bonded to aromatic rings. The intensity of these bands suggests the occurrence of strong inter- and intra-molecular hydrogen bonds, which is a reliable

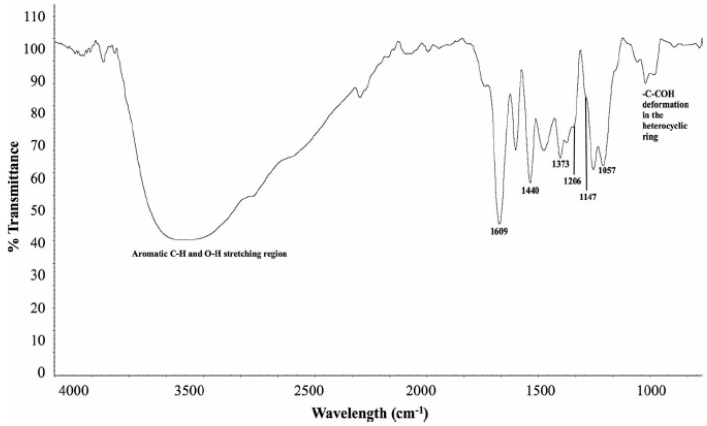

Fig. 3 ATR-FTIR spectrum of grape tannin extract (*GT*) with identifying bands labeled

hypothesis in the case of packed monomeric units of procyanidins. The 1,206 cm^{-1} peak is a combination band of C–O asymmetric stretching and –O–H in plane deformations. The same kind of combination is involved in the motion occurring as a shoulder at 1,147 cm^{-1}, with –O–H out-of-plane deformation. Even the 673 cm^{-1} band, broad and very weak, could be assigned to –O–H deformation vibrations (Silverstein 2005). In the fingerprint region, the weak signals in the region 840–795 cm^{-1} are typical patterns for the flavanol unit, in particular the –C–COH deformation in the heterocyclic ring. Being a very weak signal, the tannin is probably a mixture of *cis*- and *trans*-arrangements, with the second isomeric form being dominant. This observation is also confirmed by the attribution of the peak at 1,609 cm^{-1}, and this rigid configuration could affect the binding sites in the GT–C bonds formation (Foo 1981; Silverstein 2005).

The OH stretching occurs at relatively low frequencies compared with typical signals for phenolic compounds, suggesting the existence of an extended hydrogen bonding system that typically lowers the vibrational frequencies. The peak is centered around 3,297 cm^{-1}, but broadened consisting of an envelope of bands related to the different sites for the formation of hydrogen bonds between packed monomeric units (Pantoja-Castro and Gonzalez-Rodriguez 2011).

Because of its variability due to the formation of hydrogen linkage, the band related to –OH stretching vibrations is selected as a marker to show evidence of the interaction occurring between the polymeric film and the GT additive. An enlargement in the broad band related to –OH motions could be observed in the case of the functionalized film, being more evident in the 3 % GT-added cellulose film (Fig. 4). This is related to a broadening in the distribution of hydrogen bonds that involve different functional groups, while an interaction between ethyl cellulose and tannic extract occurs. According with the nature of this broad peak, characterized by long tails which usually occurs in the case of broad distributions of vibrational motions, a Lorentzian algorithm of deconvolution was performed to obtain the main peak components, (no baseline, FWHH = 5,000) in the region 3,545–3,120 cm^{-1} on the spectra related to polymeric film functionalized with 3 % GT extract. The resulting three main components in functionalized film are centered on 3,483, 3,394, 3,240 cm^{-1} (Fig. 5). The first peak is in good agreement with the component obtained for the simple polymeric film (3,478 cm^{-1}) and could be attributed to the stretching of –OH moieties in the ethyl acetate. The shoulder centered at 3,394 cm^{-1}, located at a wavelength too high to be attributed to tannins solely, occurs as a consequence of the binding of vacant sites in the polymer with the polyphenolic compound. The third component, also absent in the spectrum of the simple polymeric film, can be attributed to the –OH moieties in the added tannin, giving a good match with the band observed in the extract.

The region of skeletal motions (1,650–1,200 cm^{-1}) is almost invariant when comparing the simple

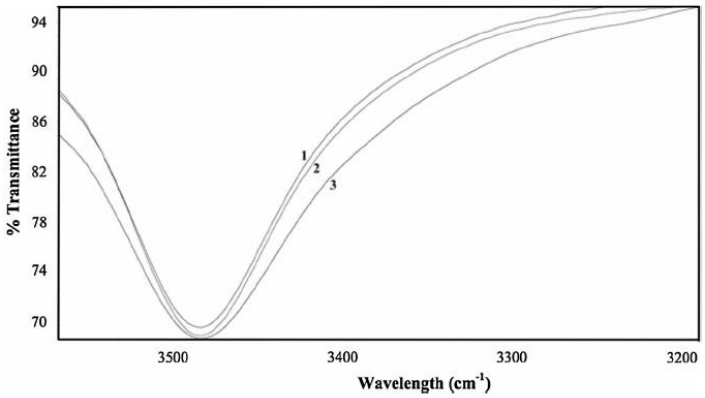

Fig. 4 ATR-FTIR spectra of: EC (*1*), EC + 1.0 % GT (*2*), EC + 3.0 % GT (*3*) displaying the changes in the aromatic C–H and O–H range 3,550–3,200 cm^{-1}. *Note* broadening and appearance of shoulder bands

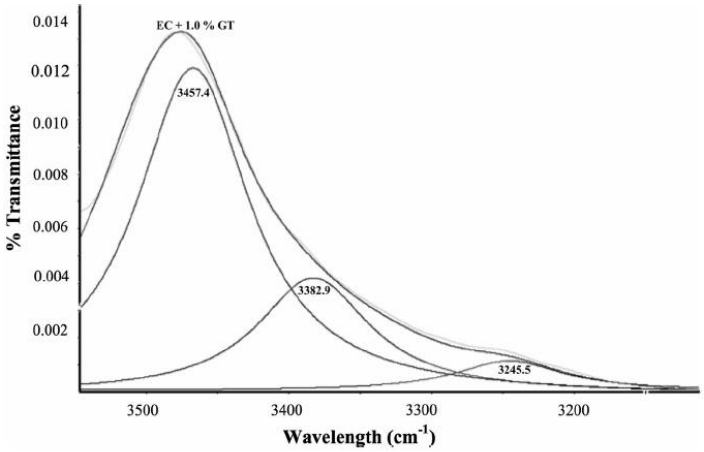

Fig. 5 FTIR spectrum of EC + 1.0 % GT, with the three main components obtained after Lorentzian deconvolution of the –OH peak in the range 3,345–3,120 cm^{-1}. The component *peaks* are centered at 3,245.5, 3,383.0, and 3,457.4 cm^{-1}, where shoulder bands appear on the FTIR spectra of the EC + GT polymer films

polymeric film (Fig. 6, curve 1) and the polymeric film added with 1 % GT (Fig. 6, curve 2). In the 3 % GT-added film, an increase in the tannin concentration lead to a band overlapping with the EC spectral features resulting in peak broadening and low spectral resolution (Fig. 6, curve 3). In both blended polymers (2–3) the region around 1,610–1,520 cm^{-1} related to the tannin functional groups (B and C ring of flavanols) underwent a variation. Motions related to EC did not significantly overlap this spectral region and the two strong peaks related to the flavanoid structure disappeared as a consequence of a chemical interaction between the polymer and the extract. A hypothesis for the decreasing intensity of the first peak could be that the hydrogen linkage formation at the heteroatom constricts the ester group motion in the rigid in-plane structure of the six-membered ring, while in the second case, the intra-molecular hydrogen bond that is formed between the –OH in the *ortho*-position is released, making the two sites available for intermolecular interactions.

In the same way, the typical motions in the fingertip region, in the range 770–840 cm^{-1}, decrease in intensity thereby resulting in an overlap with the weak vibrational motions of the polymeric film. The weak patterns of deformation of –C–C–OH groups in

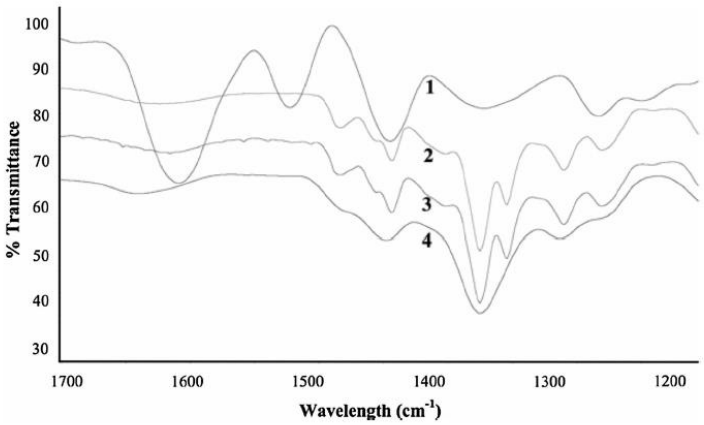

Fig. 6 Comparison of FTIR spectra for: GT (*1*), EC (*2*), EC + 1.0 % GT (*3*), EC + 3.0 % GT (*4*) in the spectral region 1,720–1,200 cm^{-1}

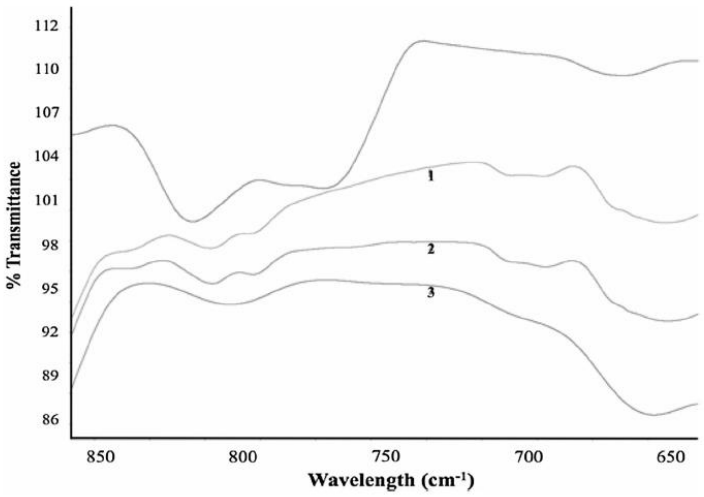

Fig. 7 Comparison of FTIR spectra for: GT (*solid line*), EC (*1*), EC + 1.0 % GT (*2*), EC + 3.0 % GT (*3*) in the spectral region 870–640 cm^{-1}

the B and C rings of the tannin extract are completely absent in both of the GT functionalized films, suggesting that the formation of a network of hydrogen bonds inhibits the weak skeletal deformations related to –OH moieties (Fig. 7).

The area of combination bands (1,210–1,100 cm^{-1}) overlaps the strong signals related to the polymeric film. For this reason, it was not taken into account, despite the presence of the important markers previously described.

The spectral interpretation confirms the presence of GT in the polymer films without any noticeable change in the chemical nature. As a result of the hydrogen bonding, GT incorporated into the polymer matrix still remains active and can offer effective antioxidant activity.

The typical hydrogen bonding formation between the chains of cellulose molecules and the plausible interaction between EC and GT are shown in Fig. 8. This molecular binding contributes towards the excellent non-

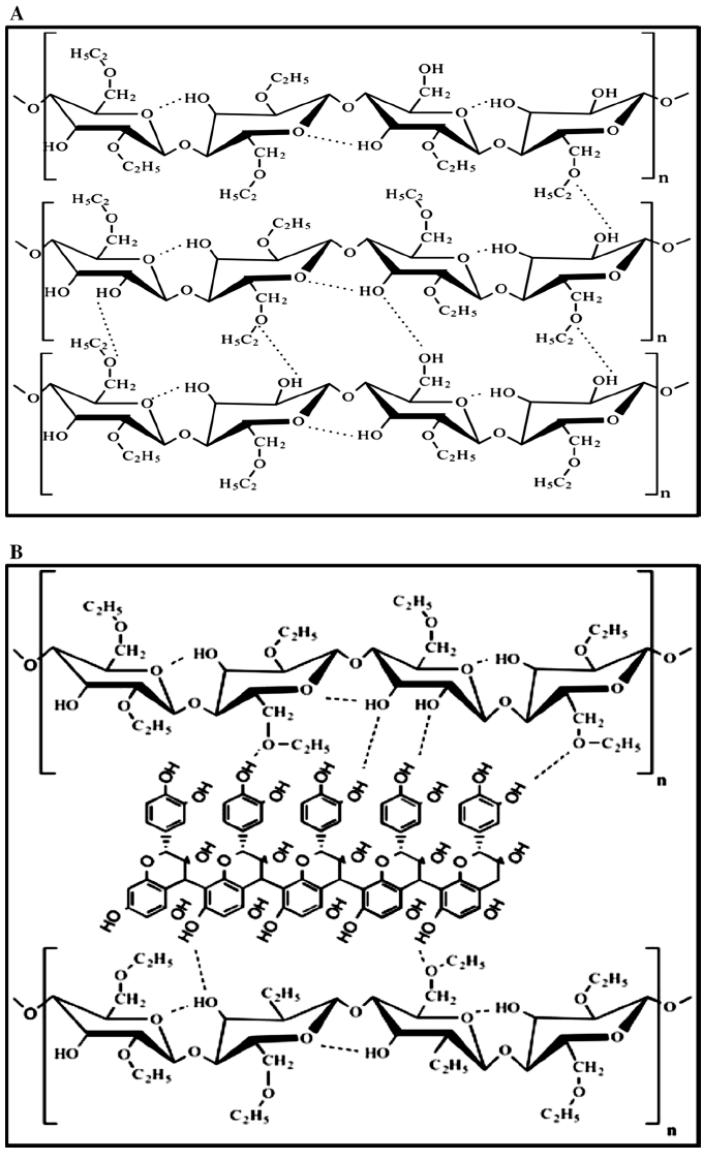

Fig. 8 Ethyl cellulose strand configuration and proposed incorporation of grape tannin into the polymer. *Dashed lines* represent potential hydrogen bonding of **a** ethyl cellulose, **b** grape tannin (represented by a 4–8 linked catechin oligomer) in ethyl cellulose

leaching phenomena of GT when blended with EC. Based on the above findings it can be hypothesized that in the EC–GT blends, GT endures intermolecular hydrogen bonding with EC and thus disrupts some original hydrogen bonding within EC matrix, which contributes towards the physicochemical properties.

Conclusions

The incorporation of GT into EC provides excellent antioxidant activity even at very low concentrations, from 0.5 % GT, as shown by the ABTS radical scavenging assay. Beyond 2 % loading GT imparts a plasticizing affect through the disruption of EC chain hydrogen bonding. The hydrogen bonding between the GT and EC provides secure molecular binding thereby providing low leaching from the matrix and a prolonged antioxidant affect. The low concentrations of GT incorporated allows for multiple applications since the polymer remains transparent and relatively unchanged in color. GT as a natural byproduct of the wine industry, which the Food and Drug Administration has classified as generally safe for use, can provide an economical source of natural antioxidant compounds for utilization within active polymers. Therefore, the present study indicates in combination with GT, EC films have potential perspectives for food packaging applications.

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Chapter 3

Monitoring the Oxygen Intake Along the Supply Chain

Chapter 3: Monitoring the oxygen intake along the supply chain

3.1. Introduction

The winemaking process following grape must fermentation implies further treatments, among other clarification, tartaric stabilisation, dosage of additives, bottling (or fining in tank/barrels prior to bottling). All these processes often involves the movement of large volumes of wine (up to hundreds of hectoliters) between the tanks in which treatments are carried out. The emptying and filling of tanks and the pumping of wine are considered critical points of the production chain, putting the wine in contact with atmospheric oxygen. Even when the company assist the wine movement with continuous nitrogen stream, it could be difficult to fully avoid it from contact with oxygen all over the process.

The development of oxygen meters with peculiar designs have enabled to measure oxygen in cellar using portable devices; different set up are provided to fit with the cellar equipment, as it was described in **section 2.5.2**. Emphasis was given to the measurement of dissolved oxygen in tank/barrels (Nevares, Del Alamo, Cárcel, Crespo, Martin, & Gallego, 2009; del Alamo-Sanza, & Nevares, 2014); the curves of oxygen

consumption measured in red and white wines stored in tank over different time scales have been previously reported (Fregoni, Fregoni, Ferrarini, & Spagnolli, 2004) and a further example of kinetic of oxygen consumption in a bottled white wine is presented in figure 3.1.

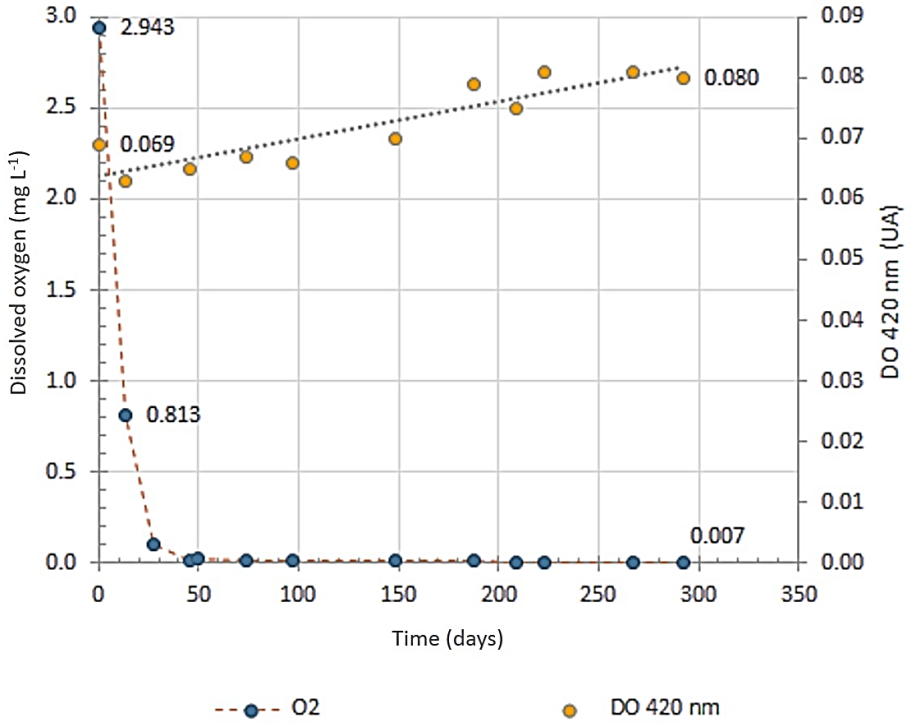

Figure 3.1. Timecourse of dissolved oxygen for a white wine and related increase in optical density for a Sauvignon blanc wine after bottling.

Nevertheless, an experimental design for the oxygen management following vinification until bottling was not provided so far, according to our knowledge.

The PhD project has involved collaborations with private companies to set up an experimental design that takes into account the production requirements and the structure of the cellar facilities to select the most appropriate sampling points. This section presents the results of monitoring the evolution of the dissolved oxygen (DO) content along the winemaking supply chain with focus on two configurations: (i) clarification, cold stabilisation, addition of oenological treatment, bottling implants; (ii) clarification, treatment with resins, addition of oenological treatment, bottling implants.

The random (or controlled, in the case of micro-oxygenation) contact with oxygen requires a continuous and systematic monitoring: an optimal oxygen management during production ensures optimal dissolved oxygen conditions at bottling, and in particular the knowledge of wine DO levels allowed to detect the critical points and to plan specific intervention to limit their occurrence.

3.2. Materials and Methods

3.2.1. Sample and Cellar Equipment

The experiment was set up in a private winery located in Emilia Romagna, Italy. An Italian table white wine, obtained from a blend of different grape varietals, was monitored from the end of fermentation until bottling/packaging.

The wine was stored in stainless steel tanks for the whole duration of the experiment. The sampling points based on the disposition of the facilities in cellar are schematically represented in **section 3.3.3**.

3.2.2. Measurement of dissolved oxygen

The dissolved oxygen (DO) content of wine was directly measured in tanks using a device which was composed by a tube to sample and to collect wine from the taps (**figure 3.2**), and a detection system based on an electrochemical cell where the sampled wine was collected (CboxQC instrument, Anton Paar, Austria). Results were compensated for temperature and expressed as ppb DO at 20°C. Dissolved oxygen was further analysed applying a fluorescent dot (PSt3 sensor, Nomacork, France) on the specula located at the exit of the tanks, and using the Nomasense O_2 P300 probe (Nomacorc, France) to collect and process the signal (**figure 3.3**). Results were expressed as ppb DO.

Figure 3.2. Attachment to tap for sampling using the QboxQC oxygen meter (Anton Paar, Austria)

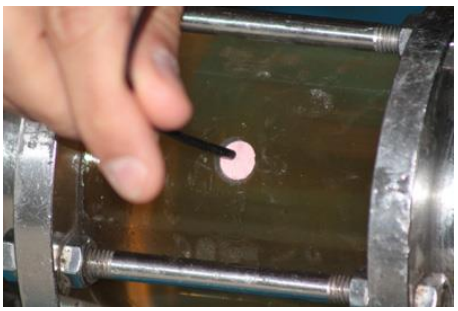

Figure 3.3. Measuring the DO through the glass of the specula using the PSt3 sensor (Nomacork, France).

Selected wine parameters: pH, free and total sulphur dioxide, total Fe, Cu, K were analyzed using a WineScan™ FT120 (FOSS, Hillerød, Denmark).

3.3. Results and Discussion

3.3.1. Definition of Optimal Measurement Conditions. A Comparative Approach

Figure **3.4** illustrates the experimental set up designed for the monitoring of oxygen in industrial tanks. An aliquot (65 hL) of white wine ready for bottling was fluxed with nitrogen to remove the CO_2 in excess then transferred in a 600 hL tank. The tap to connect the CboxQC meter was in correspondence of the liquid level, thus enabling the sampling (sample point n.1, **figure 3.4**). The specula using the PSt3 sensor was added in correspondence of the emptying of the tank (sample point n.2, **figure 3.4**). Nomasense enabled a continuous measurement during the pumping of wine from the tank to the bottling line, and the time-course for the whole process is illustrated in **figure 3.5**; the CboxQC meter allowed sampling with a frequency of 15 minutes.

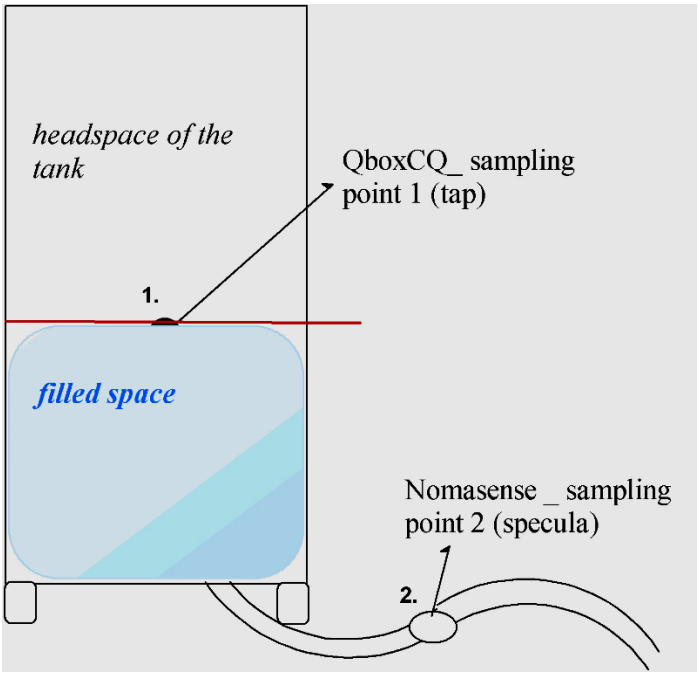

Figure 3.4. Experimental set-up for the measurement of dissolved oxygen according with the structure of the tanks commonly found in cellars.

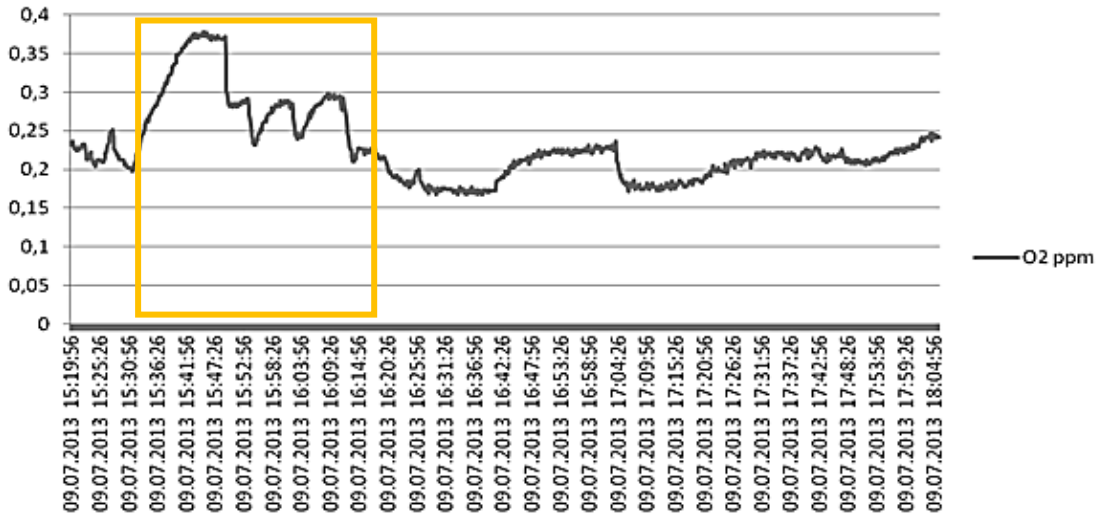

Figure 3.5. Time course of DO in the tank during the production process, measured in the specula with the fluorescent dot.

The temporary higher values (highlighted in yellow in **figure 3.5**) were due to the pumping of the wine (65 hL) into the tank. The following emptying of the tank ensured slightly constant DO values, with occasional peaks (**figure 3.5**) due to discontinuous occasional phases with time: e.g. work breaks, delays in the bottling plant, slowdown in the wine delivery. **Table 3.1** compares the DO values calculated with the two oxygen meters during the trial, from the time zero until the wine level of 55 hL; this limit is defined by the level of the tap in the tank. Thus, the positioning of potential sampling point and the volume of wine to be sampled during the specific process influences the most appropriate device for analysis.

Time	DO (ppb) QboxQC	T (°C)	DO (ppb) PSt3 sensor	T (°C)	Notes
3.11 pm	938	19	200	19	Start (level: 65 hL)
	907		250		
	1004		250		
	905		260		
4.14 pm	1048	20	210	19	Level: 60 hL
	1229		213		
	907		214		
	918		220		
6.04 pm	916	20	222	20	Wine level < 10 hL. Stop monitoring

Table 3.1. Comparative experiment for the electrochemical and fluorescence – based method for the DO monitoring.

Moreover, the different oxygen values measured by the two devices reflect the natural stratification of a gas in stationary conditions (non-mixing of the liquid mass); this is a

technical limit when large volumes of wine are involved in the monitoring processes, which can be overcome by inducing a mixing or adding of intermediate sampling points.

In general, the contribution of the technical staff working in the cellar would be necessary to build up an optimal and informative experimental design.

In the comparative experiment, wine sampled by QboxQC was near the interface of the liquid level with air, providing DO values high (although within the limit of 1.3 ppm) and unstable. The measurement of DO in specula were more stable and are less prone to fluctuations induced by pumping and interruption of the production cycle. Results were more representative of the oxygen dissolved in wine, allowing to monitor the whole process and showing that any significant oxygen intake occurred during the experiment: the slight increase of ≈200 ppb DO during the tank filling is compensated and the oxygen concentration stabilizes at lower values at the end of the experiment. We concluded that the wine reached the bottling line without undergoing undesirable oxygen increments.

The comparative approach was further applied in wines bottled and stored in the same conditions. **Table 3.2.** provides results for the measurements made on a white wine stored at room temperature (temperature fluctuations within 22-26°C). The results showed that the use of dot enabled to measure the dissolved and the head space oxygen concentrations, and consequently the total oxygen in the bottle; the electrochemical device with the sampling chamber, instead, provided results which were comparable with the parameter dissolved oxygen (DO) measured with dot.

The time required for analysis varied significantly using the two methods: the dot allowed to readily measure the DO values (estimated time: 2 minutes), while the electrochemical method provided a 15 minutes acquisition period, which can be a limiting factor when frequent samplings are needed.

Results of this preliminary trials enabled to define the field of applicability of the two gauges available for the experiment, taking advantage on their complementarity to optimize the experimental design.

			Nomasense (Nomacorc)			
Bott. N°	**vol.bott.**	**diam.int**	**T°**	**DO (ppm)**	HS (ppm)	TO (ppm)
1	0.75	20.5	22.8	0.14	0.71	0.85
2	0.75	20.5	22.8	0.12	0.75	0.87
3	0.75	20.5	22.8	0.14	0.75	0.89
			Average	**0.14**	0.74	0.87
			MIN	**0.12**	0.71	0.85
			MAX	**0.14**	0.75	0.89
			Cbox QC (AntonPaar)			
1	0.75		26.5	0.12		
2	0.75		26.5	0.11		
3	0.75		26.4	0.07		
			Average	**0.10**		
			MIN	**0.07**		
			MAX	**0.12**		

Table 3.2. Oxygen measurement data obtained with alternative devices. *DO Dissolved Oxygen; HS HeadSpace oxygen; TO total oxygen.

3.3.2. Pre-clarification of wine

Wine selected for the DO monitoring was stored in an external tank prior to delivery in cellar for oenological treatments. The clarification was performed the same day of delivery to the company then, after 3 days, the wine was filtered. **Table 3.3** lists the experimental conditions, the time-course of dissolved oxygen during clarification and the oenological parameters monitored. It could be noticed that the higher content of oxygen was detected following wine addition in the external storage tank. This is easily explained considering that the transport and transferring of wine didn't occur with inert gas protection, and that analyses were performed outdoors, enabling contact with atmospheric air oxygen. Addition of oenological products was planned by the company to protect wine in the critical passage of wine transport from outdoors to the cellar; as a consequence total sulfur dioxide increased, and the free SO_2 content changed from 9 to 13 mg L^{-1} (see **table 3.3, end of chapter**); according with theory, 1 mg L^{-1} O_2 enables the oxidation of 4 mg L^{-1} SO_2, but in this case the addition of 4 mg L^{-1} SO_2 resulted in a decrease of ≈0.6 mg L^{-1} of dissolved oxygen. The following hypotheses were formulated: (i) the reaction between O2 and SO2 did not reached the equilibrium at the time of O2 measurement (it is noteworthy that SO2 reacts slowly with O2); (ii) the presence of additional compounds that modify the theoretical stoichiometry reaction between O_2 and SO_2; (iii) the overestimation of DO parameter most probably due to the presence of atmospheric oxygen.

Sampling point	Production phase	Volume hL	Measuring device	Sampling point	Time	T °C	DO ppm	Free SO_2 ppm	Total SO_2 ppm	pH
Vinified mass storage	Wine stored in pre clarification	10.000	Immersion probe	liquid surface	09:45	17.2	1.86	9	91	3.45
		10.000	Immersion probe	intermediate	09:45	17.2	1.86			
		10.000	Immersion probe	max. depth	09:45	17.2	1.86			
		10.000	QboxQC	tank tap	09:45	19.0	1.9			
Vinified mass storage following clarification	at the end of clarification	10.000	Immersion probe	several points; av.	16:30	19.4	1.3	13	101	3.45
	after 72h clarification	10.000	QboxQC	tank tap	14:00	19.8	0.047	12	100	
Filtering process	during withdraw for filtering	n.d.	QboxQC	tank tap	14:00	20.8	0.04	12	100	3.46
	during withdraw for filtering_2	n.d.	PSt3 sensor	specula	08:30	23.1	0.5			
	beginning filtration	n.d.	PSt3 sensor	specola	09:00	21.3	0.9			
	end filtration	n.d.	QboxQC	tap	09:00	26.0	0.5			
	Filters collector	n.d.	QboxQC	tap_2	09:30	26.0	0.28	11	99	3.46

Table 3.3. Minitoring of DO and oenological parameters during the clarification process.

The dot inserted into the specula (**Figure 3.6**) allowed to monitor in continuous the flow of wine from the external container to the clarification tank, showing a systematic decrease of the amount of dissolved oxygen (**Figure 3.7**).

Figure 3.6. PSt3 sensor in specula used to monitor the DO during wine transport.

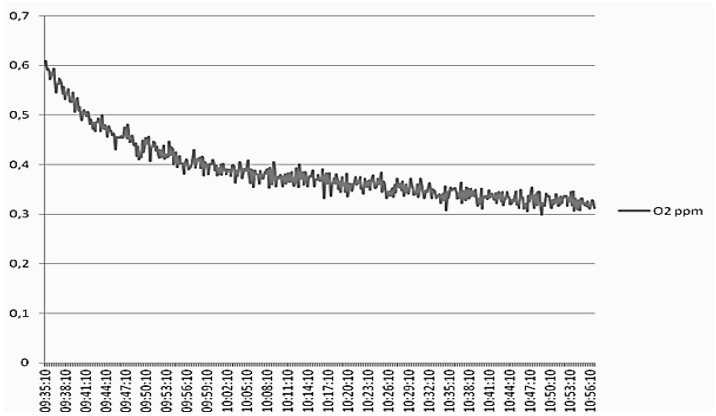

Figure 3.7. Route of the dissolved oxygen during transport as monitored by the PSt3 sensor.

The OD in wine reached very low values after 72h from the end of clarification (0.047 ppm), then underwent a slight increase after the transport and during the filtration; however, these slight fluctuations did not affect the SO_2 and pH parameter that remain constant.

3.3.3. Tartaric stabilisation, addition of oenological products, and bottling

Figure 3.8 illustrates the sampling points planned along the winemaking. The process provided the cold stabilisation of tartrates, followed by the addition of oenological treatments. The wine was moved along the supply chain using pumps and, occasionally, nitrogen flows; the pumping of wine and the sudden drop in temperature are considered critical points, due to possible exogenous oxygen intake and the increasing solubility of the gas at low temperatures. Therefore, it is important to monitor the DO concentration at the end of stabilization to add the optimal level of antioxidants before packaging.

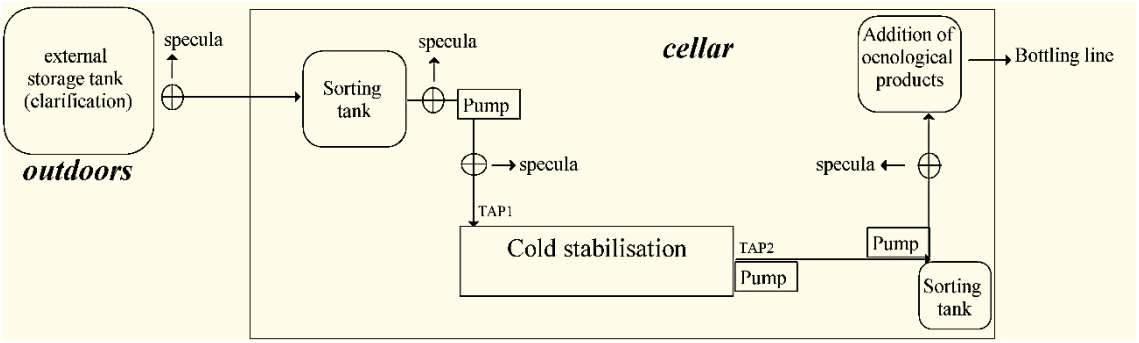

Figure 3.8. Disposition of facilities in cellar and identification of the sampling points.

The indoor monitoring provided sampling points distributed from the sorting tank to the cold stabilisation implant, then the measurement of DO in wine flowing through the joints till the tank where sulfur dioxide, ascorbic, metatartraric, citric acids, and copper metabisulfite were added. **Table 3.4** lists the results for sampling. The filling of the sorting tank with filtered wine was assisted by injections of nitrogen; nevertheless, an increase of DO was registered at this stage, reaching a value of 2.35 ppm in a total volume of 1000 hL.

The oxygen measured was stable prior the cold treatment; after this stage, DO almost doubled its value, reaching a concentration of 4.82 ppm. The oxygen is not prone to easily react with organic substrates in the absence of catalytic conditions (see **section 2.2**); it follows that the high oxygen intake at this stage is not harmful in short-time

Sampling point	Production phase	Volume hL	Measuring device	Sampling point	Time	T °C	Dissolved O_2 ppm	Free SO_2 ppm	Total SO_2 ppm	pH
Tartaric stabilisation	Storage in the sorting tank	1000 hL	QboxQC	tank tap	15:30	24.5	2.35	10	103	3.45
	Transport of wine to cold stabilisation	n.d.	PSt3 sensor	specula	15:45	25.8	2.73			
	Transport of wine after cold stabilisation	n.d.	PSt3 sensor	specula	16:30	1.5	4.82	9	100	3.44
Oenological treatments	Filling of the tank for oenological treatments	n.d.	PSt3 sensor	specula	17:00	10.6	4.5			
	Wine storage before bottling	600 hL	QboxQC	tank tap	17:45	24.0	2.4	30	135	3.39
Bottling	Delivery to the bottling line	n.d.	PSt3 sensor	specula	11:00	24.7	1.95	26	131	3.39
	Wine available at the bottling line	n.d.	QboxQC	tank tap	16:30	26.0	0.7	23	132	3.39

Table 3.3. Monitoring of DO and oenological parameters during the tartaric stabilization, addition of oenological products, bottling.

scales for the wine conservability, when the oxygen excess is removed within the bottling stage. When increasing temperature to reach room conditions, the oxygen level decreases to stabilize at DO levels < 1ppm in the absence of pumping, high values of transition metals or further temperature fluctuations. Parameters monitored at bottling are then: DO (0.7 ppm); iron (4.29 ppm); copper (0.23 ppm); potassium (710 ppm). The oxygen level at bottling was in agreement with values previously measured on wines bottled without microoxygenation pre-treatments (Castellari, Matricardi, Arfelli, Galassi, & Amati, 2000), and it has to be managed with an appropriate selection of packaging and closure (see **chapter 4**). Potassium (K) is usually present in high concentrations among major metals in wine, ranging between 10–10^3 mg l^{-1} (Pohl, 2007). Precipitation of K-tartrates induce a variation of pH in wines, which extent is related to the buffer capacity of wines. In this experiment the slight decrease in the pH parameter (-0.06 units) confirmed that wine acidity was stable after the industrial processes.

In general, the oxidation of transition metals in acidic media occurs following very slow kinetic rates, but it is likely to increase at increasing pH values, enhancing Cu and Fe oxidation or forming Al, Cu and Fe clouding. As an example, at increasing pH values the concentration of [Fe(III)] oxidised phase drastically decrease, due to hydrolysis, and Fe(II) is more likely to be oxidised. In particular, it was observed that below pH 4 Fe^{2+} is the dominant equilibrium species in solution and its oxidation rate is very low, and fundamentally independent from pH; reversely, the kinetic rate increases over the range 4<pH>6, being strongly dependent on pH value and concentration of solvated iron ions formed ([$Fe(OH)_2^0$] species). The rate constant of Fe(II) oxidation differ in the two cases by five orders of magnitude, being 6x10-5 min-1 when the Fe^{2+} species is dominant (pH<4), and increasing at increasing pH values to reach 4.3x105 min^{-1} (Morgan & Lahav, 2007).

Both metal oxidation and clouding affect wine conservation (McKinnon, & Scollary, 1997; Mozaz, Sotro, Segovia, & Azpilicueta, 1999). Cu and Fe contents have exogenous and endogenous origins: besides the concentration naturally present in grapes, technological treatment and long contact of wine with cellar equipment could increase metal concentrations (Sauvage, Frank, Stearne, & Millikan, 2002). $CuSO_4$ can be added to wine to remove H_2S, thus limiting the formation of mercaptans and the development of undesirable odours, represents an exogenous source of copper (Galani-Nikolakaki, Kallithrakas-Kontos, & Katsanos, 2002), which has to be limited to avoid excess oxidation reactions. Nevertheless, the total Cu and Fe concentrations determined at bottling ranges between the values 0.1–100 mg L^{-1} expected for table wines (Pohl, 2007), and lied in the legal limits for "quality wine" (8 mg L^{-1} for iron and 0.5 mg L^{-1} for copper) set by the European Union (Danilewicz, 2007).

Sulfur dioxide underwent a slight decrease at bottling when compared with the concentration added (total SO_2: 135 →132 ppm; free SO_2: 30 → 23 ppm) in the time – scale of 24h. The final level of free sulfur dioxide provide a potential protection from oxidation during shelf-life of a white wine, if combined with a proper packaging which regulates the OTR levels (Ugliano, Kwiatkowski, Travis, Francis, Waters, et al., 2009).

3.4. Conclusions

The monitoring of the dissolved oxygen along the supply chain was demonstrated to be an effective tool to determine the optimal concentration levels at wine bottling. The measurement of DO coupled with the monitoring of few oenological parameters (pH, sulfur dioxide, free metals) enabled to detect critical points of the production process, to limit potential sources of oxygen intake and/or contamination of wine with oxidation catalysts, and to plan the appropriate concentration of oenological products and the suitable packaging to protect wine during shelf life. A proper design of experiment requires the collaboration with the technical staff working in cellar, to optimize information provided by selected sampling points. The combined use of different devices for the measurement of oxygen levels allowed versatility in the analysis, and the identification of the most appropriate set-up was influenced by the structure of cellar facilities and the production needs. The development of fast and reliable

analytical methods would implement the application of this experimental design as a routine quality control for wine companies.

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Chapter 4

Shelf-Life Trials:

Prediction of oxidative status in bottled wines

In the last decade the production of food packaging has undergone major developments with regard to the use of new synthetic materials with high performances. The wine industry has embraced this new trend and the sector of wine closures has been particularly affected, for the chance to work alongside traditional cork closures with synthetic high-performance materials whose technological characteristics can be designed in advance.

This enables the management of oxygen in contact with wine during shelf-life; as demonstrated in the previous section, the oxygen intake is one of the main factors responsible for the exposure of wine to oxidative degradation (maderization and loss of physical - chemical and sensory properties).

Beyond the technological potential, alternative closures are becoming an important part of the global wine market: vine cultivation has undergone in recent decades a great expansion worldwide, and the industry in cork closures has responded to increased demand with an intensive exploitation of raw materials, which determined a progressive decline of its availability (Bugalho, Caldeira, Pereira, Aronson, & Pausas, 2011).

Cork remains the most exploited material for the production of wine stoppers so far, affecting 70% of the closures employed on a world scale. Europe and North Africa are the main producers of cork oak (Quercus Suber), and in particular Portugal produces 52% of the estimated 300 thousand tons per year worldwide (*source: Amorim Cork S.p.A. 2007*). The production of wine corks absorbs 69% of the cork industry production, distributed between natural technical corks. Italy ranks third among world producers, with 5.5% of total production estimated in 2007 (*source: Amorim Cork Italy S.p.A. 2007*). Sardinia is the main production site of the raw material on a national scale with a high concentration of manufacturing companies and processing industries within the region. 90%

of natural cork produced in Italy comes from the regions of Gallura, Ala and Budduso, and from the territories of Marghine land, Goceano or Sulcis (*source: United Nations Statistics Division 2006*).

Since the end of the Nineties European and Italian authorities have tried to cope with phenomena of depletion of raw material due to intense exploitation, with wooded replenishments policies supported by the European Union (as an example *Reg. EEC 2080/92* and EC *Reg. 1257/99 of the Rural Development Plan of Sardinia*, to support slow-growing tree species). Nevertheless, it is estimated very long time for reforestation to make the production of corks sustainable, and the current demand on a national scale is met in part by importing raw material to be used in the processing industry.

In addition, the cork can in some circumstances highlight the limitations of the high variability of a natural material, which was explained in section 4.4., and it susceptibility to microbial attack which can adversely affect the aging of wine with the development of mold; the so-called hints of "cork" are due to the development of 2,4,6-trichloroanisole (TCA) due to the metabolism of the fungus *Armillaria mellea*, parasite of the cork oak.

New wine producers as California, South America, South Africa, Australia, New Zealand, have given impetus to the use of synthetic corks, screw-cap, crown-cap, produced with the use of materials which are inert to the proliferation of microorganisms and free from typical phenomena of contamination and degradation of the natural materials.

The synthetic materials allows to control parameters such as compactness and porosity, and to adapt the performance of the cap to the specific needs of the product through the control of the oxygen transfer rate (OTR) in the bottle; on the other hand due to their recent introduction in the wine market, they require a careful assessment of their technical performances in

time and their tailored use in different varieties of wine (young wines, wines for aging).

It must further be considered the cultural impact on consumers; according to a survey of 2007 US consumers did not consider the screw cap a valid and stylish alternative to cork or synthetic cork (Marin & Durham, 2007), and only in recent times its use as been considered synonymous of quality in the consumer perspective (Mueller Loose & Szolnoki, 2012). According to a study by Barber et al. (Barber, Almanza, & Donovan, 2006), women consider wax seals and aluminium closures significant indication of a quality wine while men believe that the corks are important indications of the quality of a wine. In general, certain types of prejudices related to the world of wine and its consumption as an expression of social status, influence the choice of a traditional closure products over those made of plastic, by connecting them to a cheap and low quality product. According to a study by Hall et al. (Hall, Shaw, Lascheit, & Robertson, 2000), the screw caps are considered indicators of an inexpensive wine, while natural cork stoppers are the expression of a high quality product. A further factor influencing the consumer feedback is the relationship between producers, retailers and the media; by way of example, the extensive development of screw-cap in Oceanic areas (Australia and New Zealand) is the result of an intensive promotion campaign supported by academic studies (Barber, Taylor & Dodd, 2008).

In this perspective the shelf life studies, according with the model presented in this chapter enable: (i) to make sustainable production while respecting the availability of the natural product, (ii) to assign to each product packaging that is able to bring out the most of the property and (iii) to limit the economic impact of the production and trading of wines.

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4A) KINETICS AND THERMODYNAMICS OF THE BROWNING ONSET IN WHITE WINES

RESEARCH ARTICLE:

Modelling the evolution of oxidative browning during storage of white wines: effects of packaging and closures.

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(UNDER REVIEW)

4.1. Introduction

Traditional winemaking mostly use cork closure, glass bottle and sulfur dioxide (SO_2), to protect wines from oxidative damages; however, today's consumers are more concerned about the abuse of SO_2, and there is an increasing awareness of sustainability and the use of 'green' product. In this view, new packaging can represent an alternative for the wine industry to implement their "environmental friendly" products. Despite the use of different alternative packaging has been implemented in recent times, there is a lacks of a systematic study on their long-term impact in wine quality. The ideal wine packaging has to face the constant physico-chemical and sensory changes occurring in wine for the whole duration of its shelf-life; in particular phenolic and volatile constituents undergo significant modification as a consequence of ageing and oxidation processes (Lief, 1965; Styger, Prior, & Bauer, 2011); in this view, there is a great interest in the prediction of wine aging to optimize the conservation and improve shelf life.

The contribution of packaging to the preservation of wine quality is due to a complex balance between intrinsic processes occurring in wine itself and the influence of surrounding environment, mainly through temperature fluctuations and gas permeation/migration (Bakker & Clarke, 2011).

Browning is the common name for the colour modification in bottled wine, which turns to brownish hues as a consequence of oxidative stress and the formation of condensation by-products (brown pigments). The browning phenomenon affects wine conservation leading to the detrimental changes in colour, aroma and taste of wine (Godden, Lattey, Francis, Gishen, Cowey, Holdstock, et al., 2005; Li, Guo, & Wang, 2008). The changes in sensory properties mostly consist of browning occurrence, an increase in bitter taste and a loss of pleasant fruity aromas and the appearance of unpleasant notes, which have been previously classified like "honey-like", "farm-feed", "hay", and "woody-like" (Silva Ferreira, Guedes de Pinho, Rodrigues, & Hogg, 2002; Silva Ferreira, Hogg, & Guedes de Pinho, 2003).

All this considered, there is a need of further investigation to disclose the complexity of wine browning and to provide the winemakers with practical information for improving the shelf-life of their products. The method currently used to monitor the occurrence of oxidation in wine is the measurement of the increase in optical density (OD) at 420-440 nm during time, as monitored using UV-vis spectroscopy (Singleton & Kramlinga,

1976). Accelerated aging tests have been developed to artificially reproduce the wine ageing in bottled wines (Fernandez-Zurbano, Ferreira, Pena, Escudero, Serrano, & Cacho, 1995; Kallithraka, Salacha, & Tzourou, 2009; Singleton & Kramlinga, 1976). The most exploited methods consist on (i) saturating the wine bottled wines with oxygen or (ii) subjecting wine to increasing temperatures. It is well known that the kinetics of chemical reaction is affected by increasing temperature, which enhance browning of wines depending on further variables like wine composition, and packaging (Danilewicz, 2012; Robertson, 2009; Ugliano, 2013; Waterhouse & Laurie, 2006). Wine browning has been extensively described by a mechanistic point of view (Danilewicz, 2012; Li, Guo, & Wang, 2008); regarding the kinetic description, browning can resemble a zero-order (Salacha, Kallithraka, & Tzourou, 2008; Serra-Cayuela, Jourdes, Riu-Aumatell, Buxaderas, Teissedre, & López-Tamames, 2014; Sioumis, Kallithraka, Makris, & Kefalas, 2006), a first-order reaction (Berg & Akiyoshi, 1956), or a two-stage model likely described by a kinetic mixed order including both the initial formation of coloured polymeric compounds, and their dissociation yielding a decrease in colour intensity (Pérez-Zúñiga, Abad, & Cartagena, 2000).

In this study the evolution of browning in white wines from bottling (time zero) and during storage (up to 10 months) was monitored by measuring selected oenological parameters and including kinetic and thermodynamic descriptions of this phenomenon; the kinetic of browning onset was calculate using a zero-order kinetic equation which consider the increase in optical density (OD 420 nm) for the duration of the experiment. Wines were also subjected to accelerated aging under controlled temperature conditions (thermal cycles of 20 – 30 – 35 – 40 – 45 – 50 – 55 – 60° C, 10 days) and the temperature dependence of the oxidation rate constants was modelled using the Arrhenius equation, which was used to estimate the apparent activation energy for the browning onset. In addition quality parameters measured in bottled wines were compared, to establish the influence of storage conditions in the quality of Italian commercial white wines.

4.2. Materials and Methods

4.2.1. Wines and samples treatment

Six commercial white wines vintage (2012) including one Sauvignon **(A)** , two Chardonnay/Trebbiano blends **(B-D)**, one Chardonnay **(C)**, one Albana **(E)** and one Muller Thurgau **(F)** from different Italian regions and sealed in different containers were selected for this study; the codification, packaging and closures are described in detail in **Table 1**. The bottled series of each wine were kept in dark, upright and at room temperature (20±1°C) for ten months and analysed with time for the following parameters: alcohol, total polyphenolic compounds (TPC), pH, total and volatile acidity, free and total sulphur dioxide, total iron and copper, dissolved oxygen and optical density at 420 nm. The antioxidant activity (AA) in terms of ability to scavenge radicals has been measured for wines at the beginning and the end of the experiment. After the first analysis (time zero), the sampling frequency was empirically determined considering the rate of variation between subsequent measures. All samples were analysed in duplicate.

4.2.2. Chemicals and colorimetric kit

Distilled water used to prepare working solutions were obtained using a MilliQ water system (Millipore, Bedford, MA). Fuming hydrochloric acid (37%), sodium hydroxide (99%), and iodine solution (0,1 N) were purchased by Merck (Merck, Darmstadt, Germany); starch paste for sulphur dioxide titration (1% w/v) was purchased by Carlo Erba (Carlo Erba, Milano, Italy). 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical for the radical scavenging assay, gallic acid and $Fe_2SO_4 \bullet 7H_2O$ as reference standards for colorimetric determinations were supplied by Sigma (Sigma–Aldrich, Saint Louis, USA); the quantitative colorimetric kit for the determination of total iron in wine was provided by Steroglass (Steroglass, Perugia, Italy).

4.2.3. Measurement of dissolved oxygen

The dissolved oxygen in bottled wine was measured using a device which was composed by a sampling chamber, a probe to perforate the cap and to collect the sample under continuous nitrogen stream, and a detection system based on an electrochemical cell where the sampled wine was collected (CboxQC instrument, Anton Paar, Italy).

The whole system was isolated from atmospheric oxygen for the whole duration of the experiment and kept at the room temperature.

4.2.4. Spectrophotometric determination

The TPC was determined at 280 nm on a 10 mm quartz cuvette with value corrected by dilution and expressed as mg L^{-1} Gallic Acid Equivalents (GAE) (Ribéreau-Gayon, 1970). The increase in OD at 420 nm was monitored on undiluted samples using a 10 mm quartz cuvette (Sudraud, 1958), and absorbance values were used to calculate kinetic rates and activation energies for the browning process. The total iron concentration was determined using a quantitative colorimetric kit which provides the measurement of a complex formation of iron with a molecular probe, absorbing at 593 nm; results were expressed in mg L^{-1} of Fe (total) ions. The DPPH• assay was measured as an index of radical scavenging activity of wines, using the original procedure described by Brand-Williams et al. (Brand-Williams, Cuvelier, & Berset, 1995); results were expressed as % of disappearance of the radical (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). A Shimadzu UV mini 1240 spectrophotometer was used for all determinations (Shimadzu, Kyoto, Japan).

4.2.5. Copper determination

Determination of total copper concentration in wines was carried out with a PSA ION3 Stripping Electrochemical Analyser, supplied by Steroglass (Perugia, Italy) and composed by a 3 mm diameter glassy carbon working electrode, a platinum counter electrode and a [Ag /AgCl] reference electrode, all immersed in a 40 ml vessel fitted with an electrical spiral stirrer. The analyser is equipped with a NEOTES software package (Steroglass, Perugia, Italy).

4.2.6. Oenological parameters

Chemical parameters with oenological meaning were measured according to standardised methods of the "*Office International de la Vigne et du Vin"* (AOAC® Official Methods℠): alcohol content, pH, free and volatile acidity, free and total sulphur dioxide (OIV, 2014).

4.2.7. Calculation of kinetic and thermodynamic parameters

Kinetic rates. The formation of brown pigments as oxidation by-products was expressed in terms of a rate constant, *k*. The zero-order kinetic law (4a) implies a one-step reaction where the polyphenols irreversibly degrades to brown polymers, increasing the $OD_{420\ nm}$; the equation was applied to the real systems and under accelerated aging conditions, and results were expressed in A_{420nm} $months^{-1}$.

$$OD_{420nm} = kt \quad (4a)$$

Zero-order rate equations in the form (4b) were assumed to describe the consumption rate of free SO_2, in the hypothesis that the oxidizable substrates and the molecular adducts formed by sulphites during SO_2 oxidation are not themselves involved in the rate-determining step of the two processes. Results were expressed as mg L^{-1} $month^{-1}$ of titratable free sulfur dioxide.

$$\Delta SO_2 = kt \quad (4b)$$

Temperature dependence and Arrhenius activation energies. High temperatures were used to simulate the ageing of wines. To set up the experiment, 22 ml from the first sampling of each wine were poured into glass vials and sealed under nitrogen; then vials were stored in an air thermostat device at the constant temperatures of 20, 35, 40, 45, 50, 55, 60 (±1)°C up to 10 days, avoiding from wine exposure. Zero-order kinetic rates calculated for each temperature-controlled experiment were collected to estimate the apparent activation energies (Ea) of the browning process through the integrated Arrhenius equation (4c):

$$\ln k = \ln A_0 - (Ea/RT) \quad (4c)$$

where:

- A_0 is the preexponential constant;
- Ea is the activation energy for the process (kJ mol^{-1});
- R is the universal gas constant (8.314 J K^{-1} mol^{-1});
- T is the temperature (K).

Activation energies were easily achieved plotting the natural logarithm of kinetic constants (ln*k*) against the reciprocal of temperatures (1/K), and expressed as kJ mol^{-1}; high coefficients of linearity were reached for each experiment ($R^2 \geq 0.98$), confirming that the Arrhenius model could be applied to predicting the browning onset in wine.

K, the zero-order rate constants, were converted in mol^{-1} s^{-1} units for thermodynamic calculations using the extinction coefficient of pyrogallol at 420 nm (12 M^{-1} cm^{-1}) previously calculated by Saeidian under oenological pH conditions (Saeidian, 2012).

4.2.8. Statistical analysis

XLStat-PRO v. 7.5 software (Addinsoft, Ney York, NY) was used for data storage, basic statistics, and correlation and to determine the kinetic constants (*k*) and the apparent activation energies (Ea) of browning in white wines. Principal Component Analysis (PCA), an unsupervised multivariate exploratory method, was used to monitor the shelf-life performances of wines; for this purpose, the Unscrambler 9.7 software package was used (Camo ASA, Norway).

Analytical determinations were provided in duplicate.

4.3. Results and Discussion

4.3.1. Wine composition and packaging

Table 4.1. reports the properties of wine at time zero, and the trend of such parameters (↓; ↑; =) for the duration of the experiment; some variations within the same batches were observed due to practical constraints of the bottling process, including oxygen supply, occasional defective equipment or occasionally contaminations in cellar. All values respect the limit set for Italian table white wines, in particular the total sulfur dioxide is within the legal limit of 200 mg L^{-1} (Usseglio-Tomasset, 1992), with samples D and E exhibiting the highest values of the series (160 mg L^{-1}). The pH at bottling ranged between 3.2-3.4 and was unaffected by storage time for all samples, regardless the wine varietal and in agreement with total acidity and alcohol content parameters which were constant as well. Volatile acidity, mainly induced in wine by the presence of acetic acid and ethyl acetate, showed a significant increase for samples C (+53.4%) and D (+66.6%), both stored in tetra pack, the latter being an organic wine; anyhow, the final values were under the sensory threshold of 0.6-0.9 g L^{-1} reported in literature (Goode & Harrop, 2011), and within the legal limit of 1.1 g L^{-1} (Vilela-Moura, Schuller, Mendes-Faia, & Côrte-Real, 2008).The significant increase in volatile acidity for both wines stored in tetra pack is likely related to the well-known problem of oxygen permeability of composite packaging like brick and bag-in-box, as already

described in previous works (Ghidossi, Poupot, Thibon, Pons, Darriet, Riquier, et al., 2012; Revi, Badeka, Kontakos, & Kontominas, 2014) and reviewed in chapter 2; reversely, some authors reported that tetra pack packaging can provide a protection against oxygen permeation that was comparable to glass bottles up to one year storage (Buiatti, Celotti, Ferrarini, & Zironi, 1997). The systematic increase in the TPC over ten months for all series could be tentatively ascribed to a rearrangement of monomers into polymeric structures (phenolic polymers and brown pigments); the polymeric compounds occurred following hydrolysis, oxidation and condensation reactions as a consequence of chemical oxidation of organic substrates, light exposure, variations in temperature and storage time (Kallithraka, Salacha, & Tzourou, 2009; Recamales, Sayago, González-Miret, & Hernanz, 2006), and these polymerised adducts could likely exhibit higher extinction coefficient (ε) at the 280 nm wavelength. In this perspective, it has to be noticed that the variation in optical density at 420 nm during the period of the experiment (ΔA_{420nm}), which it is the main consequence of the formation of brown pigments, exhibited a significant linear correlation ($R^2 = 0.93$) with the increase in the total polyphenol content calculated with the spectrophotometric method (ΔTPC_{280nm}) over the same period (**figure 4.1)**.

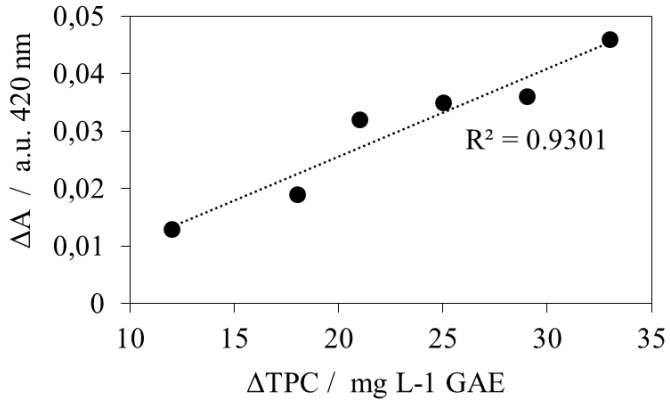

Figure 4.1. Correlation between the increase in calculated TPC and the browning occurrence in the white wines analysed in this work.

A slight decrease of the antioxidant activity (AA) in terms of DPPH radical scavenging was observed, although not significant. As the free polyphenolic compounds exhibit higher reactivity than their conjugated derivatives (Frankel, Waterhouse, & Teissedre, 1995; Vinson & Hontz, 1995) the drop of antioxidant activity after ten months could be partly due to the involvement of phenolic compounds in the formation of molecular addicts which stabilise reactive oxidation products. Furthermore, the poor correlation between the increase of TPC value and the decrease of AA during the experiment (R^2=0.62) confirmed the findings of previous experiments (Zafrilla, Morillas, Mulero,

Cayuela, Martínez-Cachá, Pardo, et al., 2003) and disclose the complexity behind the chemistry of wine polyphenolics.

Sulfur dioxide is an active antioxidant largely used in winemaking in wine, and its protective effect is also related to oxygen balance - driven by permeation and migration - which is affected by closures and packaging (see next section for details). Dissolved oxygen at time zero varied from 2.94 to 0.19 (**table 4.1**) and showed an initial rapid decrease mainly driven by law mass action, followed by a steady-state equilibrium with lack of significant difference among the tested packaging and closures after 6 months of storage.

Code	**A**	**B**	**C**	**D**	**E**	**F**
Wine	Sauvignon	Chardonnay/ Trebbiano	Chardonnay	Chardonnay/ Trebbiano	Albana	Muller Thurgau
Packaging	glass bottle	glass bottle	tetrapack	tetrapack	glass bottle	glass bottle
Closure	technical cork	screw cap	TwistCap OSO 34	TwistCap OSO 35	synthetic	natural cork
Parameter						
pH	3.38 =	3.40 =	3.20 =	3.21 =	3.22 =	3.39 =
Alcohol (% vol)	13.0 =	12.0 =	12.0 =	10.0 =	13.5 =	12.3 =
*TPC (mg/l GA)	222 ↑	234 ↑	239 ↑	199 ↑	258 ↑	201↑
Free SO_2 (mg/l)	50 ↓	36 ↓	31 ↓	30 ↓	47 ↓	55 ↓
Total SO_2 (mg/l)	104 ↓	116 ↓	105 ↓	160 ↓	160 ↓	123 ↓
*Volatile acidity (mg/l AA)	0.48 ↓	0.48 =	0.21 ↑	0.14 ↑	0.57 =	0.42 =
*Total acidity (mg/l TA)	4.73 ↑	5.25 =	5.3 =	5.3 =	6.5 =	5.7 =
Total Fe (mg/l)	5.22 ↓	10.69 ↑	3.9 ↓	15.12 ↓	4.35 =	1.73 =
Total Cu (mg/l)	0.072 =	0.417 ↑	0.095 =	0.084 =	0.127 =	0.02 =
Dissolved Oxygen (mg/l)	2.94 ↓	0.27 ↓	0.38 ↓	0.21 ↓	0.55 ↓	0.19 ↓
O.D. 420 nm (A.U.)	0.063 ↑	0.079 ↑	0.069 ↑	0.050 ↑	0.076 ↑	0.063 ↑
DPPH Radical scavenging (%)	74.7 =	80.2 =	90.6 ↓	92.0 ↓	83.5 ↓	83.9 =

*Legend: TPC: Total Polyphenol Content; GA: Gallic Acid; AA: Acetic Acid; TA: Tartaric Acid. (↓) decreasing; (=) stable; (↑) increasing).

Table 4.1. Oenological parameters of commercial white wines sampled at time zero (bottling) and their evolution in ten months.

Figure 4.2 includes PCA statistics of the six white wines at initial and final storage time modelled using selected variables of oenological interest which contribute to the development of oxidative browning. The first two components explained about 95% of variance which underline the reliability of PCA model on the evolution of white wines during storage.

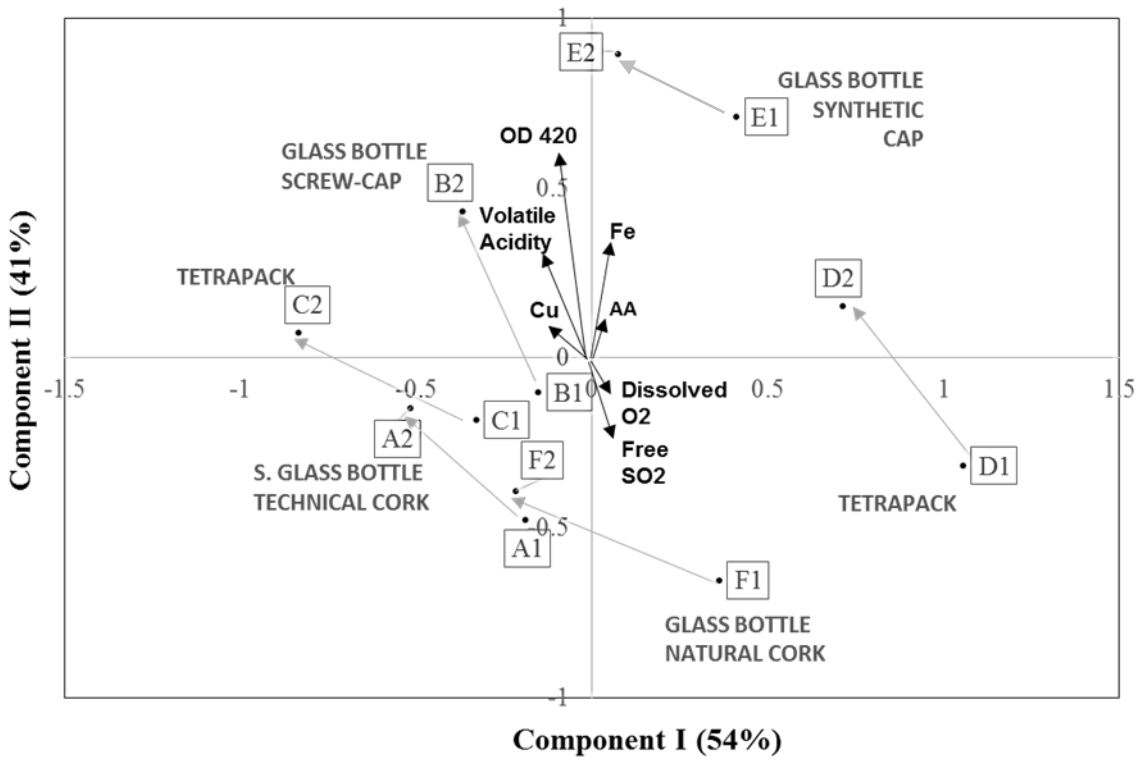

Figure 4.2. Projection of wine samples and disposition of selected oenological parameters over the first two components.

PC1 accounted for 54% of the total variance. PC2 accounted for the 41% of the variance, and variables responsible for the separation were free sulphur dioxide and dissolved oxygen values (bottom of the figure) and the increase in optical density, copper, iron and volatile acidity (top of the figure). With time all wines shifted similarly from the right-down to the left-up quadrant, thus both PCs were important in explaining the occurrence of browning. In particular, negative values on PC2 corresponded to a low oxygen consumption and generally low kinetic rate values, while the most evident browning development corresponds to more positive PC2 values.

When considering the contribution of transition metals, free iron (no limit set in EU; Chinese legal limit: 8 mg L^{-1}) and free copper (EU and Chinese legal limit: 1 mg L^{-1}) followed the same trend of the increase in OD 420 nm distributed along the PC2 component. Cu and Fe are the main catalyser of Fenton reaction, increasing the formation of reactive oxygen species (ROS) in solution (Danilewicz, 2012; Li, Guo, & Wang, 2008; Morozova, Schmidt, & Schwack, 2015). The ability of polyphenols to bind iron (and copper) in wine is considered one of the antioxidant mechanisms (besides radical quenching) exerted by these bioactive compounds.

Most of the dissolved oxygen was consumed within the first two months of storage, when stabilization mechanisms occur as a consequence of the oxygen intake during bottling; this period also resulted in an intense consumption of protective additives (i.e. SO_2). The time course of the oxygen consumption during the experiment (**figure 4.3**) disclosed a high variability in the oxygen supply at time zero for the sample series, being more evident in sample A; this variance could be ascribed to different mechanisms by which the bottling equipment are regulated, and/or occasional malfunctioning. This aliquot incorrectly introduced would be likely consumed in oxidative processes during the shelf life of wine; for this reason it is important to set up the time zero of shelf life experiment in cellar, at wine bottling.

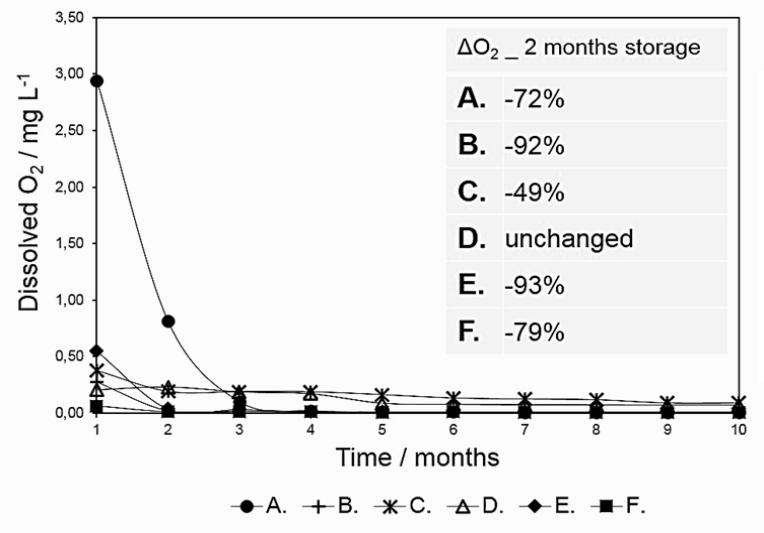

Figure 4.3. Time-course of dissolved oxygen during the period of the experiment.

4.3.2. Kinetic and thermodynamic studies

According with findings reported in the previous section, the parameters that mostly describe the browning process includes: free sulfur dioxide, dissolved oxygen, OD 420 nm. The free SO_2 consumption followed a zero-order kinetic rate (**table 4.2**), meaning that the speed-determining step was not affected by the concentration of products, and the correlation between the sulfur dioxide consumption rates and kinetic rates of the browning onset (**figure 4.4)** was limited ($R^2 = 0.62$).

	Free SO_2		Free SO_2 loss	Total SO_2 loss
Wine varietal	***k* / mg L^{-1}**	**R^2**	**%**	**%**
A. Sauvignon	1.103	0.98	36	3.8
B. Chardonnay / Trebbiano	1.682	0.89	8	3.3
C. Chardonnay bio	1.655	0.94	29	13.3
D. Chardonnay / Trebbiano	2.054	0.93	28	0.6
E. Albana di Romagna	1.830	0.93	32	5.0
F. Muller Thurgau	1.576	0.92	27	13.8

Table 4.2. Free and total sulfur dioxide consumption during ten months storage of wines.

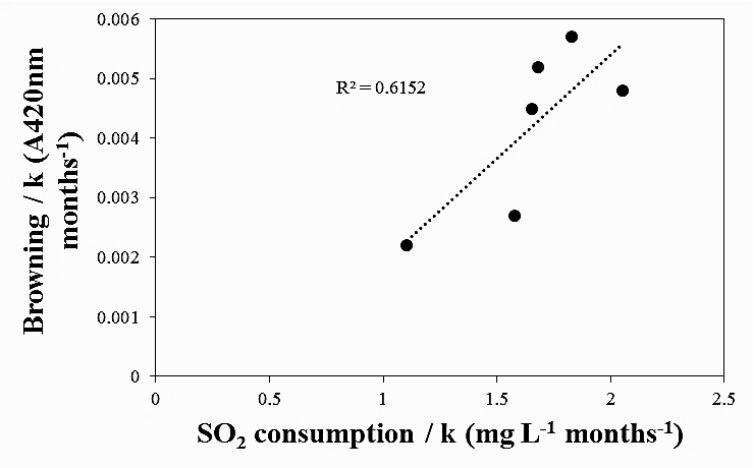

Figure 4.4. Correlation between the kinetic of consumption of free SO_2 and the rates of the browning onset measured in this experiment.

It is well known that SO_2 is oxidized to sulfate via a double mechanism: (i) the scavenging of H_2O_2 produced by polyphenols oxidation and (ii) the coupled reaction with quinones (Boulton, Singleton, Bisson, & Kunkee). SO_2 undergo different reaction paths during its oxidation, resulting in different mixtures of sulfite adducts; the consumption of oxygen is not linear respect to the consumption of free SO_2 following the formation of these molecular adducts (Danilewicz, 2007). Depending on the value of each equilibrium constant sulfite adduct contribute to the concentration of bonded SO_2 in wine, which value can fairly increase (Danilewicz, Seccombe, & Whelan, 2008; Danilewicz & Wallbridge, 2010). This mechanism can explain the moderate total SO_2 decrease in this experiment, compared to a more pronounced decrease in free SO_2 (**table 4.2**), resulting from a balance between consumption and continuous formation and replacement of SO_2 molecular adducts. The kinetic rates were calculated during the storage of wine at room temperature, whereas the apparent activation energies were calculated through accelerated aging conditions **(Table 4.3 and 4.4)**: the apparent activation energy were aligned with the averaged values of 66.4 and 74.6 kJ mol^{-1} calculated in previous experiments for white wines (Boulton, Singleton, Bisson, & Kunkee, 2013) indicating zero order rate-controlling steps in the reaction. Both kinetic

and thermodynamic results were used to evaluate the performances of different wines and packaging.

Wine varietal (present study)	packaging	closures	k / A_{420nm} months^{-1}	r^2
A. Sauvignon	glass bottle	technical cork	0.0022	0.91
B. Chardonnay / Trebbiano	glass bottle	screw-cap	0.0052	0.96
C. Chardonnay _ bio	brick	plastic twistcap	0.0045	0.93
D. Chardonnay / Trebbiano	brick	plastic twistcap	0.0048	0.94
E. Albana di Romagna	glass bottle	synthetic	0.0057	0.96
F. Muller Thurgau	glass bottle	natural cork	0.0027	0.98
Wine varietal (bibliography)	**packaging**	**closures**	k / A_{420nm} months^{-1}	**Ref.**
Table Greek white wines	glass bottle	_	0.0030	Kallithraka et al., 2009
Custoza white wine	glass bottle	natural cork	0.0022	Lambri et al., 2012
Custoza white wine	glass bottle	synthetic	0.0032	Lambri et al., 2012
Chardonnay	bag - in box	_	0.0080	Fu et al., 2009

Table 4.3. Kinetics of browning onset in white wines after 10 months storage and comparison with bibliographic data.

	Accelerated aging at 20 – 30 – 35 – 40 – 45 – 50 – 55 - 60°C (10 days)	
Wine varietal (present study)	**Ea browning/ kJ mol^{-1}**	**R^2**
A. Sauvignon	59.5	0.94
B. Chardonnay / Trebbiano	76.1	0.92
C. Chardonnay _ bio	88.3	0.87
D. Chardonnay / Trebbiano	63.9	0.85
E. Albana di Romagna	80.3	0.92
F. Muller Thurgau	46.8	0.90
Wine varietal (bibliography)	**Ea browning/ kJ mol^{-1}**	**Ref.**
Chardonnay	79.7	Fu et al., 2009
Villànyi Portugieser	90.2	Czibulya et al., 2012
Bikatory	82.1	Czibulya et al., 2012
Average white wines _glass	66.4	Berg et al., 1956
Average white wines _glass	74.6	Ough et al., 1985

Table 4.4. Apparent activation energies (Ea) calculated using the Arrhenius equation (4c) for white wines and comparison with bibliographic data.

Although Sauvignon wine stored in glass bottled with technical cork **(sample A)** showed the highest content of dissolved oxygen at bottling (2.94 mg L^{-1}), the kinetic of browning was the lowest observed among samples (0.0022 A_{420nm} month^{-1}). The oxygen consumption dropped at 72% and 99% at second and fourth month of storage, respectively, with a trend similar to previous studies, which reported oxygen ingress rates for technical corks ranging from 0.8 to 1.2 mL during the first month of storage

and 0.010-0.011 mL averaged in the following period (Lopes, Saucier, Teissedre, & Glories, 2006). Wine A showed a low intrinsic activation energy for browning (59.3 kJ mol^{-1}), meaning that the synthetic cap provided an effective barrier to gas permeation and therefore a protection against oxidation. The rate of free SO_2 consumption was also the slowest among wines (1.103 mg L^{-1} $months^{-1}$).

The Chardonnay/Trebbiano blend bottled in glass and capped with screw-cap (sample B) showed the most noticeable increase in OD_{420nm} among samples (+0.036), with 93% decrease in dissolved oxygen within the first two months, and a high browning onset kinetic rate (0.0052 A_{420nm} $month^{-1}$). Although the screw-cap is recognized to provide a moderate gas permeation (Lopes, Saucier, Teissedre, & Glories, 2006; Skouroumounis, Kwiatkowski, Francis, Oakey, Capone, Duncan, et al., 2005), the activation energy was almost the highest of the series (76.1 kJ mol^{-1}), the most probably due to a combined effect of high content in free iron (10.7 mg L^{-1}) and copper (0.42 mg L^{-1}) at bottling.

Moreover, according to literature (Ferreira, Carrascon, Bueno, Ugliano, & Fernandez-Zurbano, 2015) the moderate consumption of free SO_2 (-8%) during the ten months of storage suggested the presence in wine of other nucleophiles (e.g. gluthationes, thiols, ascorbic acids, etc.) that can compete with SO_2 for reacting with quinones; this hypothesis was not deepened in this experiment.

The apparent activation energy was high for **sample C**, Chardonnay (88.3 kJ mol^{-1}) and moderately high for **sample D**, Chardonnay/Trebbiano blend (63.9 kJ mol^{-1}), bearing a high TPC value at bottling (239 mg L^{-1}). In their respective tetra pack packaging and during 10 months, browning developed quite fast, with kinetic rates of 0.0045 and 0.0048 A_{420nm} $month^{-1}$, respectively; the kinetic of consumption of free SO_2 was especially high for sample D, 2.054 mg L^{-1} $month^{-1}$, and the ratio between O_2 consumption and O_2 inlet was almost one (consumption was compensate by oxygen permeation over the first two months of storage). The high content in iron solvated ions (15.12 mg L^{-1}), coupled with a low content in natural antioxidant (199 mg L^{-1} GAE) catalysed both oxidation of sulfites and Fenton reaction, and the browning was limited by an high activity of sulfur dioxide; the packaging appeared to have a minor contribution.

A slight lower kinetic rate for browning in sample C, coupled with a slower consumption rate of free sulfur dioxide (1.655 mg L^{-1} $month^{-1}$), could be instead attributed to an high intrinsic activation energy of the wine itself and a lower amount of

iron (3.9 mg L^{-1}), since packaging and storage conditions remained unchanged with respect to sample D. The initial copper content was very similar in both cases (C = 0.095; D = 0.084), slightly lower in sample D and didn't affect significantly the wine oxidation. Gas permeation is a problem affecting the multilayer packaging which are highly permeable to oxygen (Ghidossi, Poupot, Thibon, Pons, Darriet, Riquier, et al., 2012; Revi, Badeka, Kontakos, & Kontominas, 2014), thus influencing wine consumer's preferences: a recent study including the response of a sensory panel defined Vilana white wine (Greek wine variety) as simply "acceptable" after three months storage in glass bottles, and "unacceptable" when stored with alternative plastic multilayer materials over the same period (Revi, Badeka, Kontakos, & Kontominas, 2014).

According with the experiment presented in this dissertation, it could be concluded that tetra pack is a valid alternative for wines bearing a strong intrinsic antioxidant activity and low content in metal catalysers, and which are provided for short-terms conservation.

Sample E was an Albana wine bottled in glass and corked with a synthetic cap; against an high intrinsic activation energy estimated (80.3 kJ mol^{-1}), an high TPC content (258 mg L^{-1}) and a moderate content in iron (4.35 mg L^{-1}) and copper (0.127 mg L^{-1}), it was characterised by fast browning development (0.0057 A_{420nm} $month^{-1}$), the highest value of the series, and rapid consumption of free SO_2 over ten months (1.830 mg L^{-1} $month^{-1}$); the free SO_2 content was pretty high at bottling (47 mg L^{-1}). Despite the potentiality of wine to protect against oxidation, the permeability of closure affected wine conservation, inducing a strong oxygen consumption at the beginning of storage (-93.09% in two months). Synthetic closures allow high levels of oxygen permeation and in previous studies two different synthetic caps commercially available were assayed reaching 2.5 ml of oxygen inlet in bottle within 150 and 200 days of vertical storage (Lopes, Saucier, Teissedre, & Glories, 2006).

The Albana assayed in this experiment showed good intrinsic properties for long-term conservation (high TPC value, high free sulfur dioxide, relatively low content in transition metals, which were traduced in high activation energies for browning); in this series an improper packaging contributed to worsen the wine durability.

According with previous experiments, natural cork coupled with glass bottles reported intermediate performances between screw-caps and technical corks, in one side, and synthetic closures, with oxygen ingress values ranging from 0.8 and 1.3 mL during the

first month, and an average oxygen ingress ranging from 1.5 and 2.4 mL over 24 months of vertical storage; the study also highlighted the variability of the natural material when compared with other technologies (Lopes, Saucier, Teissedre, & Glories, 2006). In this experiment, **sample F** (Muller Thurgau wine) was packed with glass bottle and natural cork closure; the wine mass at bottling was characterised by a low content in iron (1.73 mg L^{-1}) and copper (0.02 mg L^{-1}), and a slow kinetic for the browning process (0.0027 A_{420nm} $month^{-1}$); oxygen consumed after two months was 79.20% and the dissolved oxygen at bottling was the lowest of the series (0.19 mg L^{-1}). The low activation energy barrier 46.8 kJ mol^{-1} was probably due to the low content in TPC (201 mg L^{-1} GAE) classifying sample F as wine potentially susceptible to oxidation. The free sulphur dioxide at time zero was high (55 mg L^{-1}), but its consumption rate was moderate (1.576 mg L^{-1} $month^{-1}$) when compared to the samples assayed. The closure regulated properly the gas permeation during storage, protecting an intrinsically susceptible wine and preserving the SO_2 protective effect in time.

Results obtained for the occasional **sample F** need to be accompanied by some statistical considerations related to natural cork: recent studies (Faria, Fonseca, Pereira, & Teodoro, 2011) have reported a variability factor of 1000 for the oxygen permeability of cork stoppers. It is therefore not possible to provide generalizable data (margin of error < 5%) without involving a larger number of samples in the same experiment.

4.4. Conclusions

The study of white wines stored under different packaging conditions allowed to profile the potential shelf-life durability on a kinetic and thermodynamic perspectives. The kinetic rates of the browning onset and the potential Arrhenius activation energies for the same phenomenon were successfully calculated, and results were consistent with previous findings reviewed from the scientific literature. This approach also enabled to compare the chemical parameters of oenological interest measured during the experiment with potential development of browning in wine, showing that the packaging used for wine storage significantly affects the protection of wine against oxidative degradation. This experimental approach provided preliminary screening of wine packaging performances, and there is a need to further implement increasing the

sampling and introducing targeted compositional analyses to make it effective in optimizing shelf-life durability.

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4B) THE INFLUENCE OF CLOSURES IN CHEMICAL AND SENSORY ATTRIBUTES OF WINE

RESEARCH ARTICLE:

Evoluzione delle caratteristiche chimiche e sensoriali di vini bianchi Muller Thurgau imbottigliati con diverse tipologie di tappo

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4.6. Introduction

As it was stated in the general introduction, several factors affect the choice of packaging and closures for commercial wines, ranging from the technological performances to the cultural impact.

In a technological perspective, many shelf-life studies have highlighted the effects of different closures on the evolution of commercial wines. Lopes et al. (2009) have evaluated the combined impact of oxygen intake at bottling, which is due to equipment and devices used in the cellar, and the oxygen input during storage, which is governed by a different permeability of closures. It was thus demonstrated that a variety of Sauvignon blanc has undergone in two years of storage an optimal aromatic development and reduction effects negligible, as a result of using a natural cork (Lopes, Silva, Pons, Tominaga, Lavigne, et al., 2009).

The majority of white wines currently available in the market are intended for consumption within two years after bottling; for these wines the evolution of the aromatic component in the early stages following bottling becomes a critical stage to assess the integrity of the product.

The amount and timing of the release of oxygen are different by using different materials: screw caps are characterized by greater exposure to O_2 in the first few weeks, due to higher volume generated in the headspace (Dimkou, Ugliano, Dieval, Vidal, et al., 2011).

On the contrary the cork generally releases a certain amount of oxygen in the early stages of use, followed by a slower transfer rate; in the use of natural products also becomes important the variability between corks belonging to the same lot (Godden, Lattey, Francis, Gishen, Cowey, et. al, 2005).

The synthetic material allows greater control of technological properties during the production phase, ensuring specific benefits for the different production needs; plastic closures can supply an high amount of O_2 for fining in the bottle, when needed (Lopes, Saucier, & Glories, 2005).

The purpose of this study was to evaluate the influence of three selected caps: natural cork, technical cork, and synthetic closure, in the shelf-life of a Muller Thurgau during 9 months storage. Wines were analysed according with the evolution of chemical parameters of oenological interest and profiled according with the response of a sensory panel; results are presented in a comparative perspective. A further sensory evaluation

over 12 month storage was performed to implement the ability of the sensory panel to discriminate differences between the three closures, showing how the sensory perception may occur at a later time than the first analytical evidences of browning.

4.7. Materials and Methods

4.7.1. Samples

For this study a Muller Thurgau Trentino DOC wine, vintage 2012, has been provided by the Vivallis Coop. of Nogaredo (TN).

Three experimental batches were produced at the bottling stage, one capped with a synthetic cork closure (SIN1), the second with a monolithic natural cork (SU2) and the third with a technical cork closure (SU3). The selected synthetic stopper with controlled permeability was chosen with a low oxygen transfer rate. The model used allowed an oxygen transfer rate controlled during storage of wine in the bottle: 0.37 mg after three months, after 6 months 0.64 mg, 1.2 mg and 1.1 mg after twelve months after the first year (*source: Nomacork*). It is not possible however to produce a prediction of the transfer rate for the natural cork; the producer ensures instead the maintenance of the chemical-physical and organoleptic properties of the starting material for a period of up to 24/36 months, thanks to accurate boiling and vaporization processes, TCA monitoring for the whole period of the processing, sensory analysis for the elimination of defective caps, and lubrication for vertical storage.

The production of technical corks allows to control the oxygen permeability rate to a certain extent; the commercial proposal includes two macro classes of permeability for technical cork, low (0.15 cm^3 / day) and medium (0.35 cm^3 / day). However, the specific class used to cap the wine used in this experiment has not been specified by supplier.

4.7.2. Chemical analyses

For a period of about nine months after bottling chemical analysis were carried out on the three different closing series. Chemical parameters of oenological meaning (see **section 4.2.6**) were periodically measured according with the OIV quality standards (OIV, 2014).

Further parameters have been monitored including: TPC (OD 280 nm, converted into mg L^{-1} GAE); total iron (mg L^{-1}); total copper (mg L^{-1}) (see **section 4.2.2.-4.-5.**).

4.7.3. Measurement of the dissolved oxygen

The content of dissolved oxygen (mg L^{-1}) and its decrease over time were measured by the oxygen device for still wines previously described (**section 4.2.3**), equipped with a sampling chamber in an inert atmosphere of nitrogen and an electrochemical detection system (SFD Anton Paar system , Graz, Austria).

4.7.4. Electronic nose

The characterization of the aromatic fraction at time zero and after three years of storage was performed using a PEN2 electronic nose system equipped with a desorption unit for alcoholic samples (Airsense Analytics GmbH, Germany); 10 ml of wines were added to 40 ml screw – cap vials and stored for fifteen minutes to saturate the headspace with wine aromas, then the wines were sampled against a blank solution (1% ethanol in MilliQ water). Results were extrapolated after the response of ten non-specific sensors and processed to provide the olfactory profile of wines at different storage periods.

4.7.5. Sensory analysis

Descriptive sensory evaluation of wines (Lawless & Heymann, 1998) was performed at 1, 10 and 12 months after bottling; the samples were randomized and coded, and wines were sampled on a volume of 30 ml and at a temperature of 20°C in transparent glasses (ISO 1977), and each panellist advantaged of an individual workstations illuminated with white light (ISO 1988). The following descriptors were evaluated: yellow hue, floral note, fruity note, overall smell, acidity, alcoholic note, bitter, astringent, sweetness, flavour, overall flavour, the overall persistence, overall pleasantness; the 10-cm scale provided for each descriptor is anchored at the ends by the minimum and maximum values (0 and 10, respectively), and the descriptor is provided as a coordinate between such extremes.

4.7.6. Statistical analysis

All data were stored and statistically processed using the XLStat-PRO 7.5 software (Addinsoft, Ney York, NY). Data obtained by electronic nose and sensory analysis were subjected to analysis of variance (ANOVA) and principal component analysis (PCA) using the Unscrambler 9.7 software package (Camo ASA, Norway).

4.8. Results and Discussion

4.8.1. Chemical analyses

For the whole duration of the experiment, pH and potential acidity have not been significantly affected by closures; pH values were maintained around 3.4 while total acidity lied between 5.4-5.7 g L^{-1} (tartaric acid equivalents) for the whole duration of the experiment.

The volatile acidity (expressed in g L^{-1} of acetic acid) has followed a similar trend for the three batches, with a slight increase which likely did not affect the sensory perception (*SIN1:* 0.45 → 0.51 g L^{-1}; *SU2:* 0.42 → 0.48 g L^{-1}; *SU3:* 0.45 → 0.49 g L^{-1}).

Free and total sulfur dioxide parameters instead developed differently in relation to different closures, as shown in **Table 4.5.** Although the sequence of data was not perfectly linear because of the method used for quantification (iodometric titration) characterized by low accuracy, all series underwent different percentage decreases compared to the initial concentration.

The free sulfur dioxide in sample SU2 showed the higher consumption during the experiment, with a percentage of 27.3% decrease, followed by the synthetic stopper of the SIN1 batch (-21.3%) and from the technical cork SU3 that produced minimal consumption (- 10.4%). In the case of the total sulfur dioxide SIN1 and SU2 samples followed a similar trend, with a decrease of 9.6% and 13.8% respectively, while having a significantly lower decrease for the SU3 sample when compared to the initial value (-4.8%).

In **Figure 4.5** showed the free and total sulfur dioxide decrease in time; comparison highlighted an interesting relationship between the decrease of SO_2 and the consumption of dissolved oxygen (**figure 4.6**); in particular, it can be noticed that the batch capped with technical agglomerated cork (SU3) had the higher O_2 consumption (-

98% dissolved oxygen present at bottling) but a modest decline of free (4.8%) and total (10.4%) SO_2.

	Free SO_2 (mg/L)			**Total SO_2 (mg/L)**		
	SIN1	*SU2*	*SU3*	*SIN1*	*SU2*	*SU3*
13-Mar	64	55	58	125	123	125
10-Apr	63	54	58	118	110	124
17-Apr	64	58	64	127	111	104
13-May	61	54	59	115	120	125
05-Jun	64	52	63	124	118	127
28-Jun	59	52	56	128	123	120
25-Jul	59	54	59	128	115	125
25-Sep	55	29*	51	108	95	119
09-Oct	52	50	49	119	119	120
29-Oct	55	49	52	116	113	119
28-Nov	52	42	54	115	109	119
16-Dec	51	40	52	113	106	124
Δ	**-21.3%**	**-27.3%**	**-10.4%**	**-9.6%**	**-13.8%**	**-4.8%**

Table 4.5 Variation of free and total SO_2 parameters up to 9 months monitoring.

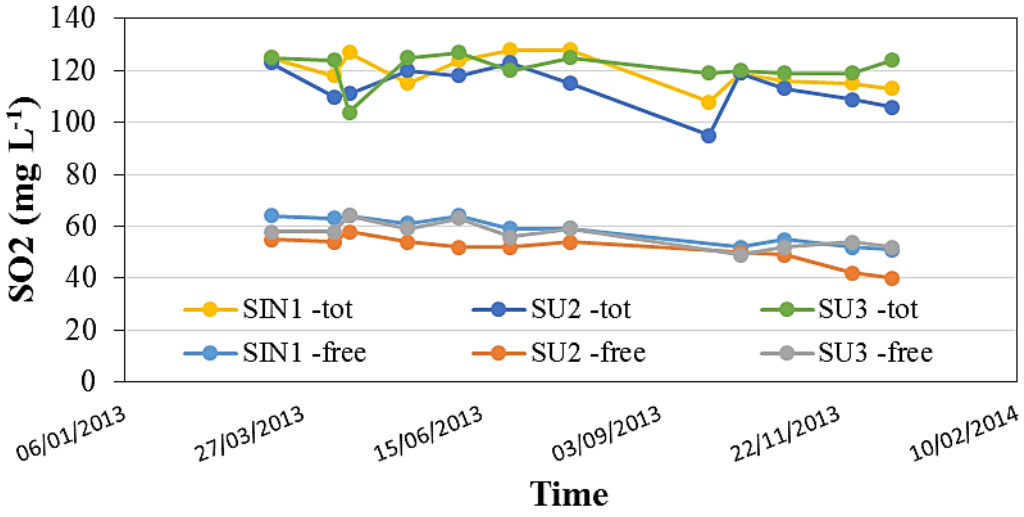

Figure 4.5. Evolution of free and total SO_2 parameters for the three series in 9 months of bottle storage. SU2 series (natural cork) underwent the most significant decrease in both parameters.

It must also be considered that the use of different bottling lines could provide significant differences in the oxygen supplied at time zero (**Figure 4.6**); the first

sampling of the SU2 batch presented a high quantity of oxygen (0.19 mg L^{-1}), when compared with the first sampling of SU3 (0.013 mg L^{-1}) and SIN1 (0.06 mg L^{-1}) batches; in particular the SIN1 sample had the minor gas intake at bottling between the three analysed series. The oxygen supply along the supply chain is an event partially controllable flushing inert gas in the bottling line or monitoring the proper functioning of the winery equipment.

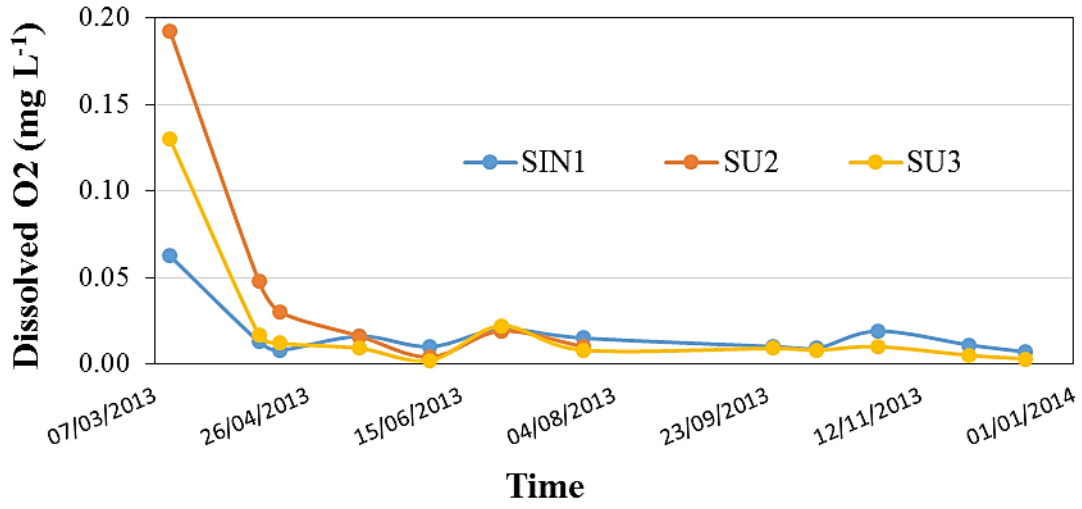

Figure 4.6. Time course of dissolved O_2 parameters in the three bottled series. The SIN 1 sample has a less noticeable percentage decrease (-88%) related with a lower content in oxygen at bottling.

The dissolved oxygen depletion monitored after bottling showed the same trend in the first month (from 12/03 to 17/04) for all three series, and then followed a more gradual decrease attributable to the type of closure used. SIN1 series had a reduction of dissolved oxygen concentration of 88%, of which 95% was consumed within the first month of storage. The SU2 series showed a decrease of 96% of the initial oxygen concentration; most of it (87% of total consumption) was used in the first month of storage, followed by a second phase for a period of about 7 weeks in which an extremely slow but constant consumption occurred, followed by a stabilisation step starting from the 11th week. The SU3 technical cork product showed a similar trend when compared to SU2, with a total oxygen consumption of 98%, 92% of which is concentrated in the first month of storage. In this case the concentration of dissolved oxygen also stabilized around the eleventh week of storage, after a slow and gradual decline.

In summary, the SIN1 batch was characterized by a lower consumption of oxygen, which mostly occurred, however, in a short period after bottling. The SIN1 and SU2

samples had the same oxygen content after the experiment (7.0 ppb), while for the SU3 sample contained 2.5 ppb of dissolved oxygen at the last sampling.

It was previously reported that the oxygen and the free SO_2 consumption in bottled wines retain a ratio of concentrations 1:4; on the basis of the measured sulfur concentrations, it can be inferred therefore that the consumed oxygen concentrations are about 3.7 mg L^{-1} for the SIN1 and SU2 samples and 1.5 mg L^{-1} for the SU3 sample, respectively.

Considering the oxygen concentration at time zero and assuming constant conditions of head volume, temperature, light exposure, it can be concluded that different consumption rates are attributable to different closures employed. A significant O_2 intake during the experimental period has been hypothesize for SIN1 and SU2; in both cases, the high porosity of the materials (synthetic and natural cork) allowed retention and subsequent slow release of oxygen following compression in the bottle neck. The SU3 technical cork, characterized by a high density of the material (which is produced by mechanical pressing) had a lower oxygen delivery during the monitoring period, which led to a moderate consumption of oxygen present bottling.

Any linear correlation was found for the three series when comparing consumed oxygen and free SO_2 parameters (*data not shown*); it seems to confirm that the consumption of dissolved oxygen is involved in a complex system of variables, including the degradative processes and the evolution of the phenolic fraction.

Instead, there is a good linear correlation between the development of brown pigments ($OD_{420\ nm}$) and consumption free of sulphur dioxide, with high R^2 coefficients (SIN1: 0.91, SU2: 0.73, SU3: 0.74, *data not shown*); the disappearance of sulfur dioxide is thus directly attributable to the formation of molecular adducts with quinones or other phenol oxidation by-products.

The main indicator of the occurrence of an oxidative degradation of wine is the colour variation following the formation of condensation coloured structures (maderization and browning phenomena); from the analytical point of view this can be expressed as an increase of the optical density at a wavelength of 420 nm. Starting from the same zero value ($A_0 = 0.062 \pm 0.001$) the increase in $OD_{420\ nm}$ followed a zero-order kinetic for the three batches analysed (linear regression between concentration of brown polymers formed and storage time: SIN1 $R^2 = 0.97$, SU2 $R^2 = 0.93$, SU3 $R^2 = 0.95$). **Figure 4.7** shows these trends, with a net increase of 420 nm optical density at the point

of final monitoring for SU2 series and the smaller increase in color intensity for sample SU3.

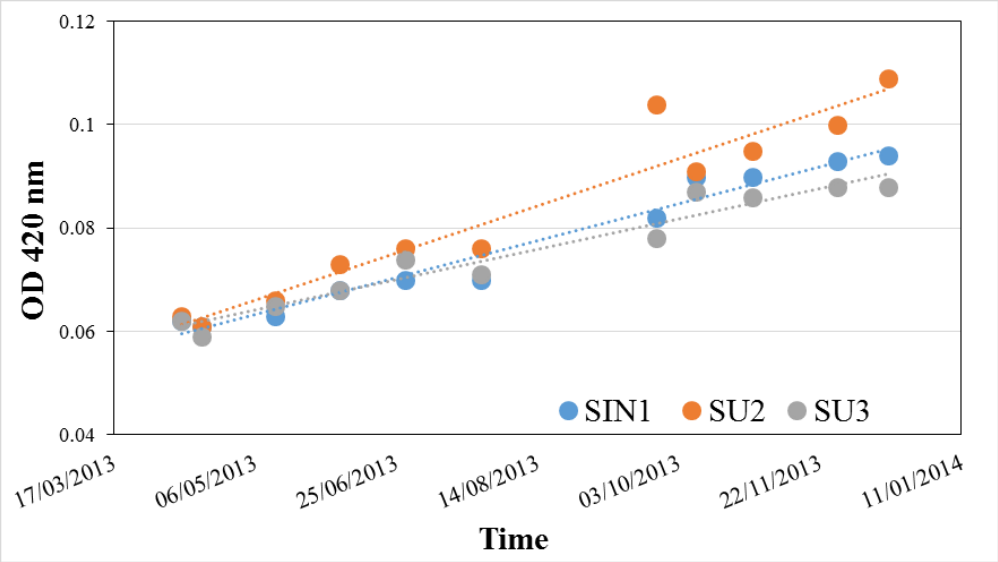

Figure 4.7. Increase in $OD_{420\ nm}$ following zero-order kinetic rates for the three wine series.

As regards the transition metals involved in the catalysis of oxidative phenomena, the values measured at the beginning and end of the experiment are reported in **Table 4.6**. The starting values presented a certain variability, which can be attributed to occasionally contamination produced by the winery equipment. After bottling, the total content of free iron resulted almost constant for SIN1 and SU3, while significantly weakened in SU2 sample; this is probably due to the formation of coordination compounds between Fe^{2+}/Fe^{3+} ions with polymeric phenols, which are efficient chelating agents. The free copper content (<0.03 ppm for all three series) presented a generic increase from the beginning to end of the experiment; This trend suggests a transfer rate of this metal by the three closures over time, not significant for SIN1 and SU2 series, more evident for the SU3 series.

Cu (mg/L)		t_0	t_n
	SIN1	0.021	0.042
	SU2	0.020	0.036
	SU3	0.001	0.065
Fe tot (mg/L)		t_0	t_n
	SIN1	1.78	1.68
	SU2	2.9	1.59
	SU3	1.45	1.54

Table 4.6 Free iron and copper concentrations. t_0; measurement at time zero. t_n; sampling at the end of the monitoring period.

The total polyphenols were calculated for each sample measuring the optical density at 280 nm; the concentration was expressed in mg L^{-1} of gallic acid using a calibration

curve (R^2 = 0.99). All three series showed an increase in the total polyphenols calculated (*data not shown*); the increase in optical density over time can be ascribed to a rearrangement of the monomeric and oligomeric fractions of polymeric material, and confirmed results for the experiment in **section 4A**.

4.8.2. Electronic nose and sensory analysis

Wines were analysed by electronic nose at bottling (_0) and after 10 (_10) and 12 (_12) months of storage. The data provided by 10 sensors have been processed through the use of PCA modelling (**Figure 4.8**). The resulting representation can be considered satisfactory with a variance explained by the first two components accounting for 84% of the total.

At bottling (_0) wines have similar characteristics and are grouped in the lower left quadrant. After the wines seem to have changed their aromatic profiles and the distances between them increase ten months of conservations (_10), although all located at the bottom right of the plot. These differences increase further after a year of conservation: the wines are placed in the upper right of the plot, and SU2_12 sample appears to have aromatic characteristics significantly different from SIN1_12 and SU3_12.

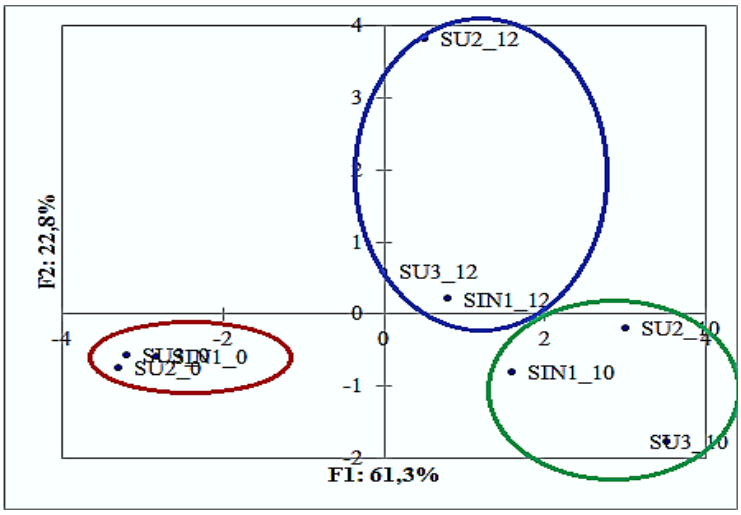

Figure 4.8. Electronic nose profile: PCA representation of changes in aromatic profile of SIN1, SU2, SU3 series starting from bottling up to a year of storage.

Distribution of the sensor responses over the PCA space can provide information about the aromatic components that "separate" samples in the space delimited by the two main components, F1 and F2 (**Figure 4.9**). In particular, the sensors 2, 6 and 9 correlates with the samples at the time of bottling, while the signals provided by the sensors 1 and 3 are

most highly correlated with the samples analysed after ten months of storage. Finally, the sensors 5 and 7 characterize the samples after twelve months bottling.

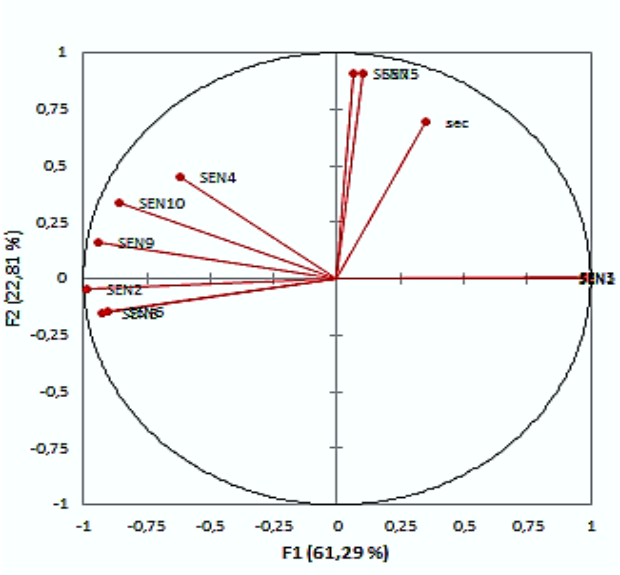

Figure 4.9. Distribution of the information provided by the sensors in the delimited by the first two principal components (F1 and F2).

The evolution of the sensory profile was monitored by quantitative descriptive analysis. The wines were evaluated by a panel a month after bottling, and subsequently after ten and twelve months. The average values of the data provided by the panel are represented by spider plot in **Figure 4.10**. At first glance the wines after twelve months of storage (SIN1_12, SU2_12 and SU3_12) exhibited higher colour intensity than the other, while the wines analysed one month after bottling SIN1_0, SU2_0 and SU3_0) and after ten months (SIN1_10, SU2_10 and SU3_10) are enriched in fruity notes and overall odour perceptions. The analysis of variance (ANOVA) performed on sensory data allowed to point out that these differences were statistically significant (*data not shown*).

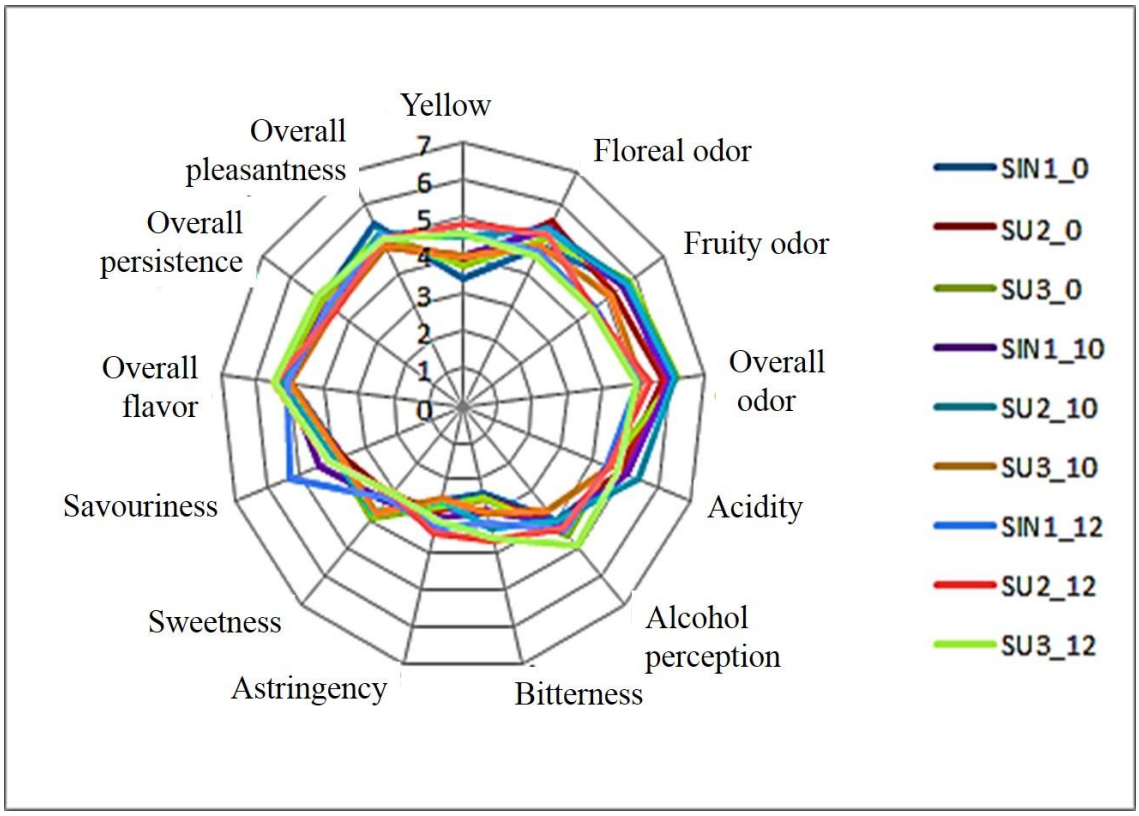

Figure 4.10. Sensory profile of SIN1, SU2 and SU3 at bottling (_0), after ten (_10) and twelve (_12) months of storage.

In particular wines valued within the first month of storage were considered to have less colour intensity than wine evaluated after ten and twelve months, although these differences are often not significant. The type of plug used, therefore, did not affect on the colour in wines kept for the same period of time, according with panellists response. The SIN1 colour intensity significantly increase after twelve months of storage (SIN1_0 vs SIN_12). The sensory analysis, however, did not fit with the instrumental responses (OD 420 nm). In twelve months storage the fruity notes decreased for all series. The overall smell decreased significantly in SU3 wine passing from the first (SU3_0) to the twelfth month of storage (SU3_12). Any significant reduction descriptor was recorded for SIN1 and SU2 wines during the 12 months of storage.

Most evident differences were revealed after 10 months of storage (_10), in particular for the SU3 sample, characterized by a loss in aromatic perceptions. Over the next two months such differences were reduced, although the panel was still able to differentiate the SU3_12 from the other "_12"samples.

4.9. Conclusions

The chemical, physical and sensory analyses of an Italian Muller Thurgau vintage 2012 performed during a hypothetical shelf-life period has allowed to characterize the performance of three different closures currently available on the market: synthetic polymer, natural cork technical agglomerate cork.

On the basis of the results obtained it was possible to conclude that the type of cap used had no significant effects on the parameters of pH, potential acidity, while the volatile acidity underwent a slight increase, similar in all three cases, without reaching sensory perceptible levels.

The level of free and total sulphur dioxide consumed was affected by closures, showing a significant lower consumption for the SU3 sample. This result can be explained by the technical characteristics of the technical cork, which produces greater stability compared to the other types analysed.

The series capped with technical cork has also introduced the smaller increase in the optical density, while the natural cork have provided a more noticeable browning phenomenon for this experiment.

The SIN1 batch, composed by wines capped with synthetic closures showed a lower dissolved oxygen concentration at bottling; this difference was attributed to the bottling system.

The free SO_2 consumption measures during the experiment allowed to conclude that the caps made of synthetic material and natural cork have allowed a higher O_2 input.

The reduction in free SO_2 content was highly correlated with the increase in colour intensity (OD at 420 nm) for all samples, while there wasn't a significant correlation between OD at 420 nm and dissolved O_2 consumption, as well as between the free SO_2 and dissolved O_2 consumption, confirming that dissolved oxygen is involved in oxidative processes occurring in wine but also in reactions that allow the evolution of the aromatic component of the wine itself.

A slight variation in the concentration of metal catalysts has been observed in three series; in particular, iron ions decreased in SU2 series, where the production of brown pigments does suggest significant chelating activity, and an increase of copper in the series SU3 was observed (although not significant, in the order of ppb), likely attributable to contaminants released by closures, which are the only variables among batches.

The electronic nose has revealed that the aromatic component of wines changed with the progress of conservation; among them, the natural cork provided more fluctuations during storage. The wines at the end of the monitoring were mainly differentiated from the time zero according to colour, fruity overall odour descriptors. In particular, the technical cork sample appeared to have reduced appreciably the overall aromatic component after 10 months of storage, and its sensory profile still differ from the other series after 12 months of storage.

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Chapter 5

Structure-Antioxidant Activity Relationship of Wine Polyphenols

Wine is a major source of polyphenols, and for this peculiarity it has been recognized to provide protection against cancer and coronary diseases, among others; the unique properties of wine phenolics as bioactive compounds has been extensively studied in the last decades (Rice-Evans, Miller, & Paganga, 1997; Villaño, Fernández-Pachón, Troncoso, & García-Parrilla, 2005).

Beyond the potential medical applications, the high content in polyphenolic compounds also protect wine from the oxidative stress, in a certain extent (**section 2.3**); for this reason, the study of the phenolic fraction in wine and the optimization of extractive procedures contribute to prevent from undesirable detrimental variations during shelf life (Castellari, Matricardi, Arfelli, Galassi, & Amati, 2000; López, Puértolas, Condón, Álvarez, & Raso, 2008).

Polyphenolic compounds are a group of plant metabolites synthesized during plant growth in response to stress and external aggressions (ultraviolet radiations and microbic aggressions, among others). Polyphenolics can be widely classified into phenolic acids and flavonoids: phenolic acids include benzoic acid, cinnamic acid and their derivatives. Flavonoid compounds include the following sub-classes: flavan-3-ols (catechins and related compounds), flavonols (quercetin and their glycosides adducts), flavones (apigenin and luteolin), flavanones (naringenin) and isoflavones (genistein and daidzein). All listed compounds have in common the presence of aromatic rings with one or more hydroxyl substituents into their chemical structures. Polyphenolic compounds naturally occur as monomers, molecular adducts (glycosylated, galloylated, methylated structures) pigments (anthocyanins and pigmented polymers) and polymerised forms (the so called “tannins”) (Bravo, 1998).

The antioxidant effect of polyphenols in wine is related to their low oxidation potential at the oenological conditions, which enable them to easily release an electron or a proton through ET and HT processes, stabilising reactive species occurring during the oxidation process, and their ability to form coordination complexes with free metal ions (**section 2.3**).

In this section, experiments aimed to find a cause-effect relationship between wine composition and antioxidant activity are presented. Such experiments disclosed the importance to profile the total content of polyphenols in wine and to determine the contribution of each class of phenolics, as experimentally determined, in the antioxidant activity.

Section 5A is devoted to the presentation of an experiment aimed to determine the correlation between maceration time, extraction of bioactive compounds and antioxidant activity of selected international wines, which was performed as part of the PhD project in collaboration with staff of the University "Goce Delčev", Republic of Macedonia.

Section 5B reports preliminary results for an innovative device optimized for the rapid and simultaneous detection of TPC and AA parameters in wine and wine products. A fast, reliable and easy to use analytical approach allows to monitor the evolution of the phenolic fraction along the supply chain (extraction of phenols in fermentation, treatments with resins, tartaric stabilization, fining) and in bottled wines.

In addendum C, a scientific paper published in collaboration with staff of the University "Goce Delčev", Republic of Macedonia, illustrates the effect of selected yeast strains in the antioxidant activity of international red wines.

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Chapter 5: Structure-antioxidant activity relationship of wine polyphenols

5A) THE EXTENT OF MACERATION TIME ON THE LEVEL OF PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF RED WINES

RESEARCH ARTICLE:

Extraction and evaluation of natural occurring bioactive compounds and change in antioxidant capacity during red winemaking

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(UNDER REVIEW)

5.1. Introduction

Red wines quality is mostly affected by the phenolic fraction extracted during fermentation. Wine polyphenolic affect the sensory properties of wines (colour, mouthfeel) and the antioxidant properties (Casassa & Harbertson 2014).

Red wines are particularly rich in natural antioxidants due to the extended contact of musts with grape skins, which contain anthocyanins, flavan-3-ols, flavonols, dihydroflavonols, hydroxycinnamoyl tartaric acids, hydroxybenzoic acids and hydroxystilbenes; in addition, they are enriched of flavan-3-ols and gallic acid from the seeds, and the hydroxycinnamoyl tartaric acids which are mainly present in the juice (Adams 2006; Doshi, Adsule, Banerjee, & Oulkar, 2015). There are several factors affecting the phenolic composition of grapes, including varietal, climate conditions, ripening stages; furthermore, the winemaking practices affect the extractability and availability of these compounds in finished wine (Sacchi, Bisson, & Adams, 2005; Kennedy, Saucier, & Glories 2006; González-Neves, Favre, & Gil, 2014). In particular, seed flavan-3-ols advantage of a long fermentation since they are protected with lipidic layer which is disrupted when appropriate content of alcohol is formed, allowing their releasing from the seeds (Sun, Sacks, Lerch, & Heuvel, 2011).

Regardless from sensory impact of these compounds on the wine, the relationship between wine composition and antioxidant activity has been investigated in many recent studies. The storage of compositional data and their correlation with the antioxidant power of wine is a powerful tool to build up a database for different wine varietals which would provide information on an annual and regional basis.

The possibility to provide to wine producers technological data on different vintages was considered a powerful tool to optimize the grape processing avoiding detrimental oxidations due to an improper intake of natural antioxidants.

A partnership with the University “Goce Delčev” of the Republic of Macedonia have allowed to profile local (Stanušina, Vranec) and international (Merlot, Cabernet Sauvignon and Shyraz) red wines produced in Macedonia according with their phenolic composition and antioxidant activity following fermentation (Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015). Among these, Vranec only was studied in relationship with the maceration time (Ivanova, Dörnyei, Márk, Vojnoski, Stafilov, et al., 2011), herein Stanušina wine, from an indigenous

Macedonian grape variety was not assayed according with its composition and antioxidant activity.

In this work Stanušina wine was studied for the first time, and the extraction of phenolic compounds during maceration was monitored with the time (3, 6 and 9 days). Results were compared with compositional data of Vranec, a well-known red wine developed in Macedonia as in the Balkan region, and Cabernet Sauvignon, an international varietal produced worldwide. Results provided in this section are extrapolated from a common work aimed to scientific publication. The draft of this research article is currently under peer-review.

5.2. Materials and Methods

5.2.1. Chemicals

The working solutions were prepared with Milli-q grade water (MilliQ water system, Millipore, Bedford, MA). Methanol, acetonitrile, perchloric acid, and formic acid for working solutions and eluents were purchased by Merck (Merck, Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) for the radical scavenging assay, as well as gallic acid, (+)-catechin, (-)-epicatechin, caffeic acid, and syringic acid standards were supplied by Sigma (Sigma-Aldrich, Milano, Italy), while protocatechuic acid, vanillic acid, p-hydroxybenzoic acid, p-coumaric acid were from Extrasynthese (Extrasynthese, Genay, France). The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox©) used for DPPH assay calibration was from Fluka Analytical (Sigma-Aldrich, Milano, Italy).

5.2.2. Grapes

Wines (vintage 2013) used in this study were obtained from *V. vinifera L.* varieties Stanušina, Vranec and Cabernet Sauvignon, cultivated in the Tikveš wine region (Republic of Macedonia).

5.2.3. Winemaking

Hand harvested grapes (100 kg) of each variety were processed separately. The must of each grape variety was collected in a plastic tank (50 L) following mechanical crusher/destemmer, added with 80 mg L^{-1} of SO_2, then inoculated with commercial dry

yeast Excellence SP Saccharomyces cerevisiae (Lamothe-Abiet, Canejan/Bordeaux, France).

Soon after the addition of SO_2 and yeast, the grape must of each variety (Stanušina, Vranec and Cabernet Sauvignon) was divided into three replicated sub-samples to monitor the extraction of phenolics compounds from skins with time, at 3, 6 and 9 days of maceration, obtaining a batch of 9 wines in total for each variety. In order to obtain representative samples for each wine variety and each maceration time, all samples were prepared by mixing wines from three tanks produced with the same technological treatment.

5.2.4. Determination of general chemical composition

Official OIV methods (OIV 2014) were used to assess the wines quality: alcohol (OIV-MA-AS312-01A), dry extract (OIV-MA-AS2-03B), specific density (OIV-MA-AS2-01A), total acidity (OIV-MA-AS313-01), volatile acidity (OIV-MA-AS313-02), total SO_2 and free SO_2 (Ivanova-Petropulos & Mitrev 2014).

5.2.5. Spectrophotometric analyses

Spectrophotometric analyses were performed after 4 months of storage of the wines in the cellar at 12–13°C. To determine the colorimetric indexes of wines, optical densities at 420 nm (browning degree), 520 nm and 620 nm (anthocyanins) were monitored using a cuvette with 1 cm optical path against the blank, i.e. water (Harbertson & Spayd 2006), thus deriving the following colour indexes (CI): colour intensity (CI = Σ 420+520+620 nm) and colour tonality (H= 420/520 nm) (OIV 2014).

TPC expressed as mg L^{-1} GAE/L was determined by reading of the absorbance of diluted samples (1:100 dilutions in distilled water) at 280 nm, and using a calibration curve of gallic acid standard solution in the range of 1.95 to 31.25 mg/L (Ribéreau-Gayon, 1970). Analytical determinations were performed using a UV-VIS spectrophotometer (Shimadzu, UV-mini 1240, Milan, Italy).

Antioxidant activity of wines was determined as a radical scavenging ability following the procedure described by Brand-Williams et al. (1995), further modified for red wines (Ivanova et al. 2015). Results were expressed as mg Trolox equivalent L^{-1} (TE L^{-1}) with a calibration curve constructed using methanol solutions of Trolox (0.19-93 mg/L).

5.2.6. HPLC analyses

High Performance Liquid Chromatography (HPLC) analyses were performed after 4 months of storage of the wines in the cellar at 12–13°C.

The HPLC system was equipped with temperature control oven, photodiode array detector (DAD) and a Chromeleon chromatography manager software v. 6.60 SP2 (Dionex DX500, Milano, Italy) and used for identification and quantification of anthocyanins, phenolic acids and flavan-3-ols in wines. The samples were filtered using 0.20 μm cellulose acetate membrane (Millipore, Milano, Italy) before direct injection into the HPLC system, kept at 30°C.

Anthocyanins and related pigments were analyzed at 530 nm with the Gemini RP-C18 column (250 x 4.6 mm; 5 μm particle size; 110Å porosity; Phenomenex, Bologna, Italy) using the following mobile phases: water/methanol (70/30, v/v) containing 6 mL/L of 70% perchloric acid (solvent A) and water/methanol (25/75, v/v) containing 6 mL/L of 70% perchloric acid (solvent B). The complete method developed by authors, including the flow rate and the gradient elution conditions have been previously published (Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015).

Hydroxycinnamic acid derivatives and flavan-3-ols were analyzed with the Aquapore ODS-300 RP-C18 column (250 x 4.6 mm; 7 μm particle size; 300Å porosity; Applied Biosystems, San Jose, CA, USA) using the following mobile phases: solvent A (water/formic acid, 98/2, v/v) and solvent B (acetonitrile/water/formic acid, 80:12:2, v/v/v). The complete HPLC method developed by authors, including the flow rate and the gradient elution conditions have been previously published (Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015).

5.2.7. Statistical analysis

To disclose the effect of maceration time (3, 6 and 9 days) on the bioactive phenolic composition and antioxidant activity of red wines (three replicated sub-samples), the one-way ANOVA, regression and statistics was performed by using TANAGRA 1.4.28 software (Lyon, France). Moreover, Principal Component Analysis (PCA) was carried out to disclosure relationship between wine samples taking into account selected variables.

5.3. Results and Discussion

5.3.1. Chemical parameters of oenological interest

The chemical parameters of wines used in this experiment have been measured four months after the end of fermentation, and results are summarised in **table 5.1**. The alcohol content, which was within the regulatory limit of 20% v/v for wines of area C III b (Reg. CE 606/2009), was increased in the case of Stanušina (13.57%), as a consequence of an higher sugar content of grape at harvest (Stanušina, 24.3; Vranec, 22.6; Cabernet Sauvignon, 20.6 °Brix, respectively). Total acidity was pretty high, thus confirming the previous values obtained for other Macedonian and Balkan wines (Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015; Rajković & Sredović 2009; Košmerl, Bertalanič, Maraš, Kodžulović, Šućur, & Abramovič, 2013). The average volatile acidity content was estimated in 0.57±0.1 g L^{-1} for all samples, without significant differentiation between samples. Free sulphur dioxide was added in a concentration range of 30-42 mg L^{-1}, while the total SO_2 ranged from 62 to 93 mg L^{-1}.

TPC ranged 733-1631 mg/L GAE, with the highest average values obtained for Cabernet Sauvignon wines (1574 mg L^{-1}), and followed by Vranec (1014 mg L^{-1}) and Stanušina (795 mg L^{-1}). The effect of skin maceration time on the total phenolics content of wines was highest at day 9, but any significant difference was observed between wines obtained after 6 and 9 days of maceration, regardless the variety (*p*-level > 0.05).

High antioxidant activity was observed for all wines, regardless the variety; the trend was similar to the TPC content, with Cabernet Sauvignon exhibiting the higher value (115 mg L^{-1}, TE). TPC content for all samples were linearly correlated with the antioxidant activity of wines (R^2 = 0.922, *data not shown*). The Cabernet Sauvignon also showed the highest average values of CI (14.51 AU), while the CI of Stanušina was the lowest (average: 1.90 AU). Peak of CI in wines were observed after 3 days of maceration, followed by color drop with time due to the copigmentation and anthocyanins precipitation, oxidative polymerization and adsorption on the pomace. The H-indexes, accounting for the hue of young red wines, ranged betweem 0.33 and 0.51 for Vranec and Cabernet Sauvignon, in agreement with values from the literature (Tsanova-Savova, Dimov, & Ribarova, 2002; Kontkanen, Reynolds, Cliff, & King, 2005, Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015).

Stanušina wine showed high hue values (1.12, on average) due to the low content of red compounds that confer a characteristic pale red colour compared to Vranec and Cabernet Sauvignon, as well as other red grape varieties (Tsanova-Savova, Dimov, & Ribarova, 2002; Kontkanen, Reynolds, Cliff, & King, 2005).

Wines	**Vranec**			**Cabernet Sauvignon**			**Stanušina**		
Skin contact (days)	**3**	**6**	**9**	**3**	**6**	**9**	**3**	**6**	**9**
Total SO_2 (mg L^{-1})	93	62	85	68	75	66	59	62	68
Free SO_2 (mg L^{-1})	45	30	35	15	23	33	15	23	42
Total acidity (mg L^{-1})	5.7	5.5	5.5	6.5	5.5	5.5	5.8	5.5	6.0
Volatile acidity (mg L^{-1})	0.60	0.56	0.48	0.68	0.56	0.48	0.52	0.63	0.68
Alcohol (%,*v/v*)	11.44	11.46	11.66	13.04	12.88	13.01	13.58	13.51	13.64
Dry extract (g L^{-1}))	22.25	22.83	23.17	29.88	30.18	29.53	27.3	26.06	26.96
Specific density	0.9933	0.9935	0.9934	0.9944	0.9947	0.9943	0.9928	0.9924	0.9926
TPC (mg L^{-1}), GAE)	913	1045	1084	1479	1612	1631	733	772	880
CI	5.81	4.24	5.51	25.20	10.20	8.13	2.11	1.75	1.85
Hue	0.44	0.53	0.51	0.33	0.47	0.47	0.9	1.17	1.29
AA (mg L^{-1}), TE)	107	109	109	113	117	115	102	100	105

Table 5.1. Colorimetric and chemical parameters of selected wines at different days of maceration. *Abbreviation: TPC- total phenols content, in Gallic acid equivalents (GAE), CI-colour intensity, AA-antioxidant activity in Trolox equivalents (TE).

5.3.2. Anthocyanins

The analysis of anthocyanins at different maceration times was determined by HPLC-DAD taking advantage of the photometric response at 530 nm (**figure 5.1.**); **Table 5.2** summarise the peak attributed to glucosides monomeric pigments.

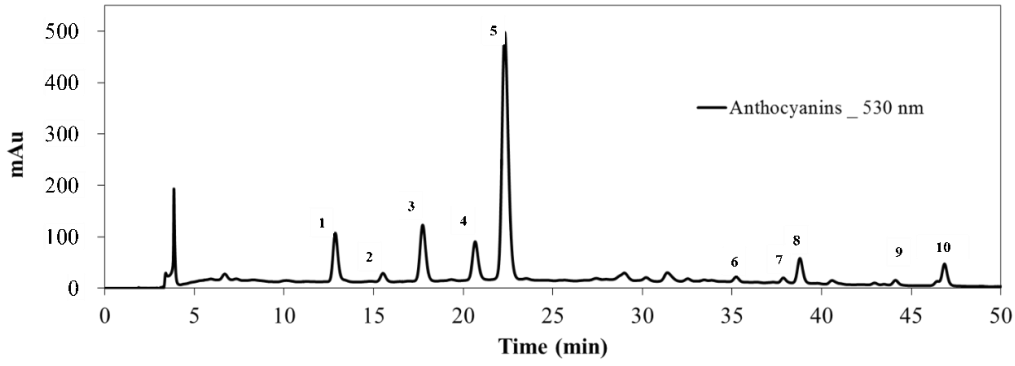

Figure 5.1. HPLC-DAD anthocyanins profile of a Vranec wine, following 9 days of maceration. Peaks: delphinidin-3-glucoside, (1); cyanidin-3-glucoside, (2); petunidin-3-glucoside, (3); peonidin-3-glucoside, (4); malvidin-3-glucoside, (5); petunidin-(6 acetyl)-3-glucoside, (6); peonidin-(6 acetyl)-3-glucoside, (7); malvidin-(6 acetyl)-3-glucoside, (8); peonidin-coumaroyl-glucoside, (9); malvidin-coumaroyl-glucoside, (10).

Nine anthocyanins derived from the different grape varietals, including four monoglucosides, three acetylglucosides and two p-coumaroylglucosides, have been identified and quantified. In all wines the malvidin-3-glucoside was the main anthocyanin regardless the variety and maceration time, as expected for *V. vinifera* varieties, followed by petunidin-3-glucoside (**Table 5.2**). Cyanidin-3-glucoside was detected but not quantified in all samples, and similarly peonidin-3-glucoside and delphinidin-3-glucoside were below the limit of quantification in Stanušina wines. Regardless the CI values calculated for wines, Vranec wines showed highest mean value of total anthocyanins (813 mg L^{-1}), followed by Cabernet Sauvignon (700 mg/L) and Stanušina (132 mg/L); furthermore, the monoglycosides were the most represented class of anthocyanins in wines, followed by acetyl derivatives and p-coumaroylglucosides. The ratio of acetylglucosides and p-coumaroylglucosides (Σ acetylated/Σ p-coumaroylated) were tentatively used to provide an authenticity index for varietal of red wines; the index for Vranec wines (mean 1.53), was in agreement with previous data for Cabernet Sauvignon (mean 6.78) and Stanušina (mean 0.57) (Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015).

Wines	**Vranec**			**Cabernet Sauvignon**			**Stanušina**		
Skin contact (days)	**3**	**6**	**9**	**3**	**6**	**9**	**3**	**6**	**9**
Dp-Glc	5.88	12.2	10.3	2.04	5.58	3.19	n.d.	n.d.	n.d.
Pt-Glc	30.4	48.6	43.2	11.7	20.5	15.5	4.34	5.13	2.62
Pn-Glc	9.13	21.1	17.8	0.35	5.70	4.31	0.00	0.00	0.00
Mv-Glc	485	649	595	251	466	436	101	115	87.8
Total Glc	**531**	**732**	**667**	**265**	**497**	**459**	**105**	**121**	**90.5**
Pt-AcGlc	3.65	5.89	5.63	2.59	12.7	10.9	n.d.	n.d.	n.d.
Pn-AcGlc	6.30	8.53	8.48	12.9	16.4	12.9	n.d.	n.d.	n.d.
Mv-AcGlc	73.3	102	94.8	157	284	255	9.92	10.4	7.88
Total AcGlc	**83.3**	**116**	**109**	**173**	**313**	**279**	**9.92**	**10.4**	**7.88**
Pn-coumGlc	3.56	6.92	5.31	0.23	0.19	0.17	0.00	1.16	0.15
coumGlc	53.5	74.1	59.2	23.9	47.2	42.0	13.7	21.6	14.5
Total coumGlc	**57.1**	**81.1**	**64.5**	**24.1**	**47.4**	**42.2**	**13.7**	**22.7**	**14.6**
Total anthocyanins	**140**	**197**	**173**	**197**	**360**	**321**	**23.7**	**33.1**	**22.5**
ΣGlc/ΣAcG	**6.38**	**6.31**	**6.12**	**1.53**	**1.59**	**1.65**	**10.6**	**11.6**	**11.5**
ΣGlc/ΣcoumGlc	**9.31**	**9.03**	**10.3**	**10.9**	**10.5**	**10.8**	**7.68**	**5.30**	**6.19**
ΣAcGlc/ΣcoumGlc	**1.46**	**1.43**	**1.69**	**7.15**	**6.60**	**6.60**	**0.72**	**0.46**	**0.54**

Table 5.2 HPLC profile of monomeric antocyanins detected in HPLC-DAD (530 nm). *Abbrevation: Dp-delphinidin, Cy-cyanidin, Pt-petunidin, Pn-peonidin, Mv-malvidin, Glc-glucoside, AsGlc-acetylglucoside, coumGlc-coumaroylglucoside. n.d. – not detected.

The anthocyanins composition of wines showed that the maximum extraction occurred after 6 days of maceration, regardless the variety. The extraction of anthocyanins occurred at an early stage of the fermentation, followed by a slight droplet in the following period (up to 9 days), due to rearrangement of reactive anthocyanins monomers and formation of pigmented polymers (Gil-Muñoz, Gómez-Plaza, Martınez, & López-Roca, 1997; Bautista-Ortín, Fernández-Fernández, López-Roca, & Gómez-Plaza, 2004; Herjavec, Jeromel, Maslov, Korenika, Marija, Mihaljević, & Prusina, 2012).

5.3.3. Simple phenolic fraction

The HPLC profiles were obtained at the two wavelength of (i) 280 nm (phenolic acids; flavan-3-ols) and (ii) 324 nm (hydroxycinnamic acids). Protocatechuic, gallic and syringic were the main hydroxybenzoic acids, as they were identified and quantified in all samples. The gallic acid showed an exceptionally high value in Cabernet Sauvignon (average: 263 mg L^{-1}), followed by Vranec (average: 139 mg L^{-1}) and Stanušina (average: 122 mg L^{-1}); these results are consistent with previous findings (Tarola, Milano, & Giannetti, 2007; Nikfardjam, Márk, Avar, Figler, & Ohmacht, 2006). Cabernet Sauvignon also contained the highest mean value of hydroxybenzoic acids (289 mg L^{-1}). Regarding the effect of extend of skin contact, the content of gallic acid increased during fermentation, with highest content in Cabernet Sauvignon and Stanušina wines at day 9, while the Vranec wine showed high content of gallic acid at day 6. In general, the content of total hydroxybenzoic acids increased during winemaking and reached highest concentration in the wines at day 9, which was in agreement with previous results (Plavsa, Jurinjak, Antunovic, Persuric, & Ganic, 2012). Five hydroxycinnamic acids were detected and quantified including caftaric, coutaric, fertaric, caffeic and p-coumaric acids. Among them, caftaric acid was the most important (average: Stanušina 409 mg L^{-1}, Vranec 170 mg L^{-1} and Cabernet Sauvignon 120 mg L^{-1}), followed by coutaric acid and caffeic acid (**Table 5.3**). Stanušina wines exhibited the highest mean content on cinnamates (492 mg L^{-1}) while the Cabernet Sauvignon showed a relatively low content in these compounds (170 mg L^{-1}); these differences were ascribed to wine varietals more than to the winemaking process. Regardless the wine varietal, hydoxycinnamates showed an extraction peak at day 3 and a subsequent decrease for the remaining period of the experiment. It is well known that

the hydroxycinnamic acids readily interact with anthocyanins during winemaking, leading to colour stabilisation processes and autoxidation of vicinal dihydroxyphenols (Schwarz, Wabnitz, & Winterhalter, 2003).

Wines	**Vranec**			**Cabernet Sauvignon**			**Stanušina**		
Skin contact (days)	**3**	**6**	**9**	**3**	**6**	**9**	**3**	**6**	**9**
Protocatecuic acid	47.6	n.d.	26.7	24.7	18.6	18.6	18.2	n.d.	15.8
Gallic acid	59.9	187	170	113.1	328	348.4	67.4	141	158
Syringic acid	32.5	24.4	37.1	n.d.	n.d.	15.5	21.8	22.0	n.d.
Total HBA	**140**	**211**	**234**	**138**	**347**	**383**	**107**	**163**	**174**
p-Coumaric acid	14.1	12.8	8.40	6.31	5.69	12.3	2.24	3.45	4.51
Caftaric acid	195	166	148	166	99.5	94.1	428	425	373
Coutaric acid	23.3	29.4	25.5	19.8	13.9	11.6	18.5	26.2	24.4
Caffeic acid	10.9	10.9	16.2	11.4	10.5	4.43	47.7	35.7	30.1
Fertaric acid	18.2	22.7	19.5	20.6	17.1	18.1	14.9	20.5	21.8
Total HCA	**262**	**242**	**218**	**224**	**147**	**141**	**511**	**511**	**454**
Total Phenolic acids	**402**	**453**	**451**	**362**	**493**	**523**	**619**	**674**	**628**
Catechin	20.5	220	251	87.4	292	375	139	214	262

Table 5.3 HPLC-DAD profile of monomeric polyphenols detected in HPLC-DAD (280-324 nm). *Abbrevation: HBA-hydroxybenzoic acids, HCA-hydroxycinnamic acids, HCAD- hydroxycinnamic acid derivatives. n.d. – not detected.

Macedonian red wines Stanušina, Vranec and Cabernet Sauvignon showed significantly high concentration of catechins (207 mg L^{-1}, on average) regardless the extent of skin contact. The averaged value of flavanol compounds was high, with a maximum for Cabernet Sauvignon (252 mg L^{-1}) and lowered for Vranec wine (164 mg L^{-1}) if compared with previous studies, (Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015), while the level of catechin in Cabernet Sauvignon was higher compared to Cabernet Sauvignon wines from the same study (mean content 170 mg L^{-1}). The content in (+)-catechin and derivatives increased with maceration time with increasing alcohol levels which are formed following fermentation, and regardless the wine variety (Glories & Saucier 2000; Sacchi, Bisson, & Adams, 2005).

5.3.4 Principal component analysis

Principal Component Analysis (PCA) was used to explore the effect of each variable (grape variety and skin contact of 3, 6 and 9 days) on the basis of HPLC results in Stanušina, Vranec and Cabernet Sauvignon wines. The first two principal components, PC1 and PC2, accounted for 79.54% of the total variance (53.36% for PC1 and 26.18% for PC2). The projection of the wine samples on the first two principal components

clearly separated the wine varieties: Vranec wines (V) were located in the down negative part of PC1 and Cabernet Sauvignon (CS) in the upper positive part of PC1, while Stanušina wines (S) were located on the left side of PC1. The plot also highlighted the effect of maceration time on the composition of red wines in the following increasing order: Stanušina, Vranec and Cabernet Sauvignon (**Figure 5.2**).

The principal components responsible for the differences in the phenolic composition are presented in the scatter plot in **Figure 5.3**. The components responsible for the separation of Cabernet Sauvignon wines were acetyl-3-glucosides of malvidin, petunidin and peonidin and gallic acid which prevailed in the positive part of the first principal component, while anthocyanin monoglucosides and *p*-coumaroylglucosides were characteristic for Vranec wines. In addition, hydroxycinnamic acids caftaric and caffeic acids were dominant compounds in Stanušina wines. In general, separation of the wines was performed according to the varietal characteristics.

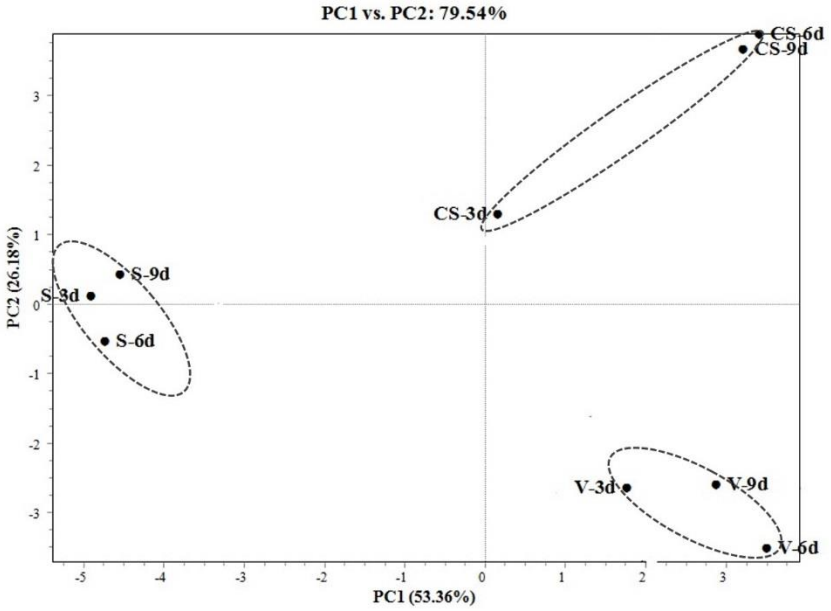

Figure 5.2. Principal Component score plot of the variables with PC1 and PC2 based on anthocyanins, phenolic acids and catechin for the Stanušina, Vranec and Cabernet Sauvignon wines macerated for 3, 6 and 9 days.

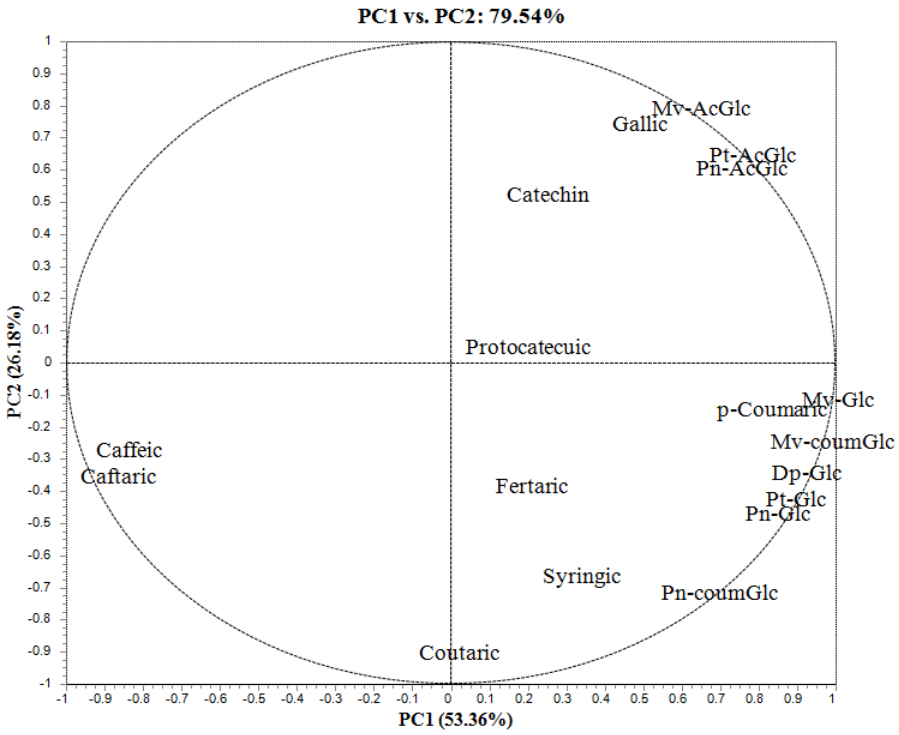

Figure 5.3. Correlation scatterplot of the variables with PC1 and PC2 based on anthocyanins, phenolic acids and catechin for the Stanušina, Vranec and Cabernet Sauvignon wines macerated for 3, 6 and 9 days.

5.3.5. Further considerations on the antioxidant activity of selected wines

The antioxidant activity in selected wines, measured in terms of free radical scavenging, was not strongly affected by the maceration time, and in particular results of the 6th and 9th maceration day were aligned, regardless the wine variety. The Cabernet Sauvignon variety exhibited the highest average AA value (115 mg L^{-1}) followed by Vranec (108 mg L^{-1}), and Stanušina (102 mg L^{-1}); as previously observed, results are in agreement with the trend of the TPC content (see **section 5.3.1**). The effect of skin maceration time on the total phenolics content of wines was highest at the 9th day of maceration, but any significant difference was observed between the 6th and 9th days; this is also reflected in the radical scavenging activity measured in the same period (*p*-level > 0.05). According with anthocyanins, they seemed to have contributed in different ways to the trend of AA in wines. In particular, a higher content in anthocyanins monoglycosides in Vranec (average: 644 mg L^{-1}) was not traduced in an improved AA, which is higher for cabernet Sauvignon regardless the lower content in glycosylated anthocyanins (mean: 407 mg L^{-1}); reversely, the acylated and cumarated glucosides content followed the

same trend of the radical scavenging activity in three wines (see **table 5.2**). Stanušina is significantly lower in anthocyanin content when compared with the other wines assayed (mean: 26.4 mg L^{-1}), but the AA, although lower, was not affected in a similar extent (see **table 5.2**).

According to the simple phenolic fraction, despite the calculated total phenolic acids are aligned with the AA calculated for each wine (**table 5.3**), different classes of compounds provided different contribution to the radical scavenging activity. Hydoxybenzoic acids and catechins mainly contributed to the high antioxidant activity of Cabernet Sauvignon wines, while the hydroxycinnamic acids, which were significantly higher for Stanušina wines (mean: 492 mg L^{-1}) followed the opposite trend of AA. This result could be partially explained with the involvement of such compounds in competitive reactions, mainly the stabilisation of wine colour through the formation of molecular adducts with anthocyanins and oligomers.

5.4. Conclusions

This work allowed to elucidate the contribution of maceration time in the extraction of bioactive compounds in Vranec, Cabernet Sauvignon and Stanušina wines. The monitoring of winemaking showed that extraction pattern depended both on skin contact period and grape variety; particularly, hydroxycinnamic acids and anthocyanins peaked at third and sixth day of maceration, respectively, followed by a slight decrease with time (9 day), due to their involvement in further reactions which enable the stabilisation of wine colour, according with well-known mechanism. Hydroxybenzoic acids and (+)-catechin content was highest at day 9 of skin maceration. Compared to Cabernet Sauvignon and Vranec, the Stanušina wines showed low level of anthocyanins, but relatively high content of hydroxycinnamic acids, such as caftaric and caffeic acids, and high antioxidant activity, despite slightly lowered when compared to the other variety assayed. The study of the simple phenolic fraction (anthocyanins, phenolic acids) coupled with the determination of the radical scavenging activity enabled the study of the contribution of each compound to the antioxidant activity of wine in the early stage of production.

5.5. Literature Cited

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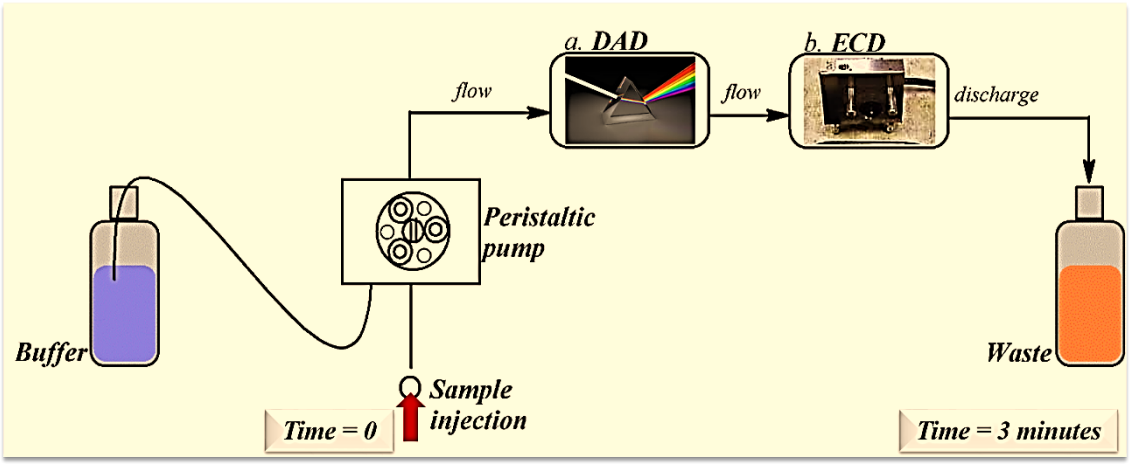
5B) A DIODE ARRAY-ELECTROCHEMICAL COUPLED DETECTION SYSTEM FOR FAST AND SIMULTANEOUS DETERMINATION OF TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN WINES AND OENOLOGICAL TANNINS



RESEARCH ARTICLE:

Method comparison for analysis of total polyphenols content and antioxidant activity using a modified FIA system with tandem diode-array and electrochemical detections

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(UNDER REVIEW)

5.6. Introduction

In section 5A it was emphasized the relationship between the content of total polyphenols and the ability to limit the occurrence of oxidative phenomena of a wine. It would be strongly recommended to monitor the extraction of bioactive compounds and the active antioxidant fraction during winemaking; unfortunately, despite the big number of analytical methods developed for these purposes, only few of them are suitable for a quick and reliable monitoring in the supply chain; wine need to be sampled by oenologists and analysed in specialized laboratories. For this reason, the development of fast and reliable analytical methods to be directly applied in cellar were considered in this thesis work an important part of the strategy for limiting the occurrence of oxidative degradation in commercial wines. In a preliminary study, a modified flow-injection analysis (FIA) system, with coupled diode-array and electrochemical detector have been designed taking advantage of the laboratory equipment, to assess the effectiveness in view of its potential applicability in the production of portable devices. Results reported in this section are extrapolated from a presentation of preliminary results which is currently under peer-review in view of its potential publication in a scientific journal.

Wine polyphenols extractability is an important technological parameter in winemaking, and their reactivity and solubility with time affect many complex physico-chemical factors (Singleton & Esau, 1969). The changes in TPC and AA parameters, in particular, provide important information on the evolution of phenolic fraction along the supply chain, but their determination is often related to complex and time-consuming analytical methods; the need for fast and combined analytical methods has stimulated the search of alternative approaches (Harbertson & Spayd, 2006; Luque de Castro, González-Rodríguez, & Pérez-Juan, 2005; Lorrain, Ky, Pechamat, & Teissedre, 2013). The use of electrochemical methods, in particular, provides further insight the redox ability of phenolic compounds that can be assimilated to the antioxidant mechanisms occurring in wine (Kilmartin, Zou, & Waterhouse, 2001; Makhotkina & Kilmartin, 2010).

Electrochemical analysis also advantage of high selectivity, which can be tailored by varying the working potential values enabling targeted analysis on specific classes of compounds. The redox properties of polyphenols are likely to reflect the antiradical capacity of the wines (Arnous, Makris, & Kefalas, 2002; Rivero-Pérez, Muñiz, &

González-Sanjosé, 2007), and the electron transfer occurring in radical scavenging seems to exhibit similar mechanisms as the polyphenolic electrochemical oxidation (Arteaga, Ruiz-Montoya, Palma, Alonso-Garrido, Pintado, & Rodríguez-Mellado, 2012; Gizdavic-Nikolaidis, Travas-Sejdic, Bowmaker, Cooney, Thompson, & Kilmartin, 2004).

The diode-array detection (DAD) has been extensively used in the determination of the total polyphenols in wine samples, particularly at the wavelength of 280 nm, where the π- system of aromatic structures (phenolic rings) exhibit a high extinction coefficient (ε). According with the Lambert-Beer law, the absorbance can be converted in analyte concentration within the linearity range of the spectrophotometer; the absorbance value of diluted wines at 280 nm is thus converted in equivalents of gallic acid (mg L^{-1}; mM GAE) building up a proper calibration curve in the linearity range (Ribéreau-Gayon, 1970).

The aim of this study was to combine advantages deriving by both detection systems, for the simultaneous determination of the total polyphenols and their radical scavenging activity. A high-pressure liquid injection system has been designed for this purpose, enabling the on-line injection of sample in the diode array detector and subsequently in the amperometry cell. The proposed flow-injection system was tested in model wine solutions of gallic acid and oenological tannins and in real samples (red and white wines), selected between Macedonian, Italian and international varieties to provide a representative sampling.

5.7. Materials and Methods

5.7.1. Chemicals

Milli-Q ultrapure water (Millipore, Bedford, MA) was used to prepare all working solutions, including model wine. Gallic acid monohydrate HPLC grade (≥98%) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) were purchased (Sigma-Aldrich, Saint Louis, MO). L-(+)-tartaric acid analytical grade (≥ 99.5%) and ethanol HPLC grade (≥ 99.9%) for working eluent and model wine were supplied by Merck (Merck, Darmstadt, Germany).

5.7.2. Wines and Oenological Tannins

Thirty-one wines from Italy and Republic of Macedonia, obtained from national and international V. vinifera grape varieties, were selected for a representative screening. Twelve oenological commercial grade tannins (HTS Enologia, Marsala, TP, Italy; Laffort, Bordeaux Cedex, France) were dissolved in model wine solution (i.e. ethanol 12%, tartaric acid 2 g/l, pH 3.6), and filtered with 0.2 µm cellulose acetate membrane filters prior to analysis.

5.7.3. Spectrophotometric analyses

The total polyphenol content (TPC) was determined at 280 nm (UV_{280nm} assay) on a 10 mm quartz cuvette (Ribéreau-Gayon, 1970) following 100 dilutions for red wines, ten dilutions for white wines, whereas tannin solution were prepared at the working concentration of 0.29 mM GAE. Results were corrected for dilution and expressed as mM GAE against a gallic acid calibration curve over the range 0–0.18 mmol gallic acid L^{-1} (R^2 = 0.9972; intercept = -0.0322; slope = 5.7378).

The radical scavenging activity was determined against the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) synthetic radical according with a method previously described (Brand-Williams, Cuvelier, & Berset, 1995), and further implemented for red wines (Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015). Red wines and tannins were diluted 40 times, while the white wines had a 5-fold dilution; results were corrected by dilutions and expressed as mM GAE using a calibration curve in the range 0–0.15 mM gallic acid/L (R^2 = 0.997; intercept = 0.8067; slope = -3.94). A Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan) was used for spectrophotometric analyses, and results were averaged over two determinations.

5.7.4. High-pressure flow injection analysis

The flow injection system was part of a Dionex high-pressure liquid chromatography system equipped with GP50 gradient pump, a PDA-100 Photodiode Array Detector (DAD) and an ED50 Electrochemical Detector (ECD) that were connected in series (Thermo Fisher Scientific Inc., Waltham, MA). The analysis was carried out as follows:

- eluent: 50 mM tartaric acid aqueous solution;
- elution: isocratic flux for 3 minutes, with a flow-rate of 1,0 ml/min;

- injection: 25 µl of sample diluted in distilled water (red wine: 40 folds; white wines: 5 folds; tannins: stock solution);

- injection system: a peak line composed of two modules, bearing different inner sections, before reaching the detectors; this allowed a stable counter pressure of 78 bar for the entire analysis.

The DAD was set-up at 280 nm, the peak was integrated baseline and the total polyphenols content was calibrated as GAE in the range 0-0.37 mM gallic acid/L (R^2 = 0.9994; intercept = 0.9607; slope = 201.38). The ECD was set at 800 mV potential to oxidize all the phenolic compounds that contribute to the antioxidant activity in wine (Mannino, Brenna, Buratti & Cosio, 1998). The anodic current under the amperometry peak was integrated to determine the total antioxidant activity of wine polyphenols using a calibration curve over the range 0-0.19 mM GAE (R^2 = 0.9984; intercept = 0.4633; slope = 189.41). The electrochemical analysis was performed in integrated amperometry mode, applying a waveform with cycles of 0.5 sec duration, divided into three regions (**Figure 5.3**): (i) the absorption of the analyte at the electrode surface; (ii) the current integration period; (iii) the cleaning steps to remove passivation layer and activate the electrode surface.

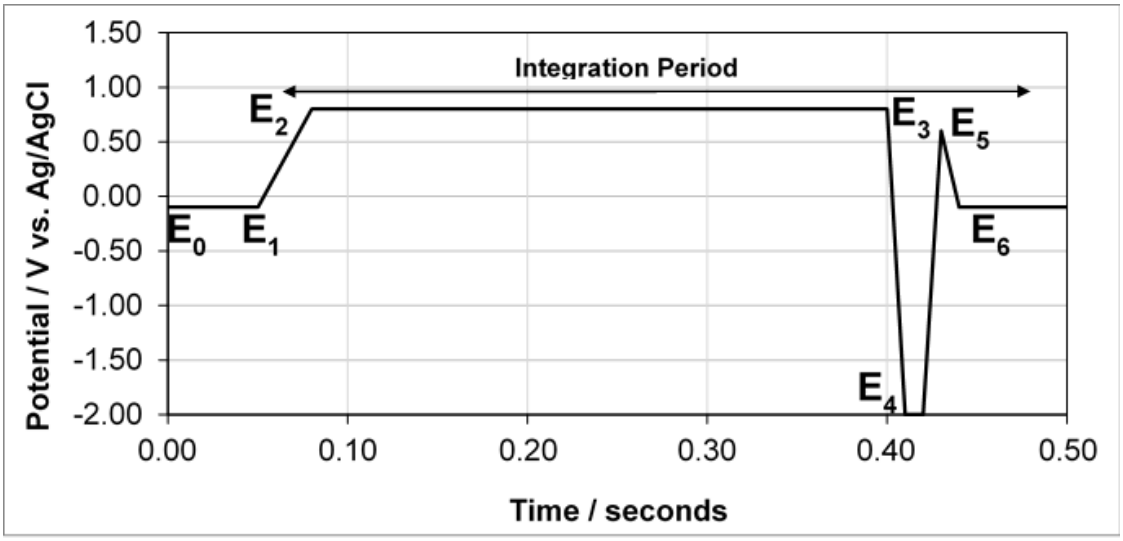

Figure 5.3. Integrated amperometry waveform used to determine the antioxidant activity of phenolic compounds in wines and model wine solutions. Detector response is the charge from integration of the phenolics oxidation current between 0.20 and 0.50 s, expressed as nC.

Samples were analysed in duplicate, and a pure tartaric acid solution was injected after each determination to clean the system. The CG electrode was also regenerated after

each sample with a polishing kit consisting of a urethane fibre polishing pad and 0.3 µm alumina powder (Thermo Fisher).

5.7.5. Data processing and statistical analysis

Univariate statistical determination were performed using Unscrambler (Unscrambler 7.6, CAMO ASA, Oslo, Norway) and Analyse-it 4.20.1 for Excel (Analyse-it Software, Leeds, UK).

5.8. Results and Discussion

5.8.1. Method validation

The FIA/DAD-ECD system was compared with spectrophotometric methods based on the optical density at 280 nm and on the removal of a synthetic radical absorbing at 517 nm; the validation included a set of samples over a wide concentration range with the aim to identify the constant and proportional systematic errors.

Linear regression coefficients allowed to detect a bias as the regression lines were significantly different from slope (β) of 1; the Bland-Altman plot was used to compare the results of each and every two methods by plotting the absolute difference (method A – method B) as a function of the measurements average [(method A + method B)/2] (Bland & Altman, 1999); the trend was significant for all methods assayed, with exclusion of the ECD vs DPPH results (*data not shown*). The difference was due to a systematic proportional difference between the compared methods which was further confirmed applying the Deming's regression ($\beta \neq 1$). The contribution of the standard used for calibration was removed using the same compounds (gallic acid monohydrate) for both FIA and spectrophotometric methods. The most reliable hypothesis about the systematic bias applied for ECD results was the variability of electrochemical detection introduced by manual cleaning of the electrode and very short reaction time for analysis; a nonadjustable instrumental bias and cell design should be further considered.

5.8.2. Wine composition and antioxidant capacity

Figure 5.4 shows the detector response curves for two selected wines (Sauvignon and Lambrusco) in DAD (**5.4a**) and ECD (**5.4b**), respectively.

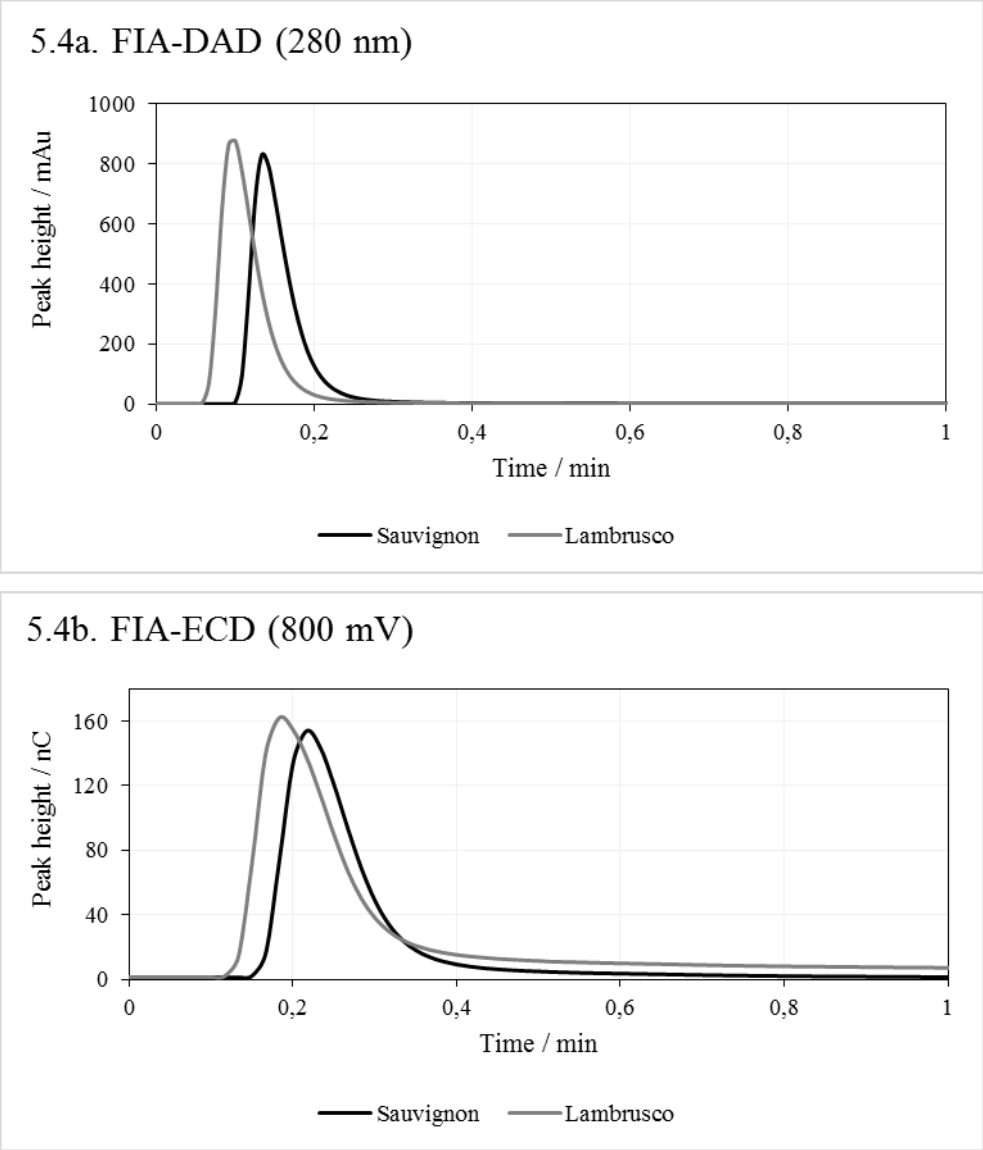

Figure 5.4. FIA-DAD (5.4a) and FIA-ECD (5.4b) experiments performed on a white wine (Sauvignon Blanc) and a red wine (Lambrusco). The same injection was used for subsequent analyses, following re-equilibration of both detection systems. Peak areas were then calculated to calibrate the response in mmol GAE L^{-1}.

The phenolic composition of 43 samples (including 31 wines and 12 commercial tannins) analysed with the FIA/DAD-ECD and the spectrophotometric methods (**Table 5.4**) was consistent with literature for the same wine varietals (Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015; Ivanova, Dörnyei, Márk, Vojnoski, Stafilov, Stefova, et al., 2011) and tannins (Magalhães, Ramos, Reis, & Segundo, 2014).

Table 5.4. Comparison between TPC and AA parameters (in mM GAE) calculated with the FIA-DAD-ECD and conventional spectrophotometric methods.

	FIA methods (mM GAE)		**Spectrophotometric methods (mM GAE)**	
Sample (wine/tannin)	$TPC_{DAD\ 280\ nm}$	$AA_{ECD\ 800\ mV}$	$TPC_{UV\ 280\ nm}$	$AA_{DPPH\ assay}$
Cabernet Sauvignon-1	7.25	3.57	10.36	3.84
Cabernet Sauvignon-2	8.93	4.65	12.18	4.59
Cabernet Sauvignon-3	9.07	4.72	12.74	4.83
Cabernet Sauvignon-4	3.59	1.93	5.92	2.09
Cabernet Sauvignon-5	7.31	4.05	10.47	3.84
Vranec-1	15.42	6.04	21.33	6.52
Vranec-2	11.07	3.21	15.48	4.46
Vranec-3	8.23	3.83	12.37	4.15
Vranec-4	6.37	3.52	9.81	3.24
Vranec-5	6.92	4.03	9.53	3.53
Vranec-6	10.60	4.43	15.60	4.38
Vranec-7	11.09	4.92	15.93	5.31
Vranec-8	7.89	4.46	11.25	4.16
Vranec-9	9.28	4.55	13.92	4.75
Vranec-10	9.67	4.12	13.92	4.96
Vranec-11	4.59	2.99	7.33	2.58
Vranec-12	4.31	3.12	6.42	2.47
Syrah-1	8.83	4.33	12.49	4.01
Syrah-2	6.91	4.46	9.37	3.34
Syrah-3	7.13	3.53	10.33	3.64
Syrah-4	6.98	3.42	10.00	3.73
Merlot-5	8.65	4.97	11.66	4.35
Merlot-6	8.03	4.72	11.20	4.23
Stanushina	6.11	3.02	8.97	3.18
Sauvignon Blanc	1.22	0.42	1.50	0.36
Lambrusco Gasparossa	1.28	0.67	1.62	0.42
Chardonnay	1.25	0.90	1.64	0.43
Chardonnay/Trebbiano-1	1.43	0.79	1.67	0.41
Chardonnay/Trebbiano-2	1.10	0.61	1.51	0.35
Muller Thurgau-1	1.09	0.82	1.49	0.42
Muller Thurgau-2	1.08	0.66	1.48	0.41
Limousin oak tannin	0.10	0.10	0.14	0.09
French oak tannin	0.08	0.09	0.12	0.08
American oak tannin-1	0.07	0.07	0.10	0.07
American oak tannin-2	0.09	0.10	0.12	0.07
American oak tannin-3	0.08	0.09	0.12	0.07
Selected oaks tannin-1	0.07	0.09	0.11	0.07
Selected oaks tannin-2	0.11	0.11	0.14	0.08
Selected oaks tannin-3	0.13	0.11	0.17	0.10
Chestnut tannin	0.14	0.16	0.19	0.11
Gallnut tannin	0.34	0.26	0.51	0.16
Grape berry tannin	0.08	0.10	0.12	0.10
Oak heartwood tannin	0.14	0.19	0.19	0.10

The FIA method coupled with DAD-ECD detection was calibrated using gallic acid as standard with satisfactory limit of quantification (LOQ = 0.012 mM (DAD); 0.005 mM (ECD)) that were suitable for the TPC and AA values expected in the oenological

samples. The quantification showed a slight improvement with respect to previous alternative analytical methods developed to quantify polyphenols, which exhibited LOQ values ranging from 0.006 to 0.25 mM for the gallic acid standard (Arce, Tena, Rios, & Valcàrcel, 1998 ; Gòmez-Alonso, Garcìa-Romero, & Hermosìn-Gutiérrez, 2007; Aid, Kaljurand, & Vaher, 2015) and compared to the differential pulsed voltammetry method for the determination of antioxidant activity, which showed a LOQ of 0.006 mM in Catechin Equivalent, CE (Šeruga, Novak, & Jakobek, 2011). Results were also improved compared to LOQ calculated for the Folin-Ciocalteu method to determine the TPC (LOQ = 0.013 mM GAE) and DPPH method to determine the AA (LOQ = 0.037 mM Trolox Equivalent, TE) (Bobo-García, Davidov-Pardo, Arroqui, Vírseda, Marín-Arroyo, & Navarro, 2014).

The TPC values obtained with the two methods, i.e. UV_{280nm} and FIA/DAD, showed a good correlation (R^2 = 0.9967), with a slight underextimation for the FIA/DAD assay (correlation line slope = 0.702). This finding can be partly explained by the different approaches in extrapolating results, i.e. Abs_{280nm} in the UV assay and the peak area calculation (mAu density/unit of time) in the case of FIA/DAD. The relationship between DPPH• and FIA/ECD amperometry was satisfactory (R^2 = 0.96, slope = 0.947 **Figure 5.7**), which slight divergence from linearity could be related to the different selectivity acting during these assays.

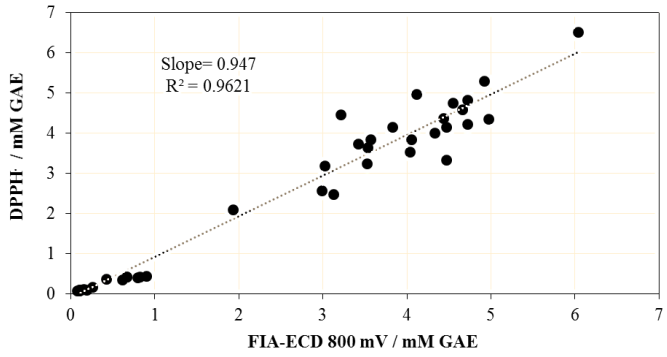

Figure 5.5. Linear regression between the FIA-ECD results and the DPPH• spectrophotometric assay to determine the antioxidant activity for the proposed series of wines and oenological tannins. Results were expressed in mM gallic acid / L for both analyses.

Most of the limitations related to the use of the DPPH assay to assass the radical scavenging activity in wines (Dicu et al., 2010; Danilewicz, 2015; (Foti & Ruberto, 2001; Litwinienko & Ingold, 2003) can be overcome by using electrochemical measurements, which allow a direct measurement of the current generated by the oxidation of phenolic compounds with no other reagents except the aqueous electrolyte.

The good correlation between the polyphenolic content and the calculated antioxidant activity values for each method ($R^2_{UV280\text{-}DPPH}$ = 0.9792, and $R^2_{DAD\text{-}ECD}$ = 0.9246) confirmed the origin of the antioxidant properties of wine that is greatly explained by the polyphenolic compounds, especially procyanidins oligomers (Muselík, García-Alonso, Martín-López, Žemlička, & Rivas-Gonzalo, 2007) and anthocyanins (Tenore, Basile, & Novellino, 2011).

5.9. Conclusions

The results obtained by the two methods of measuring the total polyphenols content and antioxidant activity of wine and oenological tannins, the FIA-DAD-ECD and the spectrophotometric assays, were in general agreement. However, the FIA method presented several advantages: the analysis was extremely fast, taking three minutes, thus enabling a large number of samples to be analysed in a short time. Furthermore, it provided the determination of both parameters in the same analysis with high sensitivity, reliability and repeatability. Both analytical approaches pointed out the dependence of wine protection against oxidation upon the content in polyphenolic compounds, even though it was observed that the DPPH assay only accounted on the molecular features having faster kinetics of reaction against radical, while the electrochemical method allowed a full screening of the overall antioxidant activity. The total polyphenol content generally provided higher values when using the optical density at 280 nm, and this is probably due to the effect of interferents contained in wine which absorb at the same wavelength; the integration of the peak produced in the FIA-DAD analysis seemed to provide a higher selectivity. The FIA-DAD-ECD method could be considered a valid alternative for the screening of TPC and AA parameters, since it allows to overcome practical problems and limitations related to the use of spectrophotometric assays.

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Addendum B.

SCAVENGING ACTIVITY OF WINE ANTIOXIDANTS AGAINST THE DPPH• RADICAL

Colorimetric methods have been the most used and implemented approaches to determine the activity of antiradical in wine; the ability of wine antioxidants to act as radical scavengers is usually investigated using stable synthetic radicals that are formed in the reagent mixtures, which are easily detected in electronic spectroscopy due to their high molar extinction coefficient (ε) in the Vis spectrum (Arnao, 2000; Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999). The decrease in absorbance of the radical species is related to the equilibrium occurring in solution between the radical scavenged and the corresponding neutral molecule that is formed, measured through the decrease in absorbance of the chromogen of the radical itself, and correlated with antiradicals present in wine. The first point to clarify is that the radical scavenging activity does not match the total antioxidant power of the sample (Huang, Ou, & Prior, 2005); although in wine the removal of reactive radicals could be considered the most efficient protection when the oxidative damages occur, the overall protective effect results from the synergy between removal of radical species, formation of stable adducts between oxidation by-products and sulphites and deactivation of metal catalysts through their chemical reduction or chelation.

Furthermore, colorimetric methods are characterised by low selectivity and provide aggregate results: to gain an information of a specific class of compounds preliminary sample treatments are required (Rivero-Pérez, Muñiz, & González-Sanjosé, 2008; Spranger, Sun, Mateus, Freitas, & Ricardo-da-Silva, 2008).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging assay is a colorimetric method routinely practiced for the assessment of antiradical properties of grape and related products (Goupy, Bautista-Ortin, Fulcrand, & Dangles, 2009; Guendez, Kallithraka, Makris, & Kefalas, 2005; Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello, & Versari, 2015; Débora Villaño, Fernández-Pachón, Troncoso, & García-Parrilla, 2005); it is based on the chromogen properties of the hydrazyl radical that exhibit a typical deep purple colour and a high extinction coefficient at the maximum absorbance value in the visible range, λ_{max} 515-17 nm, ε = 11 563 M^{-1} cm^{-1} (D. Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). The antiradical assay has been widely used since its formulation in 1995 (Brand-Williams,

Cuvelier, & Berset, 1995): the PubMed database lists up to 6000 studies involving this colorimetric method. Nevertheless, there is currently the lack of a unified protocol that regulates its application in the oenological chemistry, and its use has several limitations alongside undeniable benefits (Arnao, 2000; Arnous, Makris, & Kefalas, 2001; Kedare & Singh, 2011); several efforts have been done to elucidate possible limitations related to the use of this stable, synthetic radical, as summarised in the following sections.

3.1. Mechanisms involving DPPH• synthetic radical and main limitations in the use of the colorimetric assay

DPPH is a protocol classified as free radical-trapping method, measuring the ability of an antioxidant to intercept free radicals. The procedure allow a direct measurement of the consumption of hydrazyl radical used as a target molecule; the decrease in absorbance due to DPPH radical chromogen is subsequently converted into antiradical indexes: % radical scavenging, efficient concentration of the antioxidant necessary to decrease the initial radical concentration by 50% (EC_{50}), Trolox equivalent activity (mol^{-1} L^{-1} TE), just to list some. 2,2-diphenyl-2-picrylhydrazyl is a N-centred free radical, remarkably stable when compared to hydrazyl radicals (Foti, 2015). Its action is exerted through the stabilisation of the radical centre (R•), following Single-Electron Transfer (SET) or Hydrogen Atom Transfer (HAT) mechanisms, both summarized in **figure 5.6**. Some authors also hypothesize the occurrence of a Sequential Proton Loss Electron Transfer (SPLET) mechanism occurring in methanol solution, which is the solvent commonly used for the assay, in the case of flavonoid compounds (Foti, Daquino, DiLabio, & Ingold, 2011). Flavonoid are able to reversibly reduce quinones formed following oxidation, and a hypothesis of their reaction mechanism with DPPH is illustrated in **figure 5.7**; the oxidation of other phenolic compounds lead to complex chemical mechanism aimed to stabilise by-products through the formation of stable molecular adducts. DPPH assay aims to reproduce the variability of antioxidants chemical structures that interact in wine, using a single radical as a pro oxidant in a "one-dimensional" approach.

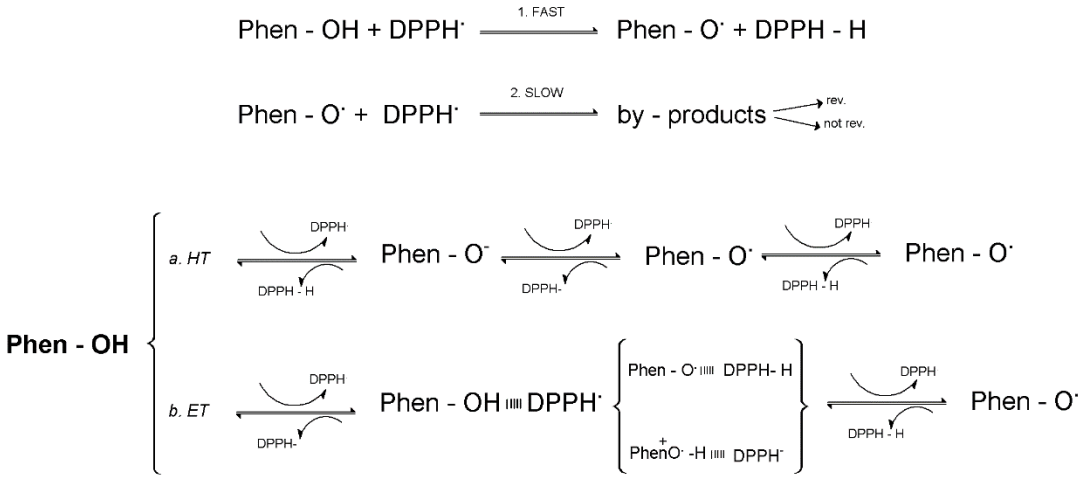

Figure 5.6. Electron transfer (ET) and Hydrogen transfer (HT) mechanisms occurring during the DPPH assay.

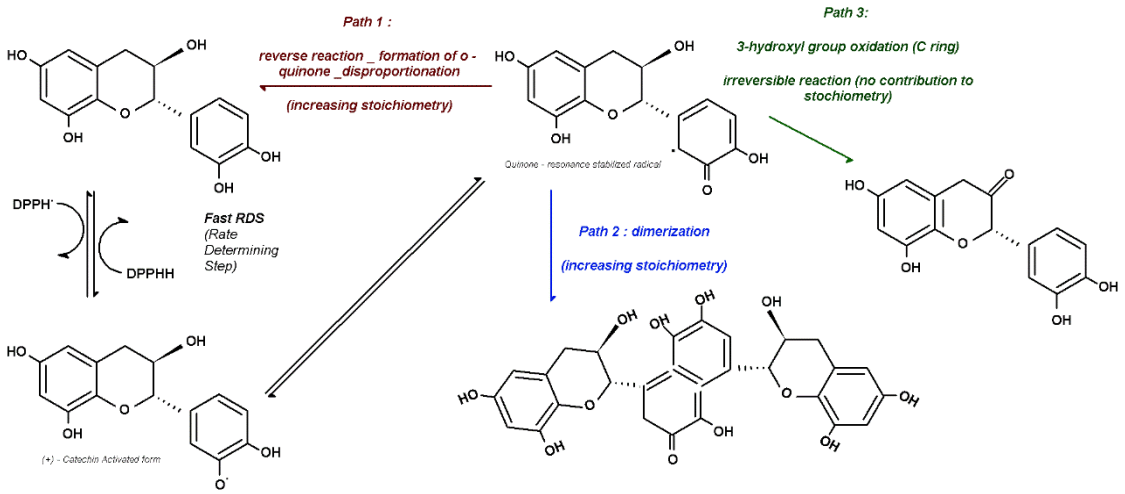

Figure 5.7. Possible reaction paths occurring between flavonoids and DPPH radicals.

One of the main concerning about the DPPH and other currently used colorimetric assay is this extremely simplified mechanism, which neglect the complexity of oxidation mechanism in wine, following several alternative reaction paths; the effectiveness of the index obtained as an index of antioxidant activity may be questioned when neglecting compositional and interfacial phenomena in complex mixtures as wine is, together with the reaction rates of antioxidants against oxygen – centred radicals, and the different stability and reaction rates involving natural and synthetic radicals (Dawidowicz, Wianowska, & Olszowy, 2012; Frankel & Meyer, 2000; Goupy, Bautista-Ortin, Fulcrand, & Dangles, 2009).

The stoichiometry of DPPH• is complicated as the redox reactions can be continued by the oxidation-degradation products the time response curve to reach the steady state is not linear with different ratios of antioxidant/DPPH; for example, tannic acid is able to reduce 50 moles of DPPH•, while caffeic acid reduces 2.6 moles only (Dicu et al., 2010). Moreover, the DPPH• assay is greatly affected by solvent impurities and change in pH (Danilewicz, 2015), and the DPPH• radical scavenging is enhanced when working with alcoholic solutions due to a partial ionization of the phenols (Foti & Ruberto, 2001; Litwinienko & Ingold, 2003).

Most of these limitations can be overcome by using electrochemical measurements, which allow a direct measurement of the current generated by the oxidation of phenolic compounds with no other reagents except the aqueous electrolyte.

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Addendum C.

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Targeted analysis of bioactive phenolic compounds and antioxidant activity of Macedonian red wines

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ABSTRACT

Phenolic composition of twenty-two Macedonian red wines, including ten autochthonous monovarietal Vranec wines produced with different yeasts for fermentation, and twelve wines from international varieties (Syrah, Merlot and Cabernet Sauvignon) from different wine regions was studied. All wines presented relatively high value of total phenols and antioxidant activity. A total of 19 phenolic compounds were identified and quantified using HPLC-DAD and among them, malvidin-3-glucoside and its derivatives were the major compounds, followed by the petunidin derivatives, while caftaric acid was the predominant cinnamic acid derivative in all wines. The anthocyanin content was mainly affected by the grape variety and to a less extent by the yeast used in fermentation. In particular, the use of locally isolated yeasts affected higher amount of anthocyanins and phenolic acids compared to the wines fermented with commercial yeasts. Principal Component Analysis showed a satisfactory grouping of red wines according to the grape variety.

1. Introduction

Wine production has a long tradition in Republic of Macedonia since the ancient Roman times and now it is the second most important export agro-food product after the tobacco, thus representing an economic opportunities for the new generation. In 2010 there were 86 registered wineries in Macedonia with a total capacity of ca. 2 million hectolitres per year and the total capacity of bottling is around 0.65 million hL per year, which is insufficient to cover the entire wine production in the country although a significant wine share (51% in 2006) is in bulk (MAFWE 2010). In 2008, 70.3 million litres were exported with value of around 39 million euros i.e. 10.4% of the total value of agri-food exports (SSO, 2009).

To increase the competitiveness of Macedonian wines on the global market there is a need to achieve distinctiveness and recognition of the products with specific quality characteristics, that origin from a particular geographical region or area (Nacka, Georgiev, & Dabovic Anastasovska, 2012). According to the climate characteristics and classification of the EU, Republic of Macedonia is classified as one geographic area for vine growing, i.e. zone III-C-b, that includes three viticultural regions divided into sixteen sub regions (districts) with specific favourable natural condition for production of quality wine. The wineries are mainly located in the region of the river Vardar valley, in particular in Skopje, Tikveš, and Gevgelija-Valandovo. Red wine represents approx. 60% of the national production and includes both autochthonous and international grape varieties such as Cabernet Sauvignon, Syrah, Merlot and Vranec. Vranec is probably the Macedonia's best known grape with dark and ruby-coloured on the vine, it produces dry, full-bodied red wines with high tannins. It is well known that polyphenolic compounds of red wine, including anthocyanins and tannins, are natural dietary antioxidant with potential health benefit and affect the quality of red wines, in terms of astringency, bitterness and colour (Mazza & Francis, 1995; Versari, du Toit, & Parpinello 2013).

Preliminary studies on the phenolic composition of wines from Macedonia have been preformed applying HPLC-DAD-MS, MALDI-TOF-MS and spectrophotometry techniques. Information is available on identification and semi-quantification of phenolic compounds of Vranec wines produced by different vinification conditions (Ivanova et al. 2011a) as well as stilbene levels and antioxidant activity of Vranec and Merlot wines, produced under different vinifications (Kostadinovic et al., 2012) applying HPLC methods. Moreover, MALDI-TOF-MS was applied for pigments

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fingerprinting of Macedonian red wines (Ivanova et al., 2011b, Ivanova Petropulos et al. 2014) and spectrophotometry was used for determination of total phenolics, anthocyanins, flavan-3-ols, flavonoids and colour on commercial wines and wines prepared under different maceration time, yeasts and different doses of SO_2 (Ivanova, Stefova, & Chinnici 2010; Ivanova, Stefova, & Vojnoski 2009; Ivanova, Vojnoski, & Stefova 2012).

However, to the best of our knowledge, there has been no report so far on the quantification of individual phenolic compounds in Macedonian red wines from different grape varieties and viticulture areas. Considering this, the objectives of the present work were (1) to characterize the flavonoid and non-flavonoid composition and to determine the antioxidant activity in red wines made from Vranec, Syrah, Merlot and Cabernet Sauvignon varieties, and (2) to assess the influence of different yeast preparations, Vinalco (Macedonian autochthonous yeast) and yeasts from Lallemand, on the phenolic composition and antioxidant activity of Vranec wines.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals and reagents were from commercial source: methanol, acetonitrile, perchloric acid, formic acid (Merck, Darmstadt, Germany), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), gallic acid, (+)-catechin, (−)-epicatechin, caffeic acid, syringic acid (Sigma–Aldrich, Milano, Italy), protocatechuic acid, vanillic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid (Extrasynthese, Genay, France). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox©) used for DPPH assay calibration was from Fluka *Analytical* (Sigma–Aldrich, Milano, Italy).

2.2. Wine samples

Twelve red wines from different *Vitis vinifera* L. grape international varieties, (Merlot, Cabernet Sauvignon and Syrah) from vintage 2103, were collected directly from the commercial wineries located in different wine regions of Macedonia, including Skopje, Gradsko, Negotino and Kavadarci.

The Vranec wines (10 samples), also collected from wineries, were produced from Vranec grapes (*Vitis vinifera* L.) grown in Tikveš region using similar winemaking protocol, as follows. Grapes were harvested at optimal technological maturity (23–24°Brix) and processed using electrical inox crusher/destemmer, then added with SO_2 (ca. 65 mg/L total concentration) before inoculation of four wines with one of the following *Saccharomyces cerevisiae* yeast strains: Clos, RC212, D254, BDX (Lallemand, Bordeaux, France), whereas other six wines were inoculated with Vinalco yeast (Bitola, Republic of Macedonia) that was isolated from the Tikveš wine region. In all trials the grape mash was macerated for 7–10 days at 23 ± 2 °C, with pumping over and delastage performed once per day during the first three days of maceration, followed by pumping over two times a day.

2.3. Proximate chemical composition of wines

The following parameters: total acidity, volatile acidity, total and free SO_2, alcohol, dry extract and specific density, were analyzed according to the official methods of analysis of wines (OIV. 2014).

2.4. Spectrophotometric analyses

Spectrophotometric analyses were performed by direct measurements of wines or using appropriate dilution of wine in distilled water when necessary, at the following wavelength: 280 nm (total phenols), 420 nm (browning degree), 520 nm and 620 nm (anthocyanins) nm with a UV–VIS spectrophotometer (Shimadzu, UVmini 1240, Milan, Italy) using a cuvette with 1 cm optical path against the blank, i.e. water (Harbertson & Spayd, 2006). Based on the Vis measurements the following parameters were calculated: colour intensity (CI = Σ 420 + 520 + 620 nm) and colour tonality (H = 420/520 nm) (OIV 2014).

Total phenols content, expressed as mg/L gallic acid equivalent (GAE/L), was determined by reading of the absorbance of the diluted samples (1:100 dilutions in distilled water) at 280 nm, and using a calibration curve of gallic acid standard solution in the range of 1.95 to 31.25 mg/L.

2.5. Determination of antioxidant activity of wines

Antioxidant activity of wines was determined as a radical scavenging ability following the procedure described by Brand-Williams, Cuvelier, and Berset (1995). Briefly, 200 μL of wine was added to 3 mL of a methanol solution of the radical DPPH with concentration of 0.025 mol/L, and measured at 515 nm after 1 h storage at dark. Antioxidant activity was calculated from a calibration curve constructed using methanol solutions of Trolox with concentrations ranged between 93–0.19 mg/L, and expressed as mg Trolox equivalent/L (TE/L).

2.6. HPLC analysis

An High Performance Liquid Chromatography (HPLC) system equipped with temperature control oven, photodiode array detector (DAD) and a Chromeleon chromatography manager software v. 6.60 SP2 (Dionex DX500, Milano, Italy) was used for identification and quantification of anthocyanins, phenolic acids and flavan-3-ols in wines. The samples were always filtered using 0.20 μm cellulose acetate membrane (Millipore, Milano, Italy) before direct injection into the HPLC system, kept at 30 °C.

Anthocyanins and related pigments were analyzed with the Gemini RP-C18 column (250 × 4.6 mm; 5 μm particle size; 110 Å porosity; Phenomenex, Bologna, Italy) using the following mobile phases: water/methanol (70/30, *v*/*v*) containing 6 mL/L of 70% perchloric acid (solvent A) and water/methanol (25/75, *v*/*v*) containing 6 mL/L of 70% perchloric acid (solvent B), at flow rate of 0.9 mL/min. The proportions of solvent B were as follows: 0 min, 0%; 23 min, 25%; 51 min, 70%; 60 min, 100%; 65 min, 0%. Anthocyanins were recorded at 530 nm.

Hydroxycinnamic acid derivatives and flavan-3-ols were analyzed with the Aquapore ODS-300 RP-C18 column (250 × 4.6 mm; 7 μm particle size; 300 Å porosity; Applied Biosystems, San Jose, CA, USA) using the following mobile phases: solvent A (water/formic acid, 98/2, *v*/*v*) and solvent B (acetonitrile/water/formic acid, 80:12:2, *v*/*v*/*v*), at flow rate of 0.5 mL/min. The proportions of solvent B were: 0–50 min, 9%; 65–70 min, 10%; 77 min, 30%; 80–97 min, 0%. Protocatechuic, *p*-hydroxybenzoic and vanillic acids were quantified at 256 nm, (+)-catechin, (−)-epicatechin, gallic and syringic acids at 280 nm, whereas *p*-coumaric acid at 308 nm, and caftaric, caffeic, coutaric and fertaric acids at 324 nm.

2.7. Statistical analysis

Statistical treatment, including Principal Component Analysis (PCA) was performed using the XLSTAT Software, Version 7.5.2, Addinsoft (Paris, France). PCA was carried out to evaluate relationships among the groups of variables, e.g. concentration of anthocyanins, flavan-3-ols, hydroxybenzoic acids and hydroxycinnamic acids and their derivatives.

3. Results and discussion

3.1. Proximate chemical composition of wines

Table 1 shows the chemical composition of all wines according to the different grape varieties (Merlot, Syrah, Cabernet Sauvignon and Vranec), the wine regions of origin (Skopje, Gradsko, Negotino and Kavadarci) and the use of different yeasts (the autochthonous yeast, Vinalco and four commercial yeasts, Clos, RC212, D254, BDX, from Lallemand) for the vinification of Vranec wines. In general, all wines showed high amount of total acidity (ranged from 5.5 to 7.9 g/L) that peaked for Vranec wine (V-Vi2) fermented with Vinalco, autochthonous yeast. These results are consistent with previous findings that found the Vranec wines characterized by high value of total acidity, typical for this variety (Košmerl et al. 2013; Rajković & Sredović, 2009). Moreover, a high value of total acids helps the microbial stabilization and the freshness of these wines. The alcohol level is always within the regulatory values of 20% for wines of area C III b (Reg. CE 606/2009), and two Vranec wines (V-V1 and V-L1) showed the highest content of alcohol (16.44% and 16.15%, *v/v*, respectively) and highest content of dry extract (36.0 and 35.3 g/L, respectively) as well. The volatile acidity showed an overall average value of 0.55 ± 0.1 g/L with no influence on the quality of wines that was protected from further oxidation and microbial contamination by the free SO_2 present in a sufficient level in the wines (14 to 28 mg/L).

3.2. Total phenols, colour, hue and antioxidant activity determined by spectrophotometry

The results for total phenols content, colour intensity, hue (colour tonality) and antioxidant activity are presented in Table 1. For all wines, total phenols ranged from 1394 to 3097 mg/L GAE (mean 2037 mg/L GAE). On average, Syrah wines contained slightly lower phenolic levels compared to Vranec, Merlot and Cabernet Sauvignon. These results are comparable to those reported for Macedonian Vranec and Merlot wines produced under different vinification conditions (Ivanova et al. 2009; Ivanova et al. 2012). Regarding the effect of yeast, it was observed that the different (yeast) strains influenced the phenolic content of wines produced under the same operative conditions, observing a higher average phenolic content in wines fermented with Vinalco yeasts, probably because this strain absorbs less phenolic compounds compared to the Lallemand ones.

All wines presented relatively high values for the antioxidant activity, ranged between 82–117 mg/L TE, regardless the variety and yeast strain used for fermentation. Plotting total phenols concentration against antioxidant activity for the Merlot, Cabernet Sauvignon and Syrah wines from different wine regions (Fig. 1a), as well as Vranec wines produces with different yeast strains (Fig. 1b), the corresponding correlation coefficients (r^2) that obtained were 0.8251 and 0.6836, respectively, suggesting that total phenols are related to the antioxidant properties of the wines.

Regarding colour intensity (CI) and hue (H), Vranec wines presented higher average values (21.04) for the CI than the other varieties (14.83), principally due to the higher content of the red compounds. Hue values were ranged between 0.52 to 0.75, characteristic for young red wines (Glories, 1984a; Glories, 1984b), as our wines were, that is consistent with values from literature (Tsanova-Savova, Dimov, & Ribarova, 2002; Kontkanen, Reynolds, Cliff, & King 2005). Hue values, which increase throughout aging, were slightly lower for Vranec wines (average 0.61) compared to the other varieties (average 0.65). It is well know that as the red wine age the absorption at 520 nm decreases whereas increases that one at 420 nm, and this explains the colour shift of wine with aging from red to orange, and finally to red brick.

3.3. Anthocyanins composition of wines

Table 2 summarizes the concentration of individual anthocyanins in the Vranec wines produced by different fermentation yeasts and red wines from different varieties (Syrah, Merlot and Cabernet

Table 1
Proximate chemical composition, total phenolics content (mg/L, GAE) colour intensity, hue and antioxidant activity (AA) of varietal red wines (Vranec, Syrah, Merlot and Cabernet Sauvignon).

Wines*	Total SO_2 (mg/L)	Free SO_2 (mg/L)	Total acidity (g/L)	Volatile acidity (g/L)	Alcohol (%,*v/v*)	Dry extract (g/L)	Specific density	TPC (mg/L, GAE)	CI	H	AA (mg/L, TE)
V–L1	55.0	28.2	7.0	0.55	16.15	35.2	0.9932	3097	23.27	0.66	116
V–L2	58.9	24.3	6.8	0.65	13.61	30.1	0.9939	1868	17.95	0.64	106
V–L3	64.0	25.6	6.3	0.54	13.13	28.6	0.9939	1394	15.43	0.58	99
V–L4	53.8	26.9	6.3	0.60	13.64	29.2	0.9935	1577	15.42	0.58	103
V–Vi1	51.2	33.3	6.9	0.63	16.44	36.0	0.9930	2257	27.47	0.60	105
V–Vi2	56.3	24.3	7.9	0.67	15.83	35.3	0.9934	2333	27.51	0.52	117
V–Vi3	55.0	24.3	5.6	0.62	13.11	28.6	0.9939	2053	24.18	0.60	111
V–Vi4	69.1	26.9	5.9	0.53	12.56	29.6	0.9966	1909	17.19	0.62	108
V–Vi5	57.6	26.9	5.8	0.45	12.78	27.6	0.9963	2224	21.71	0.62	114
V–Vi6	48.6	24.3	6.4	0.51	14.29	32.3	0.9940	2059	20.30	0.65	109
S–S	51.2	26.9	5.9	0.47	15.95	34.6	0.9933	1991	18.04	0.65	104
CS–S	52.5	24.3	6.1	0.55	15.89	36.6	0.9942	2131	17.98	0.61	111
M–S	51.2	29.4	5.8	0.43	14.35	32.0	0.9936	2564	24.20	0.57	116
S–G	55.0	26.9	5.6	0.48	13.40	35.6	0.9965	1733	11.31	0.63	91
CS–G	57.6	30.7	6.0	0.42	13.21	36.7	0.9969	1975	9.42	0.70	99
M–G	62.7	33.3	5.6	0.44	13.20	35.6	0.9966	1992	11.41	0.72	109
S–N	25.6	14.1	7.1	0.76	14.68	32.3	0.9935	1701	13.15	0.58	89
CS–N	80.6	34.6	7.1	0.64	15.30	34.9	0.9939	1791	11.03	0.66	95
M–N	34.6	20.5	6.0	0.72	14.65	31.7	0.9934	1918	12.35	0.68	100
S–K	49.9	24.3	5.8	0.51	14.38	30.7	0.9933	1612	11.37	0.75	82
CS–K	55.0	32.0	5.5	0.43	14.04	35.9	0.9952	2126	16.63	0.65	105
M–K	57.6	28.2	5.6	0.45	14.51	32.3	0.9939	2507	21.08	0.58	115

Abbreviation of wine regions: S – Skopje, G – Gradsko, N – Negotino, K – Kavadarci. Abbreviation of *Saccharomyces cerevisiae* yeasts: Vi – Vinalco; L – Lallemand yeasts. TPC – total phenols content, in Gallic acid equivalents (GAE), CI – colour intensity, H – hue, AA – antioxidant activity in Trolox equivalents (TE).

* Abbreviation of wines: V – Vranec, S – Syrah, CS – Cabernet Sauvignon, M – Merlot.

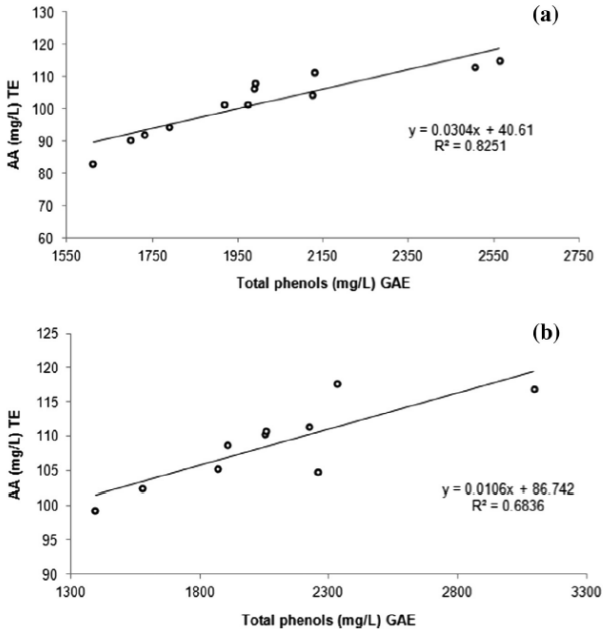

Fig. 1. Correlation between total phenols and antioxidant activity of (a) Merlot, Cabernet Sauvignon and Syrah wines from different wine regions and (b) Vranec wines produced with different yeast strains for fermentation.

Sauvignon) produced in different wine regions (Skopje, Gradsko, Negotino and Kavadarci) determined by HPLC. The chromatogram presenting the separation of anthocyanins at 530 nm is shown at Fig. 2a.

In total, 10 anthocyanins were identified and quantified in wines including five monoglucosides, three acetylglucosides and two *p*-coumaroylglucosides. The malvidin-3-glucoside was the dominant anthocyanin in all analyzed wines as expected for most of the *V. Vinifera* cultivars, followed by petunidin-3-glucoside, whereas the cyanidin-3-glucoside showed the lowest amount to below detection in some wines (Table 2). The group of anthocyanin monoglycosides represented the highest proportion of all anthocyanins in all wines, ranging from 60.3% (CS-G) to 88.5% (V-L1), followed by the acetyl derivatives ranged between 6% (V-L1) to 32.5% (CS-G) and *p*-coumaroylglucosides ranged from 5.3% (M–S) to 8.5 (V-V1). The ratio of acetylglucosides and *p*-coumaroylglucosides (Σ acetylated/Σ coumaroylated), proposed as a verification factor for varietal authenticity of red wines, was calculated. The values obtained ranged as follows: for the Vranec wines 1–1.7, for Syrah 2.6–4.8 (an highest ratio was noticed for Syrah wines from Skopje region, 4.8), for Cabernet Sauvignon 3.2–4.6 and for Merlot wine 2.3–3.8, with the highest ratio (4.6 and 3.8) for both wine varieties (Cabernet Sauvignon and Merlot, respectively) produced in the Gradsko region.

In terms of variety, the malvidin-3-glucoside was always higher in Vranec wines (range: 235–887 mg/L) compared to the international varieties Merlot, Cabernet Sauvignon and Syrah (overall range: 173–541 mg/L). In particular, the Vranec wines (V-Vi2, V-Vi3 and V-Vi1) fermented with the local Vinalco yeast strain showed the highest amount of monoglucoside, acetylglucoside and *p*-coumaroylglucoside derivatives, followed by Syrah wine from Skopje region and Cabernet Sauvignon from Gradsko and Negotino (data not shown). On average, Vranec wines contained higher amount of anthocyanins (738 mg/L) than other varieties (651 mg/L). The analyzed wines presented higher concentration of malvidin-3-glucoside as well as of anthocyanin monoglucosides compared to Cabernet Sauvignon and Merlot wines produced in Spain (Ortega et al. 2008), but similar values with Cabernet Sauvignon, Cencibel and Syrah wines produced in region of La Mancha in Spain (Hermosín Gutiérrez, Sánchez-Palomo Lorenzo, & Vicario Espinosa 2005). The results for the anthocyanins content correlate with the colour intensity values (Table 1).

It is well known that the anthocyanins content of wine can be affected by the yeast during fermentation. In this view we observed that Vranec wines, fermented with the Vinalco yeast had higher amounts of anthocyanins (on average 853 mg/L), compared to the wines fermented with the four Lallemand yeasts (on average 567 mg/L) and among them, wine V-Vi2 presented highest value (1530 mg/L). These results are in agreement with our previous findings (Ivanova et al., 2011a; Ivanova et al., 2012) and can be explained by the reversible adsorption of anthocyanins onto the yeast cell walls during the fermentation (Vasserot, Caillet, & Maujean, 1997). Moreover, it is well known that the metabolic pathways of the yeast can differ among the strains which affect the content of phenolic (e.g. tyrosol, pyruvic acid, vinylphenol) and other compounds (e.g. acetaldehyde) during maceration, and some of these compounds can react with anthocyanins modifying their adsorption properties.

Table 2
Concentration of anthocyanins (mg/L) of varietal red wines (Merlot, Cabernet Sauvignon and Syrah) from different Macedonian wine regions and Vranec wines fermented with different yeasts.

Wines*	Dp-Glc	Cy-Glc	Pt-Glc	Pn-Glc	Mv-Glc	Total Glc	Pt-AcGlc	Pn-AcGlc	Mv-AcGlc	Total AcGlc	Pn-coumGlc	Mv-coumGlc	Total coumGlc	Vitisin B	Total anthocyanins	ΣGlc/ΣAcG	ΣGlc/ΣcoumGlc	ΣAcGlc/ΣcoumGlc
V–L1	2.1	27.2	0.9	143.0	344	388	4.1	3.6	27.5	35.2	4.3	28.2	32.5	n.d.	456	11.0	11.9	1.1
V–L2	19.8	1.1	43.3	24.3	347	435	4.0	5.1	45.2	54.3	2.9	29.0	31.9	3.7	525	8.0	13.7	1.7
V–L3	63.2	5.0	99.1	40.2	335	543	9.7	5.0	27.8	42.4	6.1	29.0	35.1	0.9	622	12.8	15.5	1.2
V–L4	16.5	n.d.	41.5	19.5	355	433	4.2	3.5	44.9	52.6	4.2	30.8	34.9	4.5	525	8.2	12.4	1.5
V–Vi1	38.8	8.1	82.3	73.0	544	746	7.9	12.8	68.8	89.5	8.1	69.9	78.0	n.d.	914	8.3	9.6	1.1
V–Vi2	99.6	20.1	154	134	887	1296	14.7	15.1	96.4	126	15.3	93.5	108	n.d.	1530	10.3	11.9	1.2
V–Vi3	76.6	7.5	114	68.1	524	790	12.8	11.9	61.5	86.3	9.5	54.9	64.4	n.d.	941	9.2	12.3	1.3
V–Vi4	35.7	1.0	63.1	33.6	378	512	7.1	8.1	46.1	61.3	5.1	36.2	41.3	n.d.	614	8.3	12.4	1.5
V–Vi5	59.7	5.5	89.4	50.6	411	617	8.7	6.9	45.0	60.6	5.9	36.2	42.1	2.7	722	10.2	14.6	1.4
V–Vi6	28.9	4.8	47.4	27.9	235	345	3.2	2.6	19.6	25.4	2.8	22.6	25.4	n.d.	395	13.5	13.5	1.0
S–S	18.9	n.d.	41.3	12.8	541	614	10.2	8.4	215	234	5.2	43.4	48.6	3.1	899	2.6	12.6	4.8
CS–S	31.6	0.1	28.8	10.3	258	329	6.1	3.2	85.9	95.2	1.1	24.9	26.0	n.d.	450	3.5	12.6	3.7
M–S	50.8	2.7	51.4	23.1	251	379	9.4	5.4	52.3	67.1	3.2	21.8	25.1	2.6	474	5.6	15.1	2.7
S–G	14.9	n.d.	32.7	16.9	408	472	6.8	12.3	178	197	4.7	48.3	52.9	n.d.	723	2.4	8.9	3.7
CS–G	13.6	n.d.	17.1	4.7	472	507	4.8	3.5	265	273	0.7	59.2	59.9	n.d.	840	1.9	8.5	4.6
M–G	15.7	n.d.	27.7	17.5	397	458	5.2	9.0	168	182	2.4	45.9	48.3	n.d.	688	2.5	9.5	3.8
S–N	14.7	n.d.	29.6	15.2	316	375	6.6	11.4	136	154	5.8	54.6	60.4	n.d.	590	2.4	6.2	2.6
CS–N	26.6	n.d.	32.4	11.9	497	568	6.4	7.6	212	226	1.8	69.4	71.2	n.d.	866	2.5	8.0	3.2
M–N	26.4	0.7	41.8	24.4	408	501	7.8	10.7	141	159	4.6	65.2	69.8	n.d.	730	3.2	7.2	2.3
S–K	15.1	n.d.	34.7	13.4	489	552	9.7	11.7	185	206	4.6	63.7	68.3	n.d.	827	2.7	8.1	3.0
CS–K	27.8	n.d.	26.9	9.5	229	294	5.9	2.8	74.1	82.7	1.2	23.6	24.9	0.2	402	3.5	11.8	3.3
M–K	34.7	1.3	35.1	16.0	173	260	4.9	3.1	34.3	42.4	2.2	15.3	17.4	1.2	321	6.1	14.9	2.4

Abbreviation of wine regions: S – Skopje, G – Gradsko, N – Negotino, K – Kavadarci.
Abbreviation of yeasts: Vi-Vinalco yeast, *Saccharomyces serevisiae*; L – Lallemand yeasts. Dp – delphinidin, Cy – cyanidin, Pt – petunidin, Pn – peonidin, Mv – malvidin, Glc – glucoside, AsGlc – acetylglucoside, coumGlc – coumaroylglucoside.
* Abbreviation of wines: V – Vranec, S – Syrah, CS – Cabernet Sauvignon, M – Merlot.

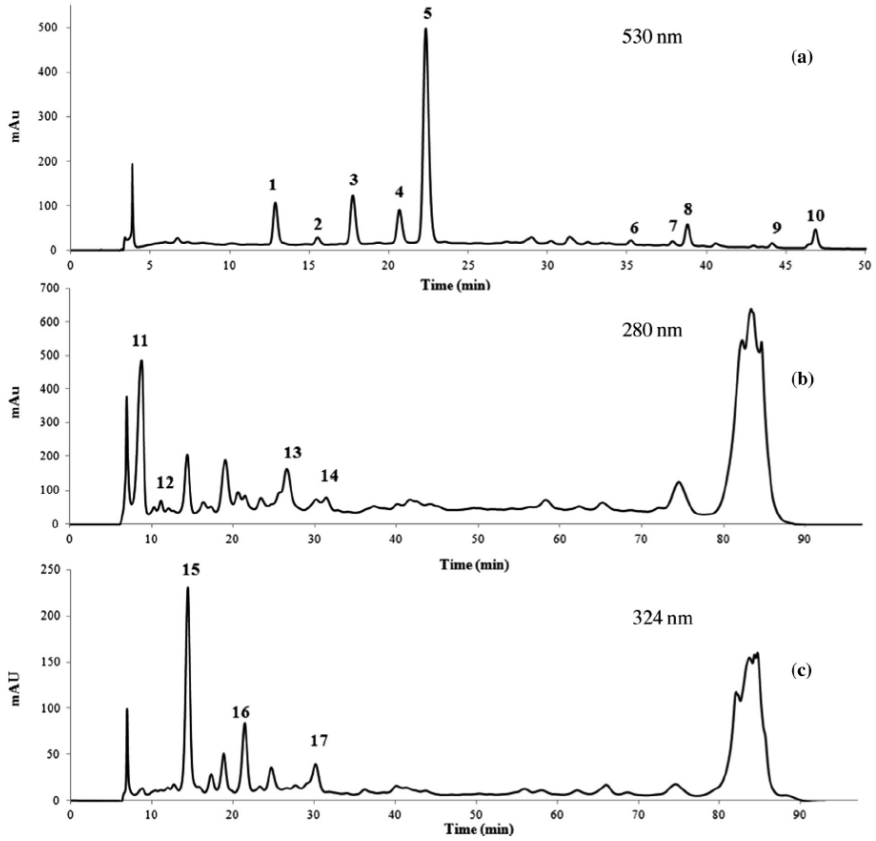

Fig. 2. UV–Vis chromatogram of V-L1 Vranec wine sample recorded at 530 nm (a), 280 nm (b) and 324 nm (c) for separation and quantification of anthocyanins, flavan-3-ols/hydroxybenozic acids and hydroxycinnamic acids/derivatives, respectively. Peak identification: delphinidin-3-glucoside, (1); cyanidin-3-glucoside, (2); petunidin-3-glucoside, (3); peonidin-3-glucoside, (4); malvidin-3-glucoside, (5); petunidin-(6 acetyl)-3-glucoside, (6); peonidin-(6 acetyl)-3-glucoside, (7); malvidin-(6 acetyl)-3-glucoside, (8); peonidin-coumaroyl-3-glucoside, (9); malvidin-coumaroyl-3-glucoside, (10); gallic acid, (11); protocatecuic acid, (12); syringic acid, (13); (+)-catechin, (14); caftaric acid, (15); caffeic acid, (16); *p*-coumaric acid, (17).

3.4. Non-anthocyanin composition of wines

Separation of phenolic acids in one Vranec wine sample (V-L1), recorded at 280 nm and 324 nm, is presented at Fig. 2b and c, respectively.

Three benzoic acids, including gallic acid, protocatechuic acid and syringic acid, were identified and quantified in the wines. Gallic acid, that originates from the grapes and also from the breakdown of both hydrolyzable and condensed tannins, i.e. the gallic acid esters of flavan-3-ols, was the dominant benzoic acid in all wines, presenting an average value of 478 mg/L in Vranec wines, mainly depending on the high contribution of wine V-L1 (Table 3). The average values of total hydroxybenzoic acids obtained for the international varieties were different, i.e. 491 mg/L for Merlot, 372 mg/L for Cabernet Sauvignon and 325 for Syrah, in accordance with other studies (Ertan Anli & Nilüfer, 2009; Mendoza et al., 2011;). The differences observed in the levels of gallic acid among the samples probably are due to the differences in techniques applied for winemaking, including the addition of enological hydrolizable tannins, whereas the contribution due to the geographical origin or the cultivar seems to play a minor effect (Nicoletti, Bello, De Rossi, & Corradini 2008; Pajovic et al. 2014; Vinković Vrček, Bojić, Žuntar, Mendaš, & Medić-Šarić, 2011). Considerable amounts were also found for protocatechuic acid and syringic acid, higher in Vranec wines (on average 48.2 mg/L and 76.3 mg/L, respectively) compared to the international varieties (on average 39 mg/L and 10.8 mg/L, respectively). Similarly as for the gallic acid, protocatechuic and syringic acids were present in highest concentration in Merlot (on average 57.2 and 14.4 mg/L, respectively), followed by Cabernet Sauvignon (on average 34.7 and 6.62 mg/L, respectively) and Syrah (on average 25.1 and 1.3 mg/L, respectively) probably as a varietal characteristic (Garía-Falcón, Pérez-Lamela, Martínez-Carballo, & Simal-Gándara 2007; Monagas, Suarez, Gómez-Cordovés, & Bartolomé 2005).

Table 3
Concentration of phenolic acids (mg/L) and catechin of varietal red wines (Merlot, Cabernet Sauvignon and Syrah) from different Macedonian wine regions and Vranec wines fermented with different yeasts.

Wines*	Protocatecuic acid	Gallic acid	Syringic acid	*p*-Coumaric acid	Caftaric acid	Coutaric acid	Caffeic acid	Fertaric acid	Total HBA	Total HCA and HCAD	Catechin
V–L1	98.1	1352	419	60.8	237	13.7	94.5	19.4	1869	425	567
V–L2	44.9	460	161	10.9	243	49.1	31.2	21.4	666	355	93
V–L3	23.7	252	0.5	8.1	176	32.2	10.7	10.7	276	237	482
V–L4	31.1	317	30.0	13.2	226	45.2	14.0	36.5	378	335	348
V–Vi1	42.1	265	66.3	3.2	206	35.1	8.3	29.7	373	282	150
V–Vi2	62.6	311	55.3	18.6	275	55.6	14.6	49.4	429	413	n.d.
V–Vi3	62.6	472	13.5	13.3	507	89.2	11.5	39.0	548	660	347
V–Vi4	29.3	291	17.4	11.0	365	68.4	10.2	38.4	338	493	271
V–Vi5	37.2	391	n.d.	11.5	384	72.9	7.54	30.6	428	507	331
V–Vi6	50.8	671	n.d.	16.3	362	67.1	31.9	43.6	722	521	284
S–S	30.8	310	n.d.	18.3	243	61.1	40.4	35.4	340	398	407
CS–S	39.1	337	n.d.	11.4	412	67.5	16.1	40.7	376	548	203
M–S	104	303	5.4	2.7	257	33.8	16.7	42.9	412	353	298
S–G	34.1	523	19.8	n.d.	147	37.2	20.5	37.2	577	242	238
CS–G	44.4	631	14.1	n.d.	244	53.9	16.4	28.6	689	343	227
M–G	26.2	847	21.2	21.9	182	25.8	19.3	34.2	894	284	649
S–N	30.3	281	25.4	14.1	243	71.2	15.6	38.4	337	382	109
CS–N	5.6	177	n.d.	n.d.	387	74.6	31.5	46.3	183	539	136
M–N	18.5	369	21.5	13.3	266	47.7	19.3	35.8	409	382	n.d.
S–K	5.1	185	n.d.	26.7	243	65.3	25.9	43.6	190	405	411
CS–K	49.7	344	12.4	3.8	387	65.3	11.1	36.7	406	504	114
M–K	80.1	445	9.6	1.1	219	31.4	11.5	42.9	535	306	209

Abbreviation of wine regions: S – Skopje, G – Gradsko, N – Negotino, K – Kavadarci.
Abbreviation of yeasts: Vi – Vinalco yeast, *Saccharomyces servisie*; L – Lallemand yeasts, HBA – hydroxybenzoic acids, HCA – hydroxycinnamic acids, HCAD – hydroxycinnamic acid derivatives.
* Abbreviation of wines: V – Vranec, S – Syrah, CS – Cabernet Sauvignon, M – Merlot.

Regarding the influence of yeast, higher proportions of gallic acid and syringic acid were present in Vranec fermented with the four Lallemand yeasts, while in two wines fermented with Vinalco yeasts (V-Vi5 and V-Vi6) syringic acid was not detected. In addition, similar contents of protocatechuic acid were found in all wines fermented with different yeast strains.

With respect to hydroxycinnamates, five compounds were detected, including caftaric, coutaric, fertaric, caffeic and *p*-coumaric acids. Caftaric acid presented highest average concentration (282 mg/L) representing 69.1% (on average) of all hydroxycinnamates, present in similar concentrations in all wines analyzed (Table 3). Cabernet Sauvignon presented highest proportion of caftaric acid (358 mg/L, on average) and Syrah lowest (219 mg/L, on average). Coutaric and fertaric acids also had an important contribution (mean content 52.9 mg/L [13.02%] and 35.5 mg/L [9.13%], respectively). Differences between varieties were observed for caffeic and *p*-coumaric acids too.

In terms of the influence of yeast, Vranec wines fermented with Vinalco contained higher content of caftaric acid, coutaric acid and fertaric acid (mean value 350, 64.7, and 38.5 mg/L, respectively) compared to wines fermented with Lallemand yeasts (mean value 221, 35.1, and 22 mg/L, respectively). According to Balik et al. (2008) the *Vitis vinifera L.* varieties contain the highest level of caftaric acid in the shoots (474–2257 mg/kg) and the leaves of grape (278–914 mg/kg), which extract's represent a valuable source of bioactive compounds with further application in the food or pharmaceutical industries (Fernandes et al., 2013).

Concerning flavan-3-ols, only (+)-catechin was identified and quantified in wines. Its content was relatively high and different among varieties and wine regions, ranged from 92.9 to 567 mg/L. In particular, the highest average levels of catechin were found in Vranec wines (319 mg/L) similarly to Merlot wines (316 mg/L), followed with Syrah (291 mg/L) and Cabernet Sauvignon (170 mg/L). With regard to the yeast influence on catechin content, wines fermented with Vinalco yeasts contained lower levels (mean content 277 mg/L) then other wines fermented with Lallemand yeasts, Clos, RC212, D254, BDX (mean content 373 mg/L), probably because of a lower adsorption of catechin on the yeast cell walls.

3.5. Principal Component Analysis

Principal Component Analysis was used to explore the contribution of each parameter on the clustering among the wines. Projection of the wines on the first two principal components (explained variability: 59.95%) showed a separation mainly according to the variety (Fig. 3a). Thus, wines from Cabernet Sauvignon variety were clearly separated from the other wines, located in the negative part of PC2, and Syrah wines formed a group in the positive part of PC1. Vranec wines were grouped and further divided in subgroups according to the yeast used for fermentation. Thus, wines fermented with Vinalco yeast were located in the upper positive part of PC1 while wines fermented with Lallemand yeasts were not clearly separated and were located near the centre, with exception of V-L1 which was placed in the upper negative part of PC1, separated from all other wines.

PCA results of the variables used for characterization of the wine samples displayed into the first two principal components are presented in Fig. 3b. It could be noticed that anthocyanins and hydroxycinnamic acid derivatives prevail in the positive part of the first principal component, while catechin, caffeic acid, *p*-coumaric acid and hydroxybenzoic acids prevail in the negative part of PC1. Wines located in the upper right quarter are richer in anthocyanins, which can be related to varietal character.

4. Conclusions

Chemical analysis on Macedonian quality wines are important to support the viticulture and wine strategy aimed at brand development particularly from local grape variety. In this view, comparing Vranec wines with other varieties from Republic of Macedonia, such as Merlot, Cabernet Sauvignon and Syrah, it was found as

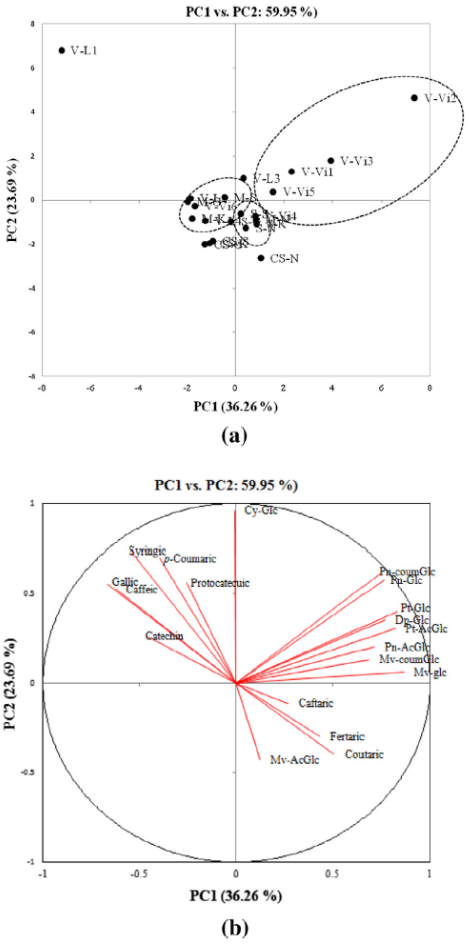

Fig. 3. Principal component score plot (a) and correlation scatterplots (b) of the variables with PC1 and PC2 based on anthocyanins, phenolic acids and catechin for the Vranec, Syrah, Merlot and Cabernet Sauvignon wines.

richer in total acids and polyphenols, especially anthocyanins that make this variety deeply coloured, fresh and stable, appropriate for long term aging and production of high quality wines. Investigating the effect of yeast, the locally isolated yeast strains seem to improve several polyphenolic components, such as higher levels of total phenols, anthocyanins, colour, phenolic acids and high value of antioxidant activity in Vranec wines.

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Chapter 6

Exploitation of the Structure and Antioxidant Capacity of Oenological Tannins

Tannins are natural extracts bearing unique properties (radical chain breakers, reducing agents, effective metal chelators and complexing agents for biological molecules), and they can be effectively derived by their botanical sources in an industrial scale using "green" extractive methods, with low impact in term of costs and environmental pollution.

For their unique properties, they are greatly considered in oenology, both increasing the extraction of grape procyanidins during fermentation and adding them as exogenous products. There is an awareness that their tailored addition would limitate the use of traditional oenological additives, potentially harmful for human's health (Versari, du Toit, & Parpinello, 2013; Cheynier, Dueñas-Paton, Salas, Maury, Souquet, et al., 2006).

Despite the chemical properties of tannins have been extensively studied in recent decades, poor attention has been devoted to their study in view of their use in food technology, and in particular in oenology. The regulation currently in force provides guidelines on analytical characterization (OENOLOGICAL TANNINS INS N°: 181 (Oeno 12/2002 modified by Oeno 5/2008, 6/2008 and OIV-Oeno 352-2009)) and regulate their use in winemaking (Resolution OIV -OENO 417- 2011 – Tannin Addition (16/70); Resolution OIV-OENO 554-2015), but the information on the composition of commercial formulations and the sensory impact of their use is fragmentary. A systematic characterization of oenological tannins currently used may provide guidelines to take advantage of their benefit properties without affecting sensory attributes like aroma, bitterness, and astringency (Harbertson, Parpinello, Heymann, & Downey, 2012; Puech, Feuillat, & Mosedale, 1999).

Part of the PhD project has been devoted to evaluate the use of oenological tannins as a sustainable alternative to protect wine from oxidizing agents. A detailed investigation would require to monitor the long-term impact in

bottled wines, then during the PhD project only explorative experiments were performed. This provided the structural characterization of commercially available products, followed by the measurement of reducing, redox, and antiradical activities.

Tannins were provided by suppliers and dissolved in model wine (pH 3.6, 12% EtOH) or directly analysed in their lyophilized form, without any purification and extraction treatment. They were subjected to analytical characterization then ranked according with their performances, as analytically determined.

This preliminary screening, which is presented in this chapter, allowed to select some extracts on a structural and mechanistic perspective, to be applied in future experiments in real wines (see chapter 7). Results are extrapolated from a scientific work aimed to publication, which is actually under peer review.

In addendum, two published works are presented concerning: (i) the rapid classification of tannins according with their botanical sources, disclosing structural differences and providing to the wine industry a potentially effective tool against adulteration (Addendum E); (ii) the structural characterization of a chestnut tannins, antioxidant extract used in winemaking as a coadjutant of clarification, which emphasizes the potential sensory impact related to the peculiar composition and introduces further experiments of tannin addition in wine.

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6A) STRUCTURE-ANTIOXIDANT ACTIVITY OF COMMERCIAL TANNINS USED IN OENOLOGY

RESEARCH ARTICLE:

Characterization of oenological tannins as bioactive by-products with multiple structure-antioxidant relationships using a combined analytical approach

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(UNDER REVIEW)

6.1. Introduction

Oenological tannins are plant polyphenolic compounds extracted from different botanical components (e.g. gallnuts, chestnut, oak, quebracho, grape seeds) and are composed of different chemical structures. These include gallic and ellagic acids in polymerised forms (hydrolysable tannins) or proanthocyanidins (condensed tannins). The tannins are commonly used in winemaking to facilitate the clarification of wines and musts (OENOLOGICAL TANNINS, INS N°: 181, Oeno 12/2002 modified by Oeno 5/2008, 6/2008 and OIV-Oeno 352-2009) and as flavourings for food and beverage production (EC No 1334/2008, EU Regulation No. 872/12). In particular, oenological practices take advantage of these compounds to enhance the formation of pigmented polymers, to improve the structure of light-bodied wines and to provide additional protection against oxidation, thus minimizing the need of additional chemicals (mainly sulfur dioxide) during the vinification process. A high content of polyphenols is commonly associated with an improvement in wine quality and shelf-life durability, with important considerations including the makeup of the simple phenolic fraction and the extent of polymerization. It was reported that a good balance in polymerized phenolic compounds results in a significant increase in wine quality, with development of typical varietal flavours and aroma and an enhancement in antioxidant capacity (Versari, du Toit, & Parpinello, 2013).

Commercial oenological tannins are supplied with only general information on the botanical source and recommended dosage. There is thus a need for an improved understanding of the chemical composition and properties of tannins to optimize and tailor their use for specific applications. In particular, the analytical characterization of the composition of commercial tannins provides useful information on the structure-activity relationships of these phytochemicals (Rice-Evans, Miller, & Paganga, 1996). Several colorimetric methods are available to obtain a preliminary index of total polyphenols and tannins in a complex mixture (De Beer, Harbertson, Kilmartin, Roginsky, Barsukova, Adams, & Waterhouse, 2004; Sarneckis, Dambergs, Jones, Mercurio, Herderich, & Smith, 2006) and total antioxidant capacity (Hagerman, Riedl, Jones, Sovik, Ritchard, Hartzfeld, & Riechel, 1998; Vinson & Hontz, 1995; Yokozawa, Chen, Dong, Tanaka, Nonaka, & Nishioka, 1998). On the other hand, structural chemical information requires separation techniques, which are useful tools to discriminate the single phenolic compounds and the extent of polymerization of larger

structures (Barak & Kennedy, 2013). The most widespread method to determine the simple phenolic fraction is HPLC with diode-array (DAD) or electrochemical (ECD) detection, which allows quantification of the monomeric polyphenols, and provides additional information on the polymerized fraction (Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2007; Kilmartin, 2001; Versari, Boulton, & Parpinello, 2007).

Phenolics are able to be readily oxidized, producing stable intermediates and preventing oxidative detrimental effects in wine. At the same time, some wine spoilage, browning and removal of varietal thiols, for example, proceeds via polyphenol oxidation steps. By removing oxidised polyphenols, using hyperoxidation, this can be minimised, but at the expense of some loss of varietal aroma. From this perspective, it becomes important to evaluate the redox activity of phenolic compounds in order to elucidate their antioxidant activity and any pro-oxidant effects.

In addition to conventional spectrophotometric assays, a further evaluation of phenolic compounds involves the characterization of their redox properties as antioxidants. Among the electrochemical methods a particular emphasis has been given to the use of cyclic voltammetry, being an attractive analytical option for the rapid screening of single compounds and complex matrices (de Queiroz Ferreira, Greco, Delarmelina, & Weber, 2015; Kilmartin, Zou, & Waterhouse, 2002; Sia, Yee, Santos, & Abdurrahman, 2010).

Antioxidant activity is commonly evaluated in terms of ability to scavenge the DPPH• free radicals (DPPH• assay), reducing power against Fe(III) salts (FRAP assay), and oxidability seen via redox activity during cyclic voltammetry. The latter approach was found to be an alternative and effective method to assess the antioxidant power of phenolic compounds, in addition to its ability to discriminate between reversible and non-reversible redox processes (Kilmartin & Hsu, 2003; Kilmartin, Zou, & Waterhouse, 2001).

Therefore, in the present work a selection of commercial oenological tannins was analysed using a combined analytical approach focusing on their phytochemical composition and antioxidant activity. The relationship among tannins and various parameters was evaluated to explain their ability to prevent the oxidative stress under oenological conditions.

6.2. Materials and Methods

6.2.1. Samples and Chemicals

Thirteen samples of commercial oenological tannins were provided by suppliers (Enologica Vason, Verona, IT; HTS enologia, Marsala, IT; Laffort, Bordeaux Cedex, FR; AEB Group, Brescia, IT) as lyophilized powders; a description is provided in **table 6.1**. Eight of the samples were expected to have a high content of procyanidin condensed tannins (labelled "Pr"), while a further five samples derived from oak and other woods were expected to be high in hydrolysable tannins (labelled "Hy").

Table 6.1. List of commercial tannins used in this work and their related use in oenological applications.

Samples code	Description	Oenological applications
Pr1	Leaves of *Vitis vinifera* red grapes	Antioxidant
Pr2	Grape seeds	Antioxidant; color stabilization
Pr3	Grape berry	Antioxidant
Pr4	Grape skins and seeds	Fining and clarifying agent
Pr5	White grape seeds	Antioxidant; color stabilization
Pr6	Grape seeds	Color stabilization
Pr7	Malbec red grape seeds	Fining agent
Pr8	Unfermented grape skins	Antioxidant; fining agent
Hy1	American Oak	Fining agent
Hy2	Limousin Oak	Fining agent
Hy3	French Oak	Fining agent
Hy4	Selected *Quercus* woods	Fining agent
Hy5	Red fruits trees wood	Clarifying agent for red wines

The tannins samples were dissolved in a model wine solution, made up of 12% ethanol in distilled water, with addition of L-tartaric acid 0.033M and NaOH to reach pH 3.6 (L-tartatic acid (≈100%) and pure ethanol (> 99%) from Merck Darmstadt, DE). Stock solutions of the tannins were prepared at 1 g/L in the model wine solution, equivalent to 3.45 mmol (+)-catechin/L, for the same mass of catechin. The Folin-Ciocalteu's phenol reagent was also supplied by Merck. Sodium carbonate (anhydrous, ≥99.5%) used for the Folin-Ciocalteu's assay, bovine serum albumin (BSA, fraction V, lyophilized powder), sodium dodecyl sulfate (SDS; lauryl sulfate, sodium salt, 95%), triethanolamine (TEA, 98%), $FeCl_3.6H_2O$ (98%) used for the Adams-Harbertson's assay, and $FeCl_3$ anhydrous and 2, 4, 6-tris-pyridyl triazine (TPTZ) for the FRAP assay were supplied by Sigma (Sigma-Aldrich, Saint Louis, MO, US). Standards of (+)-catechin monohydrate (98%) and $FeSO_4.7H_2O$ (≥ 99%) were used for the calibration of Folin-Ciocalteu and FRAP assays (Sigma-Aldrich, Saint Louis, MO, US). For HPLC

analyses, eight phenolic standards: gallic acid, caffeic acid, ferulic acid, syringic acid, (+)-catechin, (–)-epicatechin, epicatechin gallate (ECG) and rutin were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, acetonitrile, monosodium and disodium phosphate were obtained from Scharlau (Sentmenat, ES). Ortho-phosphoric acid was purchased from Ajax Finechem Pty Ltd (Sydney, NSW, AU) and 18-Ohm purified water was produced with a Barnsted Nanopure water system (Thermo Scientific, Waltham, MA).

6.2.2. Total Polyphenols and Tannins: Folin-Ciocalteu and Adams-Harbertson assays

Stock solutions were used to determine the composition in phytochemicals of the commercial extracts. Total polyphenols were determined with a well-established method routinely used in oenological analyses (Singleton & Rossi, 1965), and quantified as mmol (+)-catechin equivalent (CE)/L. Tannins were also quantified as mmol CE/L using the method by Harbertson et al. (Hagerman & Butler, 1978; Harbertson, Kennedy, & Adams, 2002). Briefly, 500 μL aliquots of the stock tannin solutions without dilutions were added to the reaction mixture to assess the reactivity of each compound against the Bovine Serum Albumin (BSA) protein. The obtained solutions were incubated at room temperature for the whole duration of the experiment and the absorbance was read at 510 nm against a TEA buffer blank using a Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan).

6.2.3. HPLC Analysis of Simple Phenolics

Phenolic monomers were assessed using the method described in Olejar, Fedrizzi, and Kilmartin (2015). Briefly, samples and standards were weighed on a Shimadzu UW2200H analytical balance (Kyoto, Japan) and diluted in model wine solution to achieve a concentration of 0.1 mmol (+)-catechin equivalent (CE)/L (29 mg/L) for the tannins samples, while the standards covered the range between 0.1-100 mg/L. Samples and standard solutions were then filtered through a 0.45 μm syringe filter prior to assay on an Agilent 1100 HPLC (Agilent Technologies Inc., Santa Clara, CA) equipped with column heater and ESA CouloChem III electrochemical detector (Thermo Fisher Scientific, Waltham, MA). Separation was undertaken using a Supelco Ascentis RP-Amide column, 3.0×100 mm, 3 μm (Sigma-Aldrich, St. Louis, MO) at 42°C. The

system operated in the gradient mode with mobile phase A being 30 mM phosphate buffer at pH 2.6, and mobile phase B being a 10:60:30 v/v/v ratio of methanol, acetonitrile, and 100 mM phosphate buffer. Compounds of interest were detected at 450 mV and 750 mV and the chromatograms were processed with Agilent OpenLAB CDS ChemStation Edition software [version C.01.06 (61)].

6.2.4. Ferric-Reducing Antioxidant Power: FRAP assay

The ability of tannins to reduce Fe(III) ions in acidic conditions was assayed according to the original colorimetric method proposed by Benzie et al. (Benzie & Strain, 1999). Samples were diluted in model wine to reach a 0.2 mmol CE/L concentration (58 mg/L for each tannin sample), then 100 µL solutions were added to 1900 µL of FRAP reagent; the resulting mixtures were stored at 37°C; FRAP values were obtained after 30 minutes using a Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan) and expressed as mmol FeSO4.7H2O/L using a calibration curve of the salt in the range 0.02 – 0.31 mM.

6.2.5. Radical Scavenging Activity: DPPH• assay

The DPPH• assay was performed using the original procedure described by Brand-Williams et al. (Brand-Williams, Cuvelier, & Berset, 1995) and further developed by Villaño et al. (Villano, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). For the radical scavenging assay, 100 µL of tannin solutions 0.2 mmol CE/L were added to 2.9 mL of 200 µM DPPH• solution in methanol. Solutions were incubated in dark and at room temperature for the time required to reach the steady-state of reaction, then absorbance was measured at 517 nm in 10 mm plastic cuvettes against pure methanol using a Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan) and expressed as percentage of inhibition (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009), using the following formula:

$$\% \text{ inhibition} = [(A_b - A_s)/ A_b] \times 100 \quad (6A)$$

where A_b = absorbance of the reagent blank, and A_s = absorbance of the sample. The absorbance values have been further used to estimate the total stoichiometry of the radical scavenging reaction for tannins, as calculated using the formula (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007):

$$n = [(A_b - A_s)/\varepsilon] \times c_0 l \quad (6B)$$

where A_b = absorbance of the reagent blank, A_s = absorbance of the sample at the steady state, c_0 = the initial concentration of antioxidant (0.2 mmol CE/L), l = optical path length (1 cm) and ε = the molar extinction coefficient of DPPH• at the working conditions (ε = 11563 M^{-1} cm^{-1}), as calculated by Villaño et al. (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007).

6.2.6. Redox Activity: Cyclic Voltammetry

Cyclic voltammetry (CV) of tannins in model wine solution (0.2 mmol CE/L) was performed according with the method previously described by Kilmartin et al. (Kilmartin, 2001). A Bioanalytical Systems (BAS) 100A electrochemical analyser was used to record the voltammograms. The BAS C2 electrochemical cell stand was used with a 3 mm glassy carbon disk electrode (BAS M-2012), a platinum counter electrode and an Ag/AgCl reference electrode (+207 mV versus SHE). Prior to each experimental run, the glassy carbon electrode was polished with 3 μm alumina powder (PK-4 polishing kit), then rinsed with ultra-pure water. The voltammograms were recorded at a scan rate of 100 mV/s and a sensitivity of 1 μA. A blank run was recorded daily in the model wine solution, and was used to subtract away the background current. The initial scan was taken from -200 to 500 mV, in order to record the first anodic peak. Further scans were taken from -200 to 1000 mV to detect subsequent oxidation peaks. Each experiment was run in triplicate and the average value of the current integrated under the anodic peaks were expressed in mmol CE/L, using a calibration curve of (+)-catechin monohydrate standard over the concentration range 0–1.0 mM for the first anodic peak, and integrating the charge obtained under the voltammogram to 500 mV. A second calculation was made based upon the integral of the total anodic current to 1000 mV.

6.2.7. Statistical analysis

Microsoft Excel was used for data entry, whereas derivatives of the cyclic voltammograms were calculated using OriginPro 8 (Origin Lab Corp., Northampton, MA), and statistical analyses was performed with Unscrambler X.1 (Camo ASA, Oslo, NO) and XLSTAT (Addinsoft 40, Paris, FR) software. All analyses were performed in triplicate and results were provided as average values.

6.3. Results and Discussion

6.3.1. Phytochemical Composition

Table 6.2. summarizes the effective content in polyphenols and tannins for the commercial formulations and the concentration of simple monomers. With regard to total polyphenols, a range of 1.17 to 2.77 mM CE was obtained, while the estimation of the polymeric fraction varied between 0.71 and 1.62 mM CE; these values correspond to 34% to 80% of a theoretical maximum if they were completely catechin units (3.45 mM). This broad range of phenolics content could be related to parameters like the botanical source, the purity of commercial formulation and the effectiveness of extraction methods (Bautista-Ortín, Cano-Lechuga, Ruiz-García, & Gómez-Plaza, 2014; Neves, Spranger, Zhao, Leandro, & Sun, 2010; Spigno, Tramelli, & De Faveri, 2007).

White grape extracts have attracted particular attention in the valorisation of both fermented and unfermented skins, due to their high content in active phenolics and stilbenes (Chidambara Murthy, Singh, & Jayaprakasha, 2002; Das & Singh, 2004; Katalinić, Možina, Skroza, Generalić, Abramovič, Miloš, et al., 2010). In winemaking, these extracts are commonly used as fining and clarifying agents, due to their high content in flavonoid glycoside structures. They play a key role in the complexation of biological molecules responsible for colloidal suspensions in wines (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006) and contribute to the co-pigmentation process, increasing the colour intensity of red wines (Boulton, 2001; Cheynier, Dueñas-Paton, Salas, Maury, Souquet, Sarni-Manchado, & Fulcrand, 2006) and affecting their sensory properties (Brossaud, Cheynier, & Noble, 2001; Harbertson, Parpinello, Heymann, & Downey, 2012).

Among the series of commercial extracts based on procyanidins (Pr) investigated in this work, a high content of total polyphenols was found for the unfermented grape skin (Pr8) extract, coupled with a significant content in flavonol-glycosides; sample Pr8 contained 2.48 mM of total polyphenols, 1.09 mM of which were tannins, and the following content in flavonoids: quercetin glucoside 0.024 mM RU, (+)-catechin 0.043 mM, (–)-epicatechin 0.037 mM, ECG 0.005 mM. Sample Pr8 also exhibited a high content in syringic (0.026 mM) and ferulic acids (0.012 mM). Sample Pr4, a blend of grape skins and seeds extracts, showed the highest TPC value of the tannin samples of

	TPC	Tannins	Gallic acid	(+)-Catechin	Syringic acid	(-)-Epicatechin	Ferulic acid	ECG	Quercetin glycoside
	(mM CE)	*(mM CE)*	*(mM)*	*(mM)*	*(mM)*	*(mM)*	*(mM)*	*(mM)*	*(mM RU*)*
Pr1	1.17±0.0	0.71±0.03	0.0086±0.0001	0.0087±0.0001	n.d.	0.0124±0.0004	0.00500.0004	0.0090±0.0011	n.d.
Pr2	2.64±0.09	0.82±0.01	0.0083±0.00	0.0269±0.0002	0.01720.0002	0.0181±0.0003	0.0087±0.0007	0.0066±0.0005	n.d.
Pr3	2.11±0.01	1.03±0.01	0.0083±0.0017	0.0137±0.0001	0.0144±0.00	0.0102±0.0002	n.d.	0.0048±0.00	0.0097±0.0027
Pr4	2.75±0.01	1.16±0.04	0.0248±0.0001	0.0111±0.0005	0.0135±0.0001	0.0136±0.0001	0.0081±0.0036	0.0060±0.0009	0.0177±0.0012
Pr5	1.94±0.01	1.05±0.04	0.0093±0.00	0.0356±0.0010	0.0180±0.0006	0.0362±0.0011	0.0054±0.0003	0.0101±0.0022	n.d.
Pr6	2.33±0.01	1.08±0.04	0.0101±0.0004	0.0200±0.0005	0.0143±0.0002	0.0211±0.0005	0.0051±0.0003	0.0065±0.0009	n.d.
Pr7	2.33±0.02	1.60±0.01	0.0049±0.0002	0.0201±0.0010	0.0235±0.0007	0.0161±0.0008	0.0105±0.0006	0.0081±0.0003	0.0242±0.0111
Pr8	2.48±0.02	1.09±0.01	0.0042±0.0001	0.0428±0.0017	0.0261±0.0017	0.0368±0.0010	0.0119±0.0001	0.0051±0.00	0.0119±0.0047
Hy1	1.42±0.00	1.06±0.03	0.0166±0.0001	0.0046±0.0004	0.0122±0.0004	n.d.	0.0063±0.0005	0.0028±0.00	n.d.
Hy2	2.23±0.01	1.27±0.05	0.0212±0.0001	0.0050±0.0002	0.0122±0.0004	n.d.	0.0070±0.0001	0.0030±0.0002	n.d.
Hy3	1.77±0.01	1.15±0.04	0.0153±0.00	0.0047±0.0001	0.0127±0.00	n.d.	0.0093±0.0010	0.0029±0.0001	n.d.
Hy4	2.77±0.01	1.35±0.02	0.0134±0.00	0.0166±0.0002	0.0153±0.0002	0.0135±0.0007	0.0067±0.0004	0.0209±0.0013	n.d.
Hy5	1.91±0.00	1.62±0.00	0.0147±0.0001	0.0045±0.00	n.d.	n.d.	0.0050±0.00	0.0028±0.00	n.d.

Table 6.2. Phytochemicals characterization of tannin samples: total polyphenols, effective tannic fraction and monomers. *RU = Rutin Equivalent; n.d. = concentration was below the limit of quantification.

the "Pr" (condensed) series (2.75 mM CE) with a significant tannin fraction (1.16 mM CE) and content in flavonol glucosides (0.018 mM RU). These results are in good agreement with previous work by Katalinic et al. (Katalinić, Možina, Skroza, Generalić, Abramovič, Miloš, et al., 2010), where grape berry skin extracts obtained from seven white and seven red *Vitis vinifera L.* cultivars from Croatia were analysed according with their content in polyphenolic compounds. They obtained average total polyphenol values of 3.01 mM CE in white grape skin extracts (value averaged over seven Croatian white grape varietals), among which 0.19 mmol CE/kg were made up of flavonoid compounds (Katalinić, Možina, Skroza, Generalić, Abramovič, Miloš, et al., 2010).

Sample Pr4 also exhibited the highest content in gallic acid monomers among the extracts (0.024 mM), followed by the hydrolysable Limousin oak tannin, labelled Hy2 (0.021 mM). Despite the high antioxidant potential of these compounds (see section 2.3), particular attention has to be paid when adding them to wine, as previous studies have shown the contribution of gallic acid to bitter and astringent sensory perceptions (Robichaud & Noble, 1990).

Extract Pr5 from white grape seeds provided the highest content in flavan-3-ol compounds, namely (+)-catechin (0.035 mM), (–)-epicatechin (0.036 mM), and ECG (0.01 mM), while quercetin glycosides were not detected for this tannin. The lack in this flavonol confirmed results of a previous study performed on seed extracts from selected white grapes varietals (Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006). The grape seed samples Pr5 and Pr7 showed similar results to each other with the exception of increased levels of catechin and epicatechin in Pr5 and a quercetin glycoside in Pr7 (**see table 6.2**).

These differences remain when compared to the other seed extracts, Pr2 and Pr6. The monomeric results begin to highlight the differences that extraction technique, varietal, genus, and component extracted can make. This was previously studied by Pinelo et al. (Pinelo, Arnous, & Meyer, 2006), who demonstrated the differences that varietal and the components extracted make to the concentrations of phenolics extracted. Yilmaz et al. (Yilmaz & Toledo, 2004) further demonstrated the differences that genus can make on the levels of gallic acid and flavonoids when comparing *Rotundifolia* and *vinifera* seed extracts.

The condensed extracts assayed in this work included an extract from *Vitis vinifera* leaves, namely sample Pr1. Tannins derived from this source were reported to be rich in flavonol glycosides and hydroxycinnamic acids, and were recommended for oenological applications as antioxidant agents (Monagas, Hernández-Ledesma, Gómez-Cordovés, & Bartolomé, 2006; Ramalhosa, Valentão, Andrade, Andrade, Bento, & Pereira, 2013; Souquet, Labarbe, Le Guernevé, Cheynier, & Moutounet, 2000). The commercial sample Pr1 can likewise be recommended as an antioxidant agent, despite the lower content of total polyphenols (1.17 mM CE) and tannins (0.71 mM CE) for this extract. Further, no flavonol glycosides and hydroxycinnamic acids were detected by HPLC for Pr1, while the dominant monomers were epicatechin and ECG (0.012 and 0.009 mM, respectively) followed by catechin (0.0087 mM) and gallic acid (0.0086 mM).

The hydrolysable tannins series (Hy) was composed of extracts from tree woods. Samples Hy1, 2 and 3 were ellagitannins from American, Limousin and French oak, respectively, which are routinely used to produce barrels, chips and exogenous tannins to be used in winemaking. European oak extracts exhibited a significantly high content in polyphenols and tannins, in agreement with previously results by Chatonnet et al. (Chatonnet & Dubourdieu, 1998), where the European species (*Q. robur* and *Q. Petraea*) exhibited the highest content in water-soluble extractables (140 and 90 mg/g, respectively) and total polyphenol indexes, 30 and 22 respectively (as calculated from the optical density at 280 nm (OD 280)); the highest polyphenol content was found in *Q. robur*, which is typical of the Limousin forests. In the same experiment, the *Q. alba* American white oak showed the lower content in extractables (57 mg/g) and the OD 280 index was 17 (Chatonnet & Dubourdieu, 1998).

The trend of oak extracts analysed in the present study was consistent with those findings: the ellagitannins extracted from Limousin and French oaks (Hy2 and Hy3,

respectively) exhibited a high content in polyphenols (2.23 and 1.77 mM CE) and tannins (1.27 and 1.15 M CE, respectively), while American oak (Hy1) had the lowest total polyphenols and tannins content between the oak extracts assayed (1.42 and 1.06 mM CE, respectively). A significant tannic fraction was detected in extract Hy4, composed by a blend of selected *Quercus* woods (1.35 mM CE) that accounts for almost 48.7% of the total content in polyphenols (2.77 mM CE). The detection of a significant amount of (–)-epicatechin monomer (0.014 mM) and the high content of tannins present in Hy4 suggested that the sample contained a portion of bark in the blend. The same consideration could also been extended to sample Hy5, an extract from fruit tree wood recommended as a clarifying agent for red wines. Hy5 showed the highest concentration in tannins in the "Hy" series (1.62 mM CE), which accounted for 84.8% of the total polyphenols content (1.91 mM CE), and contained a high concentration of (–)-epicatechin (0.01 mM) and ECG (0.015 mM). The (–)-epicatechin is usually found in the barks of oak and not heartwood along with a significant portion of tannins (Pallenbach, Scholz, König, & Rimpler, 1993). A comparison of Hy5 with the oak samples shows minimal differences, with the exception of syringic and ferulic acids that were not detected in this extract.

6.3.2. Cyclic Voltammetry

Cyclic voltammetry provided electrochemical properties of the tannin molecules, which can be related to the electrochemistry of the phenolic antioxidants present, as described in previous works that have mainly dealt with wine samples (Kilmartin & Hsu, 2003; Kilmartin, Zou, & Waterhouse, 2001; Kilmartin, Zou, & Waterhouse, 2002; Roginsky, Barsukova, Hsu, & Kilmartin, 2003; Sousa, da Rocha, Cardoso, Silva, & Zanoni, 2004). By contrast, little research has been published directly on the electrochemistry of tannins (Hagerman, Riedl, Jones, Sovik, Ritchard, Hartzfeld, & Riechel, 1998; Zou, Kilmartin, Inglis, & Frost, 2002). **Figure 6.1** shows a cyclic voltammogram of an oak tannin from the "Hy" series; **figure 6.2** represent the voltammogram of a procyanidins ("Pr" series). The electrochemical parameters that were derived from the voltammograms are presented in **Table 6.3**.

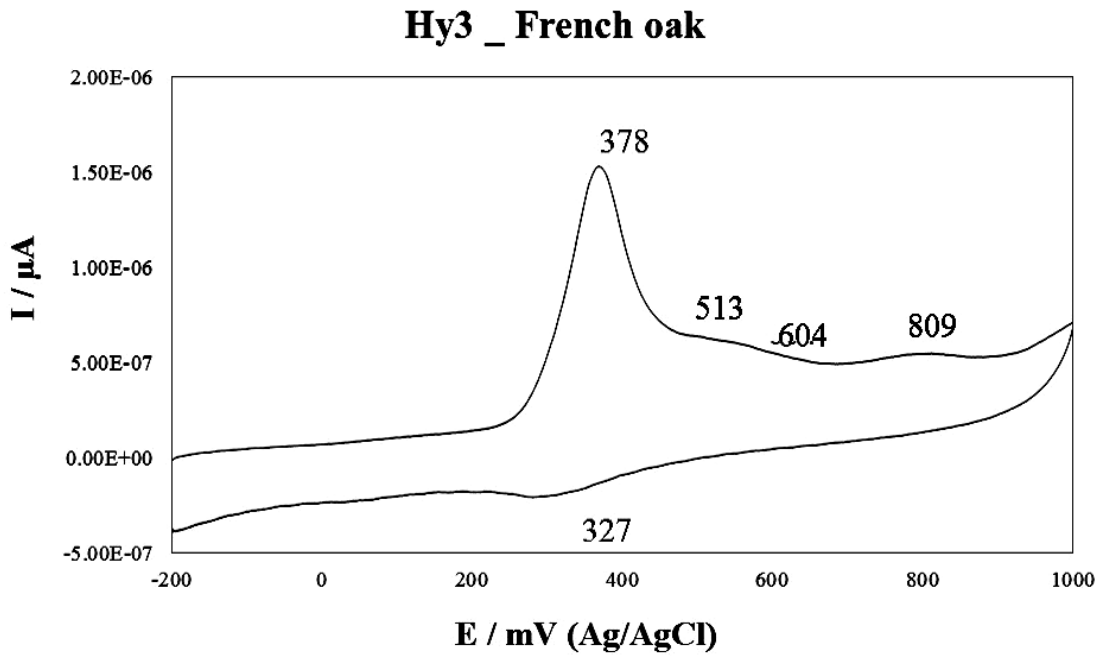

Figure 6.1. Cyclic voltammograms (background subtracted) of a French oak tannin (sample Hy3) in model wine solution, measured at 100 mV s^{-1} above the potential range: -200 to 1000 mV, at a 3 mm glassy carbon electrode.

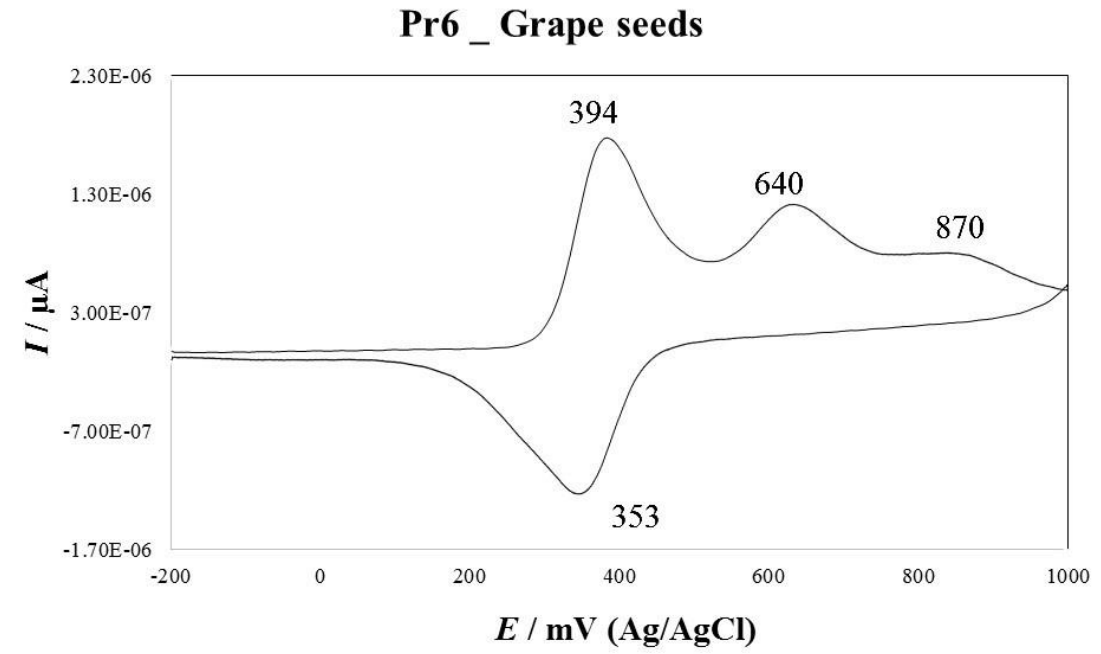

Figure 6.2. Cyclic voltammograms (background subtracted) of a grape seed tannin (sample Pr6) in model wine solution, measured at 100 mV s^{-1} above the potential range: -200 to 1000 mV, at a 3 mm glassy carbon electrode.

	E in mV (vs. *Ag/AgCl*)															
Sample	$E_{p,a}$											$E_{p,c}$			$E_{p,a}$-$E_{p,c}$*	E_{mid}
Pr1	310		***410***	_	_	_	638	_	_	_	878	_	***370***	_	40	390.0
Pr2	_	_	***391***	_	_	_	637	_	_	_	874	_	***357***	_	34	374.0
Pr3	_	_	_	***424***	_	_	_	670	_	_	888	_	***351***	_	73	387.5
Pr4	_	_	***400***		_	_	647	_	_	_	877	_	***353***	_	47	376.5
Pr5	_	_	***390***	_	_	_	635	_	_	_	877	_	***350***	_	40	370.0
Pr6	_	_	***394***	_	_	_	640	_	_	_	870	_	***353***	_	41	373.5
Pr7	_	_	***400***	_	_	_	648	_	_	_	880	_	***353***	_	47	376.5
Pr8	_	***378***	_	_	_	_	635	_	766	_	872	_	***350***	_	28	364.0
Hy1	_	***380***	_	_	506	606	_	_	_	802	_	***330***	_	_	50	35.0
Hy2	_	***383***	_	_	506	612	_	_	_	801	_	***330***	_	_	53	356.5
Hy3	_	***378***	_	_	513	604	_	_	_	809	_	***327***	_	_	51	352.5
Hy4	_	***380***	_	_	506	601	_	_	767	_	871	***338***	_	_	42	359.0
Hy5	_	_	***395***	_	535	_	647	_	_	_	869	_	***350***	_	45	372.5

Table 6.3. Anodic and cathodic peaks in the order they occur in the respective voltammograms; approximate values are given for broad peaks. In italic are reported the redox couples from semi reversible processes.

Anodic oxidation peaks, which were largely irreversible and located around 380 mV on the voltammograms of the Hy series with hydrolysable tannins, were attributed to gallate (and ellagic acid) groups attached to central sugar moieties. Small amounts of flavanol compounds contribute to the small return cathodic peak in each case (see **Table 6.3**), while the peak separation values ($E_{p,a} - E_{p,c}$) were also much larger than the 29 mV expected for a fully reversible system (Kilmartin, Zou, & Waterhouse, 2001). The further presence of an irreversible oxidation peak in the range 506–510 mV for the Hy extracts was ascribed to the presence of hydroxycinnamic acids such as ferulic acid, as shown to be present by the HPLC analyses. Samples Hy4 and Hy5 exhibited specific peaks at slightly higher potentials (871 and 869 mV, respectively), consistent with the occurrence of (–)-epicatechin in these extracts and attributed to the second oxidation peak of this flavan-3-ol compound.

Although the ECG compound, with a gallate group, was also detected in the condensed tannins "Pr" series, this anodic peak was likely overlapped to the main anodic potential in the range 390–410 mV, resulting from the oxidation of catechol ring of catechin (epicatechin) units for proanthocyanidins and of the triphenol of gallic acid present (Kilmartin, Zou, & Waterhouse, 2001; Kilmartin, Zou, & Waterhouse, 2002). A prominent reverse cathodic peak, typical of catechin or epicatechin, was always observed (**figure 6.2**), consistent with the oxidation of ortho-diphenols to quinones being followed by the reverse process on the return scan (Janeiro & Oliveira Brett,

2004; Pietta, 2000). The presence of an earlier well–defined peak at 378 mV for sample Pr8 suggested a predominance of galloylated flavanols in the larger oligomeric phenolic structures of the unfermented grape skin extract. This hypothesis was confirmed in previous works by HPLC and MALDI-TOF analyses on crude grape skin extracts (Katalinić, Možina, Skroza, Generalić, Abramovič, Miloš, et al., 2010; Monagas, Quintanilla-López, Gómez-Cordovés, Bartolomé, & Lebrón-Aguilar, 2010), and also suggested by authors as a cause of the high antioxidant activity of the analysed unfermented grape skin extract (see **section 6.3**). The occasional sample, e.g. Pr1, looked like having a significant early shoulder or peak typical of a flavonoid galloyl group or perhaps myricetin, which was absent in other samples. At the same time, some other compounds might have been extracted from the leaves with the Pr1 sample. Further anodic peaks or shoulders around 650–670 mV could be due to malvidin-type anthocyanin units, or to stilbenes, which can produce peaks at potentials greater than those seen for the catechol-containing phenolic compounds (Corduneanu, Janeiro, & Brett, 2006). All condensed tannin extracts showed an oxidation peak in the 870–888 mV range, attributable to second oxidation peaks of catechin–based compounds.

6.3.3. Antioxidant Activity

Table 6.4 summarizes results for the antioxidant assays, alongside the TPC and tannin content. Information about reducing, redox and radical scavenging activities, related to effective electron and hydrogen-transfer processes, were combined to provide the total antioxidant capacity of the extracts. In the Pr series, sample Pr8 showed the highest radical scavenging percentage, followed by Pr2 and Pr4 (DPPH• = 76.7%, 73.9% and 72.9%, respectively), which correlated well with the redox activity given by the peaks seen at potentials less than 500 mV (CV_{500mV} = 0.240, 0.229 and 0.221 mM CE, respectively). The reducing power given by the FRAP assay was high (FRAP = 0.628, 0.638 and 0.624 mM $FeSO_4.7H_2O$, for Pr8, Pr2 and Pr4, respectively), but the values were not significantly different when compared with the other Pr samples. Samples Pr6 and Pr7, procyanidins from grape seeds, were effective reducing agents according with the FRAP assay results (0.662 and 0.641 mM $FeSO_4.7H_2O$, respectively), with good redox properties according with the cyclic voltammetry response (CV_{500mV} = 0.227 and 0.200 mM CE, respectively; CV_{1000mV} = 0.748 and 0.738 mM CE, respectively). On the other hand, the low concentration in phenolic compounds in sample Pr1 lead to a low

response according to all of the antioxidant mechanisms investigated (DPPH• = 22.4%; FRAP = 0.52 mM $FeSO_4.7H_2O$; CV_{500mV} = 0.15 mM CE; CV_{1000mV} = 0.70 mM CE).

	TPC	Tannins	Radical scavenging (DPPH)	Reducing power (FRAP)	Redox activity/ 500mV	Redox activity/ 1000mV
	(mM CE)	*(mM CE)*	*(% inhibition)*	*(mM $FeSO_4.7H_2O$)*	*(mM CE)*	*(mM CE)*
Pr1	1.17±0.000	0.71±0.029	22.4±0.20	0.520±0.007	0.148±0.000	0.697±0.007
Pr2	2.64±0.09	0.82±0.012	73.9±0.30	0.638±0.001	0.229±0.002	0.802±0.006
Pr3	2.11±0.009	1.03±0.015	44.9±0.20	0.628±0.002	0.176±0.006	0.744±0.004
Pr4	2.75±0.004	1.16±0.039	72.9±0.30	0.624±0.003	0.221±0.004	0.773±0.011
Pr5	1.94±0.009	1.05±0.037	55.6±0.30	0.513±0.004	0.185±0.007	0.693±0.006
Pr6	2.33±0.004	1.08±0.037	68.6±0.30	0.662±0.023	0.227±0.009	0.748±0.002
Pr7	2.33±0.018	1.60±0.010	63.0±0.40	0.641±0.007	0.200±0.004	0.738±0.013
Pr8	2.48±0.022	1.09±0.015	76.7±0.40	0.628±0.007	0.240±0.008	0.747±0.009
Hy1	1.42±0.00	1.06±0.029	45.8±0.30	0.504±0.004	0.175±0.015	0.714±0.022
Hy2	2.23±0.004	1.27±0.049	77.4±0.20	0.642±0.003	0.233±0.004	0.737±0.012
Hy3	1.77±0.009	1.15±0.037	63.6±0.40	0.614±0.004	0.201±0.000	0.735±0.006
Hy4	2.77±0.013	1.35±0.019	76.6±0.51	0.650±0.006	0.256±0.003	0.734±0.010
Hy5	1.91±0.004	1.62±0.002	72.0±0.20	0.592±0.005	0.239±0.005	0.725±0.006

Table 6.4. Antioxidant activity of oenological tannins determined with multiple analytical approaches.

The "Hy" series was characterized by a high antioxidant activity for samples Hy2 and Hy4, which were rich in phenolic compounds. Sample Hy2 was especially effective as radical scavenger (DPPH• = 77.3%) with the highest redox activity of the series (CV_{500mV} = 0.256 mM CE), while the reducing power ($FRAP_{Hy2}$ = 0.642 mM $FeSO_4.7H_2O$; $FRAP_{Hy4}$ = 0.650 mM $FeSO_4.7H_2O$) and redox activities over the 1000 mV potential range (CV_{Hy2} = 0.737 mM CE; CV_{Hy4} = 0.734 mM CE) were quite similar for the two compounds. The Hy1 sample, American oak, exhibited the lowest antioxidant activity, in agreement with the lowest content in phytochemicals (DPPH• = 45.8%; FRAP = 0.504 mM $FeSO_4.7H_2O$; CV_{500mV} = 0.175 mM CE; CV_{1000mV} = 0.714 mM CE).

A Pearson correlation matrix allowed a simultaneous determination of correlation coefficients for similarity occurring between different methods, and results are reported in **table 6.5.** The results showed that the DPPH• assay was more suited to selectively characterize phenolics that are stronger reducing agents and are rapid free radical scavengers. The main molecular features responsible for the DPPH• radical scavenging are the B-ring in monomeric units of condensed tannins and the benzoate of gallic acid and its dilactone (ellagic acid). It has been shown how ortho -di and trihydroxyl

substitutions on flavonoids and benzoic acids increase the antioxidant power of these compounds (Leopoldini, Marino, Russo, & Toscano, 2004; Rice-Evans, Miller, & Paganga, 1997).

	TPC (mM CE)	Tannins (mM CE)	Radical scavenging (% inhibition)	Reducing power (mM FeSO4.7H2O)	CV_500mV (mM CE)	CV_1000mV (mM CE)
TPC	1					
Tannins	0.781	1				
Radical scavenging	0.811	0.808	1			
Reducing power	0.791	0.659	0.704	1		
CV_500mV	0.779	0.817	0.949	0.704	1	
CV_1000mV	0.739	0.393	0.591	0.714	0.525	1

Table 6.5. Pearson correlation matrix combining phenolic and tannin content and antioxidant activity determined with different analytical approaches.

This aspect was further confirmed in this work, as the DPPH• assay exhibited a good correlation with cyclic voltammetry when using the current integrated under the anodic peak to 500 mV (r = 0.95). The anodic current to 500 mV accounted for the antioxidant effect of chemical moieties such as catechol and galloyl groups having faster reaction rates with the DPPH• radical, itself a weak oxidising agent (Kilmartin, Zou, & Waterhouse, 2001). Furthermore, galloylation introduced on the flavanol monomers increase the effectiveness of phenolic compounds as radical scavengers (Rice-Evans, Miller, & Paganga, 1996; Yokozawa, Chen, Dong, Tanaka, Nonaka, & Nishioka, 1998), and this structure is likely to occur in oligomeric procyanidins derived from grape sources, but to be less prominent in highly polymerised tannins (Chira, Zeng, Le Floch, Péchamat, Jourdes, & Teissedre, 2015; Kuhnert, Lehmann, & Winterhalter, 2015). The correlation was worse when comparing the removal of DPPH• radical and the full anodic current calculated to 1000 mV curve (r = 0.59), since it included phenolics that are weaker reducing agents, with more isolated phenolic groups, and which are not readily oxidised by the DPPH• radical. On the other hand, the cyclic voltammetry response to 1000 mV provided a good correlation with total polyphenols (r = 0.74), but a low coefficient of similarity with tannins (r = 0.39), suggesting that the polymerised tannin fraction underwent oxidation at lower potential values (<500 mV, see **table 6.3**). The FRAP assay correlated well both with the anodic current up to 500 mV (r = 0.70) and to 1000 mV (r = 0.71), and the most of the correlation was likely provided by the current in the 200 – 700 mV range. Fe^{3+}, the ionic species that is reduced during the ferric-reducing antioxidant assay, has a cathodic potential of 180 mV vs SHE (Firuzi,

Lacanna, Petrucci, Marrosu, & Saso, 2005); in our working conditions none of the phenolics oxidise at a potential low enough to couple with the Fe(III) present (or generated from coupled oxidation processes). As a possible explanation for the occurrence of a redox process involving Fe(III) and phenolics during the FRAP assay it has been recently considered that the formation of coordination complexes and subsequent ligand environment makes the Fe(III) more reactive (Danilewicz 2015), increasing its potential and allowing effective conversion of Fe(III) to Fe(II); in particular the reduction potential was raised above 1.0 V, making Fe(III) a stronger oxidant in the FRAP assay system.

The FRAP assay also provided information that was related to the electron transfer process involving the most active phenolic molecular features, this can explain a satisfactory Pearson coefficient between DPPH• and FRAP mechanisms ($r = 0.704$). Discrepancies between the two methods were due to lability of DPPH reagent in the working solution against the increased stability of the reagent Fe(III)/TPTZ (FRAP reagent) in the buffered model wine solution (Danilewicz, 2015).

Table 6.4 shows that the TPC values calculated using the Folin-Ciocalteu method are significant lower of CE values obtained scanning to 1000 mV; these results are not consistent with previous works aimed to compare cyclic voltammetry and Folin-Ciocalteu results, where results were very close when using the two methods for red and white wine (De Beer, Harbertson, Kilmartin, Roginsky, Barsukova, Adams, & Waterhouse, 2004). As a possible explanation, we considered the nature and of commercial formulations, which could contain impurities as lignin degradation products, resinous substances and reducing sugars, and artificial food additives like dextrin, starch, and other vegetal amides (Villavecchia & Eigenmann, 1977). It was demonstrated that the Folin-Ciocalteu method is affected by low selectivity when applied to complex matrices like wine (Danilewicz, 2015), and the intentional or natural exogenous compounds listed above and possibly contained in tannins could induce an overestimate overestimate of the effective content in polyphenolic when using the colorimetric assay. In this perspective, the cyclic voltammetry signal above 1000 mV seemed to provide an improved selectivity for the quantification of polyphenols in commercial oenological tannins, but this hypothesis needs to be further confirmed.

All analytical methods used to determine the activity of tannins showed a significant contribution of the low-molecular weight fraction to the antioxidant properties of these extracts. In each case the correlation coefficient of antioxidant assays with total

polyphenols are improved when compared with their correlation to the estimated tannin content (**table 6.5**). It can be hypothesized that the steric hindrance of highly-polymerised compounds had a significant role in the electron and hydrogen-transfer mechanisms involved in the antioxidant assays.

Extracts were characterized by their total antioxidant stoichiometry that was given as the number of radical molecules reduced by one molecule of antioxidant, calculated using equation (**6B**). T**able 6.6** shows the radical scavenging stoichiometry calculated for tannin extracts and compared with results obtained by Villaño et al. (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007) for the monomeric constituents of tannins (gallic acid and flavan-3-ols derived structures).

Sample code	Sample description	n_{tot}
Pr1	*Vitis vinifera* leaves	2.7
Pr2	Grape berries	9.0
Pr3	Grape	5.4
Pr4	Grape skin and seeds	8.8
Pr5	White grape seeds	6.7
Pr6	Grape seeds	8.3
Pr7	Malbec red grape seeds	7.6
Pr8	Unfermented grape skins	9.3
Hy1	American oak	5.5
Hy2	Limousin oak	9.4
Hy3	French oak	7.7
Hy4	Selected *Quercus* wood	9.3
Hy5	Red fruit trees wood	8.7
	Gallic acid	5.6
	(+)-Catechin	4.5
	(–)-Epicatechin	6.6
	(–)-Epigallocatechin	5.4
	(–)-Epicatechin gallate	7.3
	(–)-Epigallocatechin gallate	7.9
	Procyanidin B1	7.6
	Procyanidin B2	7.4
	Ascorbic acid	2.3
	Trolox	2.0
	Resveratrol	1.0

Table 6.6. Stoichiometry calculated for the scavenging of DPPH• radical with tannins (n_{tot}) compared to results of Villano et al. (2007) for monomeric and oligomeric phenolic compounds (n_{tot}*). Values from bibliography are highlighted in yellow in the table

Although the monomers exhibit a relationship between the number of –OH substituents and the ability to effectively scavenge radicals, it has been already noticed in previous

experiments and it was further confirmed in this work that the increase of hydroxyl groups following polymerization do not provide an additive effect in terms of availability in the proton-transfer process. The occurrence of polymerisation slightly increased the stoichiometry of the radical scavenging reaction when compared to the reaction of phenolic monomers against the DPPH• radical. Two hypothesis were considered to mainly influence the availability of hydroxyl groups to participate in the radical scavenging reaction: (i) the steric arrangement following the polymerisation process, and (ii) the stability of intermediate products, such as dimers or quinones, which could further react with the radical increasing the stoichiometry. It is likely to hypothesize that the compounds exhibiting higher stoichiometry are characterised by an extended polymerization of flavonoid monomers and/or a high degree of galloylation, since it has been observed that structures with hydroxyl groups in the *ortho*- position of the aromatic ring provided a higher n-value, as well as gallic acid-like structures, since gallic acid has three –OH groups and the n-value calculated for the reaction with DPPH• radical was 6 (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007).

The Pearson similarity correlation matrix was also calculated to correlate stoichiometry with total polyphenols and tannins content (*data not shown*). The high correlation between total polyphenols and n_{tot} ($r = 0.811$), which decrease considering the similarity between tannin content and stoichiometry coefficients calculated through the DPPH• method ($r = 0.510$), suggested a high incidence of the low-molecular weight fraction in the activity of these extracts. The lower correlation could indicate that the polymers need a longer incubation time to fully accomplish their antioxidant activity. These results highlighted the influence of working conditions in the determination of antiradical activity as a possible limitation in using the synthetic radical to reproduce the mechanisms of action of natural antioxidants against free radical species.

6.4. Conclusions

A multiple analytical approach allowed to profile a selection of commercial tannins commonly used in winemaking. HPLC–ECD analysis and spectrophotometric assays allowed determination of the effective fraction of tannins against the total polyphenols content, showing a great variability over the composition of samples. This could be

ascribed to the degree of purity of the commercial formulations and the effectiveness of different extraction procedures. The cyclic voltammetry responses provided typical profiles according with different botanical sources, showing the potentiality to be used as a fast and effective method for authentication and characterisation of these compounds. Furthermore, additional information could be extrapolated from the electrochemical data, ranging from the antioxidant activity to the total polyphenol content. In particular, the cyclic voltammetry method showed high linear correlation with the radical scavenging (DPPH•) assay, when considering the integrated current under the first anodic peak (to 500 mV), related to the most reactive species (catechol ring of flavonoids and trigalloyl moieties of gallic acid–based compounds). The total current resulting from the whole potentials range considered (to 1000 mV) provided a good correlation with the total polyphenol content, as calculated using the Folin-Ciocalteu colorimetric assay. Further significant correlations between chemical and parameters were highlighted using the Pearson correlation matrix multivariate approach. In general, the content of tannin determined the effectiveness of the samples as antioxidants, although all extracts showed effectiveness against the main oxidative mechanisms (radical chain propagation, oxidation of transition metals). The sample derived from Limousin oak and from selected *Quercus* woods showed a higher content in active compounds and a stronger total antioxidant capacity. When considering the condensed tannins series, the extract from unfermented grape skins showed the highest performances, despite a content of phenolic compounds that is lower than other "Pr" samples, probably due to the high occurrence of flavonoid gallate structures that implement the antioxidant mechanisms. The stoichiometry of radical scavenging reactions was not additive with respect to the related monomers, suggesting a contribution of the spatial arrangement in the availability of active functional groups.

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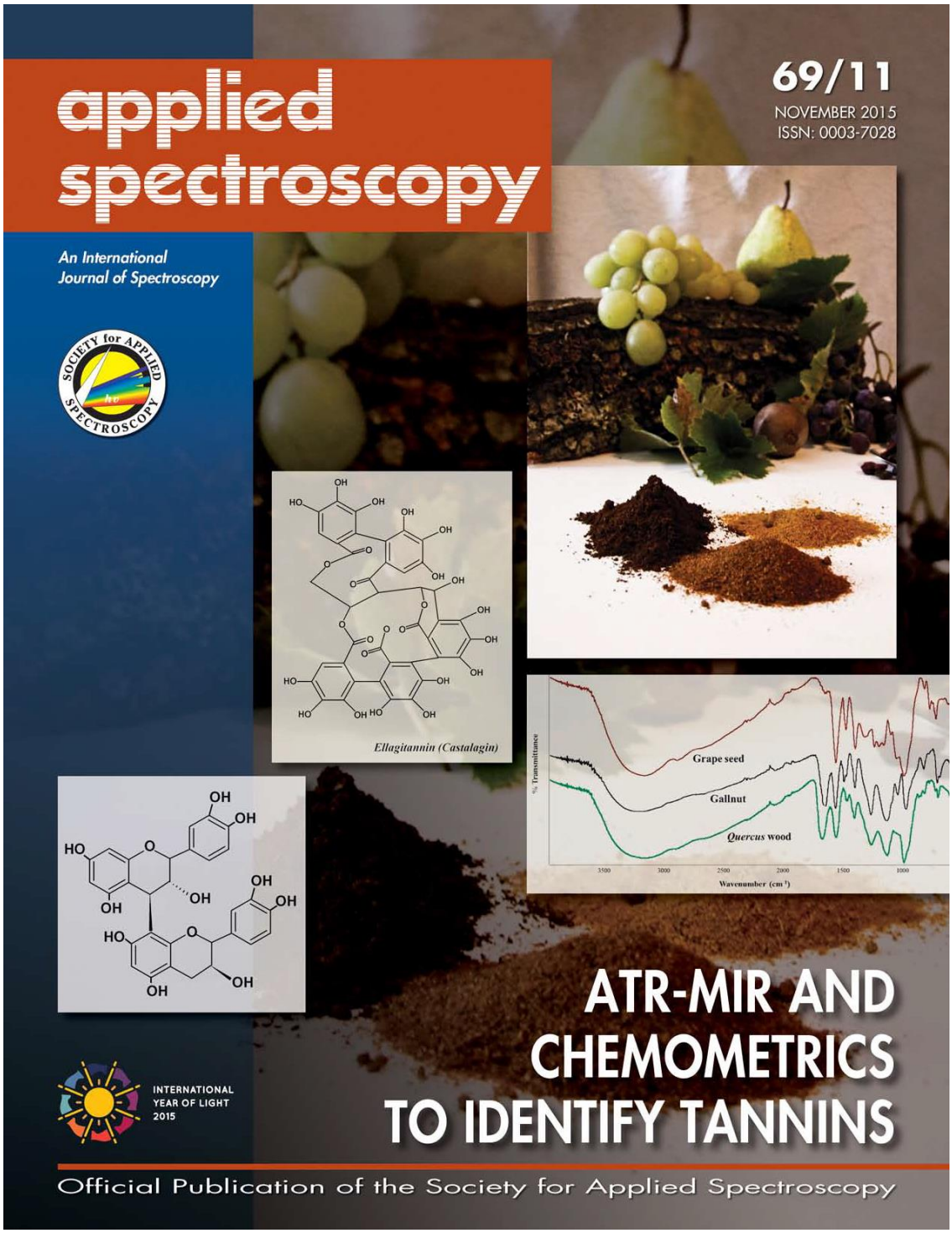
Addendum D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Ellagitannin (Castalagin)
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INTERNATIONAL YEAR OF LIGHT 2015
ATR-MIR AND CHEMOMETRICS TO IDENTIFY TANNINS
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The Use of Attenuated Total Reflection Mid-Infrared (ATR-MIR) Spectroscopy and Chemometrics for the Identification and Classification of Commercial Tannins

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In this paper attenuated total reflection Fourier transform infrared (FT-IR) spectroscopy was used to characterize 40 commercial tannins, including condensed and hydrolyzable chemical classes, provided as powder extracts from suppliers. Spectral data were processed to detect typical molecular vibrations of tannins bearing different chemical groups and of varying botanical origin (univariate qualitative analysis). The mid-infrared region between 4000 and 520 cm^{-1} was analyzed, with a particular emphasis on the vibrational modes in the fingerprint region (1800–520 cm^{-1}), which provide detailed information about skeletal structures and specific substituents. The region 1800–1500 cm^{-1} contained signals due to hydrolyzable structures, while bands due to condensed tannins appeared at 1300–900 cm^{-1} and exhibited specific hydroxylation patterns useful to elucidate the structure of the flavonoid monomeric units. The spectra were investigated further using principal component analysis for discriminative purposes, to enhance the ability of infrared spectroscopy in the classification and quality control of commercial dried extracts and to enhance their industrial exploitation.

Index Headings: **Fourier transform infrared; FT-IR; Condensed tannins; Hydrolyzable tannins; Principal component analysis; PCA.**

INTRODUCTION

Tannins are naturally occurring bioactive compounds, produced as secondary metabolites by plants. According to their botanical origin, tannins can be divided into two main classes: (1) hydrolyzable tannins, further divided in ellagitannins (lactones of ellagic acid) and gallotannins (natural polymers of gallic acid esters), which are mainly derived from woody part of plants and fruits, and (2) condensed tannins, commonly known as procyanidins, composed of flavanol monomeric units (catechins) containing galloyl moieties (prodelphinidins) or catechols (proanthocyanidins), which are mainly derived from grape, stone fruits, cocoa, coffee, mimosa, and quebracho tree species.[1,2] Figures 1 and 2 show the molecular structures of monomeric units and related polymers cited in this work.

Tannins find application in different industry settings, including wine making,[3] water purification,[4] ink manufacture, leather and dye industries,[5] synthesis of metal nanoparticles,[6] and manufacture of adhesives, surface coatings[7,8] and plastic resins.[9] In particular, tannins act as natural preservatives, and blending them with adhesives and plastics enhances the technological properties of innovative polymers doped with these natural extracts.[10–12] For example, proanthocyanidins from the common grape (*Vitis vinifera*) were shown to be particularly effective in providing antioxidant activity while not affecting the mechanical properties of ethyl cellulose films.[13]

In addition to their multiple functional properties, a further advantage of tannins is their large availability due to the widespread use of traditional agricultural practices and an increasing interest in the recovery of agro-wastes for the production of "green" derivatives.[14,15] Despite the large availability of different commercial formulations, there is a need for (1) further information on their chemical composition and (2) rapid analytical techniques for effective discrimination between tannins derived from different botanical sources to further enhance and tailor industrial applications of tannins. Alternative analytical methods, both for hydrolyzable as well as for condensed tannins, such as matrix-assisted laser desorption–ionization time-of-flight mass spectrometer[16–19] (MALDI-TOF), ^{13}C nuclear magnetic resonance[20] (NMR), and ^{31}P NMR[21,22] are widely used to get further chemical information on the structure of tannins.

In this context, attenuated total reflectance (ATR) Fourier transform infrared (FT-IR) spectroscopy, being a simple and sensitive technique, has been widely applied in the field of quality control in industry. The versatility of this technique is also improved by its ability to operate with different matrices (both solid and liquid) without the need for sample pretreatments.[23] Furthermore, ATR infrared (IR) spectroscopy allows analysis of powders directly over the internal reflection element, without dilution in an IR-transparent medium (KBr, Nujol, etc.). The product as provided by suppliers can be directly analyzed in a few seconds.

The data elaboration of a single sample in molecular spectroscopy can be considered as a univariate approach to the study of chemical differences between tannins belonging to different botanical species. This is expensive in terms of time compared with fast and efficient methods required for industrial processes. On the other hand, FT-IR analysis is very effective for discriminative purposes when coupled with multivariate chemometric approaches.[24,25]

The aim of this work is to identify IR spectral patterns that typify representative commercial tannins used in

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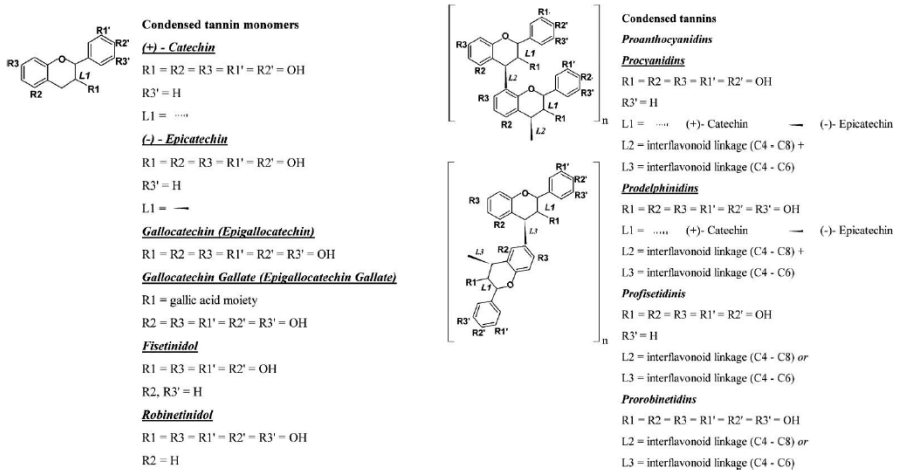

FIG. 1. Condensed tannins and their monomeric units.

industrial applications (univariate approach) and to evaluate the performance of classification algorithms across the selected samples. The effectiveness of spectral and statistical analyses provides a valid contribution in evaluating the authenticity and the degree of purity of these compounds in a fast, reliable, and nondestructive way.

EXPERIMENTAL

Samples Description. Gallic acid, (+)-catechin monohydrate, and ellagic acid standards used for reference spectra were purchased from Sigma (Sigma-Aldrich, Castle Hill, Australia). Forty commercial powder tannins (Table I) were analyzed as provided by suppliers (Enologica Vason, Verona, Italy; HTS Enologia, Marsala, Italy; Laffort, Bordeaux Cedex, France; AEB Group, Brescia, Italy) in order to ascertain the ability of infrared spectroscopy to classify and describe the different samples based on their chemical composition and declared botanical origin.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy. Attenuated total reflectance FT-IR analysis was carried out using a diamond ATR Smart Orbit accessory (Thermo Optec), equipped with a deuterated triglycine sulfate detector and a KBr window for measuring in the medium infrared (MIR) region; the incident beam had a 45° geometry with respect to the
?2 diamond surface, yielding 25 internal reflections. Tannins were analyzed without any pretreatment or purification; a few milligrams of the powder was placed over the macro ATR crystal, and a slight pressure was applied over the sample to maximize the surface of contact. MIR spectra range from 4000 to 520 cm^{-1} and were averaged over 128 scans with a resolution of 4 cm^{-1}.

Data Processing and Multivariate Analyses. The ATR spectra in the MIR region were processed using principal component analysis (PCA) and a cluster analysis (CA) unsupervised method as classification tools based on the relationship between botanical sources and molecular structure of the selected tannins. The raw spectroscopic data were subjected to mathematical elaboration: all values reported are from triplicate spectral analyses, every single spectrum was processed performing a linear algorithm for baseline correction and a Savitzky–Golay smoothing filter (nine points). The results were mediated to obtain an average spectrum for peak assignments and subsequent multivariate elaboration. The collection and preprocessing of the spectral data was performed using the Omnic SPE 7.2 software (Thermo Fisher Scientific).

The averaged spectra were subjected to multivariate analysis using the The Unscrambler 9.7 software (Camo Software AS). The different spectral regions were tested for chemometric elaboration, and ultimately the whole spectral region was found to be the best option to maximize the information.

The validation method selected for PCA was the leverage correction on a full model size. The cluster analysis was performed using the correlation algorithm over the vector-normalized spectra. According with the similarity between data, the optimal number of clusters was six, with a satisfactory sum of distances value of 0.24.

RESULTS AND DISCUSSION

Figure 3 shows the spectral profiles in the MIR region for the basic monomeric unit of tannins: (+)-catechin,

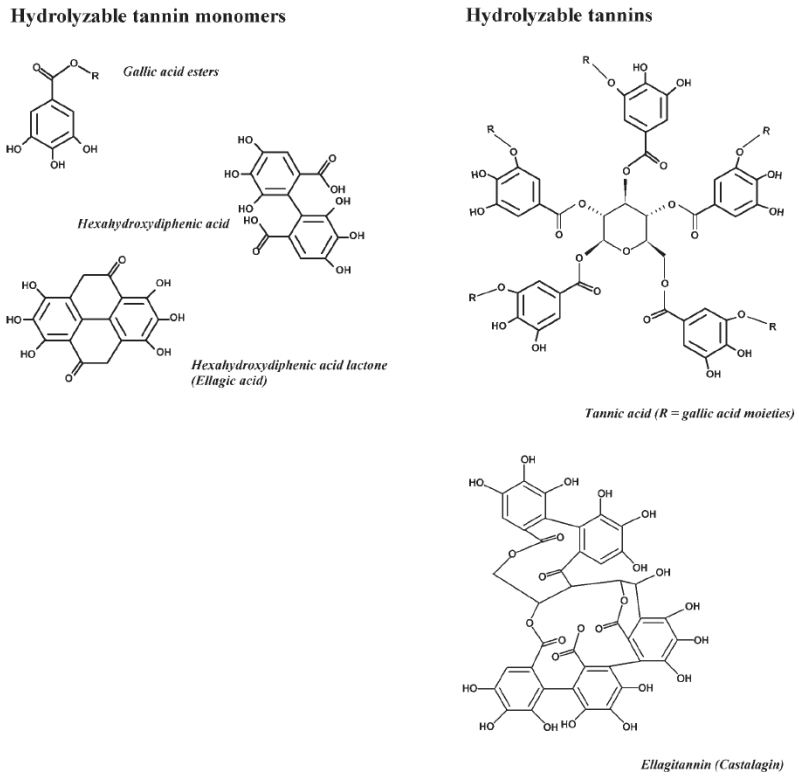

FIG. 2. Hydrolyzable tannins and their monomeric units.

gallic acid, and ellagic acid. Some differences due to the typical structure of these compounds were found.

The vibrational spectrum of catechin-based tannins showed a complex envelope of strong signals in the region 1250–900 cm^{-1}, where band occurrence and intensity is sensitive to the number and position of –OH aromatic substituents (Fig. 3a). Additional information on galloyl moieties and galloylation of monomers could be derived especially from this spectral region. In particular, flavan-3-ol–based structures have a strong peak around 1610–1620 cm^{-1} as a doublet, due to in-plane bending of the benzene ring for the catechol moieties (ring B) and to the same combination in the resorcinol moieties.[26,27] The relative intensity of this spectral feature is sensitive to elongation of the C4–C8 or C4–C6 interflavonoid linkages, so that it could be used as an index of the degree of polymerization.[28]

The MIR spectra of gallic acid (Fig. 3b) and ellagic acid (Fig. 3c) showed some similarities, since they belong to the same class of hydrolyzable compounds. At the same time, there were some slight differences that can be used to discriminate the subclasses, as follows. A diagnostic region for hydrolyzable tannins is within 1750–1680 cm^{-1}, where the stretching of the C=O group of the carbonyl moiety occurs, giving a strong peak.[29,30] This band is usually quite weak in condensed tannins, except if oxidation and rearrangement of OH group occurs as a consequence of extraction processes with proanthocyanidins.[31] A specific peak around 1717 cm^{-1} was assigned to the interaction of condensed tannins with some additives, usually cellulose.[32]

A strong peak was present at around 1318 cm^{-1} for the spectrum related to ellagic acid (Fig. 3c), and there is a strong doublet for gallic acid in the same spectral region. This signal could be considered a fingerprint band of the

TABLE I. Summary of commercial tannins classified according with chemical and botanical origin.

Sample code	Botanical origin	Chemical description (C, condensed; H, hydrolyzable; M, mixture)
PR1	Green tea	C
PR2	*Vitis vinifera* leaves	C
PR3	Grape fruit	C
PR4	Grape fruit	C
PR5	Grape skin and seeds	C
PR6	White grape seeds	C
PR7	Grape seeds	C
PR8	Malbec red grape seeds	C
PR9	Quebracho	C
PR10	Not fermented grape skin	C
PR-HY1	Red fruits tree wood	M
PR-HY2	White fruits tree wood	M
PR-HY3	N/A[a]	M
PR-HY4	N/A	M
PR-HY5	N/A	M
PR-HY6	N/A	M
PR-HY7	N/A	M
PR-HY8	Oak wood and grape	M
HY-OA1	Oak	H
HY-OA2	Oak	H
HY-OA3	Oak	H
HY-OA4	Oak	H
HY-OA5	Oak	H
HY-OA6	Oak	H
HY-OA7	Oak	H
HY-CH1	Chestnut	H
HY-CH2	Chestnut	H
HY-AD1	American Durmast	H
HY-AD2	American Durmast	H
HY-AD3	American Durmast	H
HY-FD1	French Durmast	H
HY-FD2	French Durmast	H
HY-FD3	French Durmast	H
HY-FD4	French Durmast	H
HY-FD5	French Durmast	H
HY-GEN1	N/A	H
HY-GEN2	N/A	H
HY-GEN3	Red fruits tree wood	H
HY-GEN4	N/A	H
HY-GALL1	Gallnut	H

[a] N/A, information not available.

phenolic ring, and several authors have observed that it is a typical feature for hydrolyzable tannins.[33,34] This finding is also highlighted in Fig. 4, where the spectra of a mixture of ellagitannins (Fig. 4c), a gallnut extract (Fig. 4b), and a proanthocyanidin extract from grape seed (Fig. 4a) are compared. By contrast, there are few signals of interest in this region for the spectrum of flavonoids, with only weak to very weak bands for catechin (Fig. 3a).

The region 1250–900 cm^{-1} showed diagnostic bands related to the hydroxylation patterns of aromatic rings present in tannins; the analysis of these spectral features confirms the information reported in the literature. A strong band around 1037 cm^{-1} is diagnostic for the chestnut tannin extracts (C–O stretch of phenolic O–H groups) and is often present in oak tannins at a strong intensity. The 1145–1150 cm^{-1} band is common to hydrolyzable compounds and is attributable to a combination of C–O stretching and –OH deformation motions of the carboxyl group. Quebracho tannin exhibits typical bands at 1160, 1114, and 1032 cm^{-1}, which allows it to be distinguished from the resorcinol group in grape tannins. Here the change in the monomeric unit, single OH substituent on the A ring in quebracho, produces a slight change in the hydroxylation pattern.[35,36] Condensed tannins also contain an oxygenated heterocyclic ring (ring C) that produces characteristic vibrations: a peak around 1280 cm^{-1} due to the asymmetric stretching vibration of the pyran ring. When this signal is coupled with strong bands at 1162–1155, 1116–1110, 976, and 844–842 cm^{-1} the extract can be unambiguously assigned to the condensed tannins group.[35,36]

The univariate detection of diagnostic spectral features for industrial extracts was followed by a multivariate analysis of vibrational frequencies in the mid-infrared region. The PC1 component shows the highest explanation of differences between the chemical composition of the extract (78%), followed by PC2 (10%), and so these two variables were taken into account for clustering. Figure 5 shows the PCA–CA diagram with the recognition of the six obtained clusters. The first cluster contained six samples, including all tannins derived from American oak, together with one French oak extract and two hydrolyzable tannins derived from oak. The commercial denomination of European oak concerns two botanical species, *Quercus petraea* and *Quercus robur*, whereas American oak generally refers to the white oak (*Quercus* ssp. and *Quercus alba*). It is noticeable that from the botanical point of view, American and European oaks exhibit different MIR properties, which reflects their technological applications. American oak is richer in aromatic precursors such as the cis–trans isomers of 13-methyl-γ-octalactone, but the quantity of extractable polyphenols is very low compared with the European variety. Furthermore, the European variety *Quercus robur* is particularly high in ellagitannin content, and the quality could be considered higher according with the specific technological application.[37] From this perspective, the ability of the multivariate model to discriminate the two species could be useful to avoid adulteration or dilution in the extracts.

The second cluster (Fig. 5) grouped the gallnut tannins coupled with a hydrolyzable tannin of undeclared origin (sample HY-GEN2), a vegetable extract with high antioxidant activity, most probably with gallic acid monomers arranged around a simple sugar (tannic acid–like structure). Figure 6 shows how the ATR FT-IR spectra of these two extracts overlapped in the fingertip region (1820–520 cm^{-1}), confirming a perfect match for the two spectral profiles.

The CA approach was particularly effective for the discrimination of condensed tannins. All procyanidins derived from *Vitis vinifera* (fruit pulp, seed, skin) were grouped in cluster 3, together with a sample described as a mixture of ellagic and catechin tannins (Fig. 5), of unknown purity and ratio of the two components. The robustness of the CA model was further confirmed by its ability to distinguish in a group, namely, cluster 6, the condensed tannins derived from exotic botanical sources, such as green tea (PR1) and quebracho (PR9). It is well known that the extent of polymerization and spatial arrangement of condensed tannins are strongly sensitive to the botanical source.[38] The most common condensed tannins are procyanidins and prodelphinidins

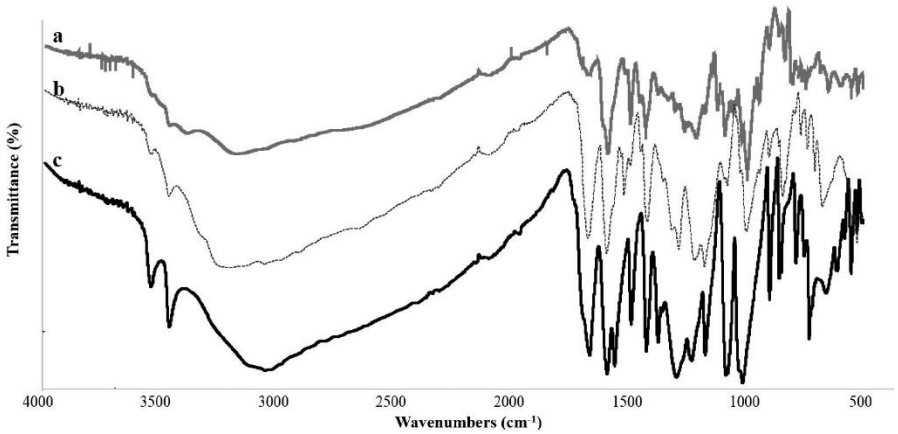

FIG. 3. ATR FT-IR spectra of the most common monomeric units of tannins. (**a**) (+)-catechin; (**b**) gallic acid; (**c**) ellagic acid.

from *Vitis vinifera*, and profisetinidins and prorobinetinidins for quebracho. The content and relative ratios of these compounds provide useful information about the botanical origin of commercial tannins. Procyanidins are found in grape skins and seeds, whereas prodelphinidins are present in grape skins only.[39] In particular, the fisetinidol and robinetinidol subunits of quebracho polymers differ from grape monomers, since they are missing a phenolic group in the C5 position of the resorcinol ring.[40]

Quebracho tannin is mainly composed of profisetinidin, a linear polymer resulting from a combination of catechins and gallocatechins, namely, a proanthocyanidin blended with hydrolyzable tannins, as observed through MALDI-TOF analyses.[41] Therefore, quebracho also contains some gallic acid, which could explain the inclusion in cluster 6 of the samples PR-HY1 and PR-HY7.

Grape tannins are random polymers mainly composed of procyanidins and galloylated procyanidins, having an average degree of polymerization of up to five for seeds

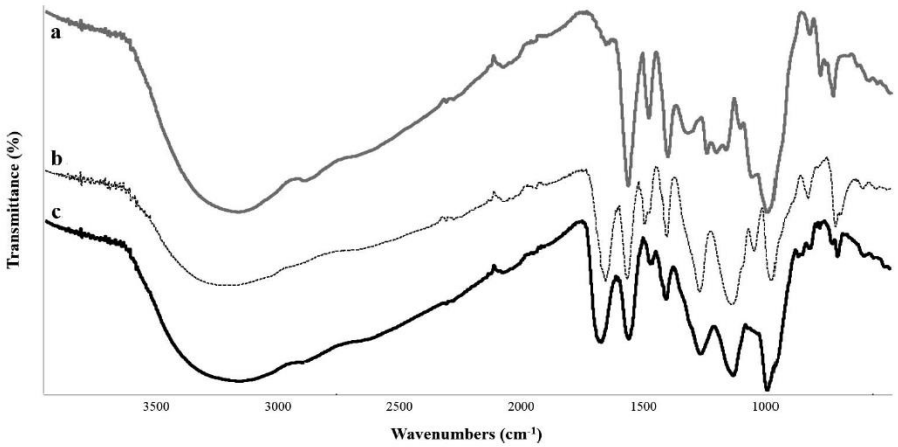

FIG. 4. ATR FT-IR spectra of three industrial tannins. (**a**) Proanthocyanidins from grape; (**b**) gallnut extract; (**c**) ellagitannins from red fruit tree wood.

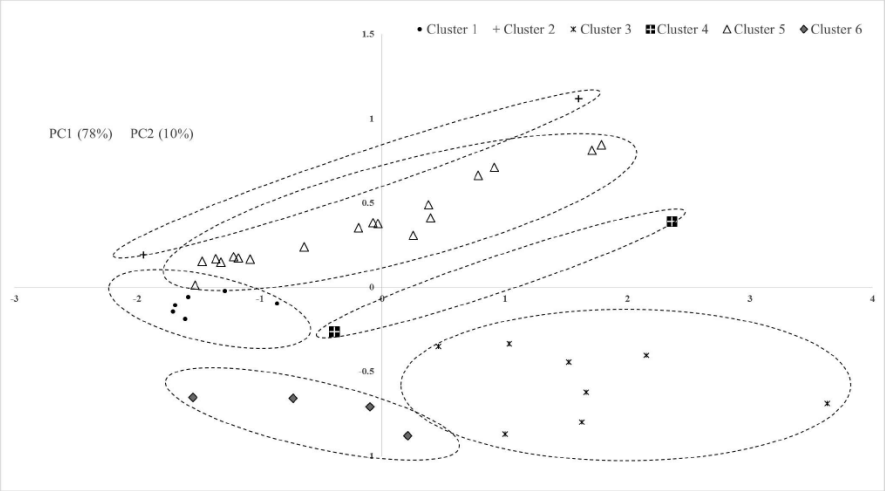

FIG. 5. PC1/PC2 score plot using PCA analysis and sample grouping based on cluster analysis.

and higher for skin extracts.[42,43] Polymerization usually follows a linear C4–8 sequence (interflavonoid linkage), but branching of monomeric units through the lateral moieties (usually C4–6) can occur.[44]

Green tea is mainly composed of monomeric units of epigallocatechin gallate (EGCG), followed by epicatechin-3-gallate, epigallocatechin, epicatechin, and catechin.[45,46] Based on the chemical structures, the grouping

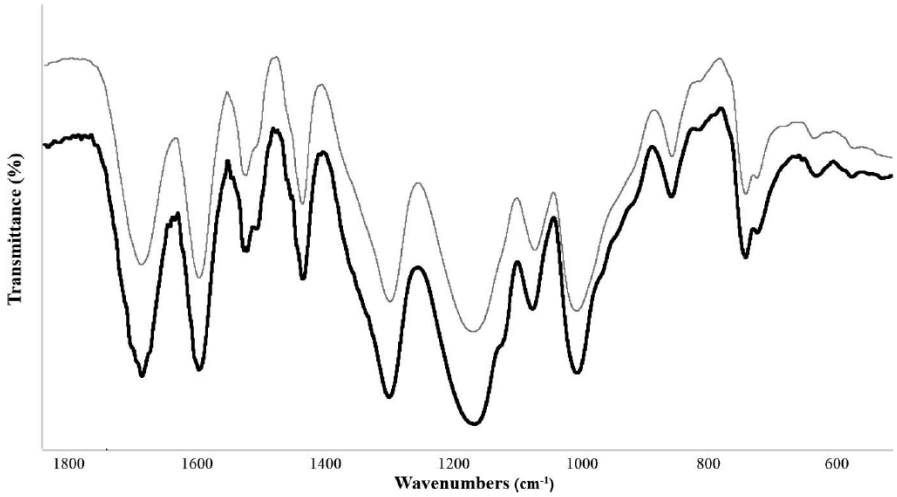

FIG. 6. Comparison between gallnut extract (gray line) and sample HY-GEN2, a hydrolyzable tannin (black line).

of tea tannins with quebracho tannins was unexpected and supports the idea that gallic acid, galloyl, and gallate structures were present in both quebracho and tea. The spectral similarities between the quebracho and the green tea tannins over the region 1300–1000 cm^{-1} could then be related to the hydroxylation patterns of aromatic molecules. At the same time, the absence of tannic acid–like structures in green teas makes the above hypothesis somewhat controversial.[46]

Cluster 4 included two odd tannins: a catechin-based extract from *Vitis vinifera* leaves coupled with an ellagitannin from toasted oak (Fig. 5). Since there is no clear link between clustering and available information, the two tannins of cluster 4 should presently be considered as outliers.

Cluster 5 was the most abundant, including eight ellagitannins, three French oak tannins (HY-FD2, 3, 4), two chestnut tannins (HY-CH1 and 2), and six mixtures of condensed and hydrolyzable tannins. Grouping of these samples indicates that hydrolyzable are the prominent tannins.

CONCLUSIONS

Forty commercial powder tannins were analyzed with ATR FT-IR spectroscopy, and the resulting MIR spectra provided valuable chemical information on tannin composition. This analysis was effective as a univariate approach, with the detection of typical vibrational bands related to specific molecular features, and multivariate analysis, for classification purposes. The univariate approach focused mainly on the fingertip region (1800–520 cm^{-1}), where diagnostic peaks were identified for discriminative purposes. The spectral region 1750–1680 cm^{-1} contains strong bands that are unambiguously attributable to stretching vibrations of carbonyl moieties that are typical of hydrolyzable tannins. Condensed tannins do not show significant bands in this region, while they exhibit a typical doublet around 1610–1620 cm^{-1}, related to catechol and resorcinol moieties. The region around 1250 to 900 cm^{-1} provides information about hydroxylation patterns, so that it could be used both to discriminate between hydrolyzable and condensed tannins and to obtain further discrimination between catechins and gallate catechins. principal component and clustering analyses were found to be effective tools for the classification of tannins based on chemical and botanical sources, especially in the case of proanthocyanidins from *Vitis vinifera*, profisetinidin from quebracho, and ellagitannins from white oak. The minor concerns about the nature of green tea tannins and the presence of a few outliers that were not properly described by the model would require additional samples of known origin to further improve this preliminary dataset.

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Addendum E

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Short communication

Spectroscopy analysis of phenolic and sugar patterns in a food grade chestnut tannin

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ABSTRACT

Tannin of chestnut (*Castanea sativa Mill.*) wood, commonly used in winemaking was characterised with a spectroscopy qualitative approach that revealed its phenolic composition: several vibrational diagnostic bands assigned using the Attenuated Total Reflectance-Infrared Spectroscopy, and fragmentation patterns obtained using the Laser-Desorption–Ionization Time-of-Flight technique evidenced polygalloylglucose, e.g. castalagin/vescalagin-like structures as the most representative molecules, together with sugar moieties. The implication of these findings on winemaking application and the potential influence of the chemical structure on the sensory properties of wine are discussed.

1. Introduction

The wood of *Castanea sativa Mill.* European (i.e. chestnut) is approximately composed of 40% cellulose, 20% hemicellulose, 25% lignin, and 10% ellagitannins, which are hexahydroxydiphenic acid molecules esterified with glucose (Mosedale, Charrier, & Janin, 1996; Nonier et al., 2005). Although the tannin fraction exhibits high variability between botanical species (Nonier et al., 2005; Prida & Puech, 2006), the chestnut extractable fraction is mainly represented by monomeric and oligomeric phenolic compounds, mostly vescalagin and castalagin, grandinin and roburin E (Salminen, Ossipov, Loponen, Haukioja, & Pihlaja, 1999), together with galloyl glucosyilated phenolic compounds (Lampire et al., 1998).

The chestnut tannin is currently used in industrial application (Navarrete, Pizzi, Pasch, Rode, & Delmotte, 2010; Ping, Pizzi, Guo, & Brosse, 2012), and it is commonly added in winemaking as fining coadiuvant (OIV 2009). The chemistry of hydrolysable tannins in wine is mainly affected by their solubility in water system, e.g. wine, their reaction with oxygen (Versari, du Toit, & Parpinello, 2013), and interaction with proteins (Mané et al., 2007). The sensory impact of chestnut tannins in wine is controversial as some chemical compounds derived from ellagitannins influences the composition of wine itself; in particular, there is a need for a detailed characterisation of the molecular structures responsible for wine bitterness and astringency (Puech, Prida, & Isz, 2007).

In this preliminary study a new commercial food grade ellagitannin extracted from chestnut wood was analysed for its composition in monomers and polymeric fractions (phenolics, polysaccharides, lignin residues) with the combined use of Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) spectroscopies, thus allowing to recognize molecular patterns which influences sensory perception. Preliminary results aim to propose an effective analytical approach for the study of the impact of natural extracts in wine, to provides complementary information to analytical techniques routinely used, including HPLC (Mattivi, Vrhovsek, Masuero, & Trainotti, 2009), in view of future and more extended characterisation of oenological tannins commercially available.

2. Materials and methods

A food grade commercial tannin powder from wood of *C. sativa Mill.* (i.e. chestnut) was divided into three sub-samples and each replicate analysed with ATR-FTIR and MALDI-TOF spectroscopies according to our procedures previously described in details (Ricci, Parpinello, Olejar, Kilmartin, & Versari, 2015; Lagel, Pizzi,

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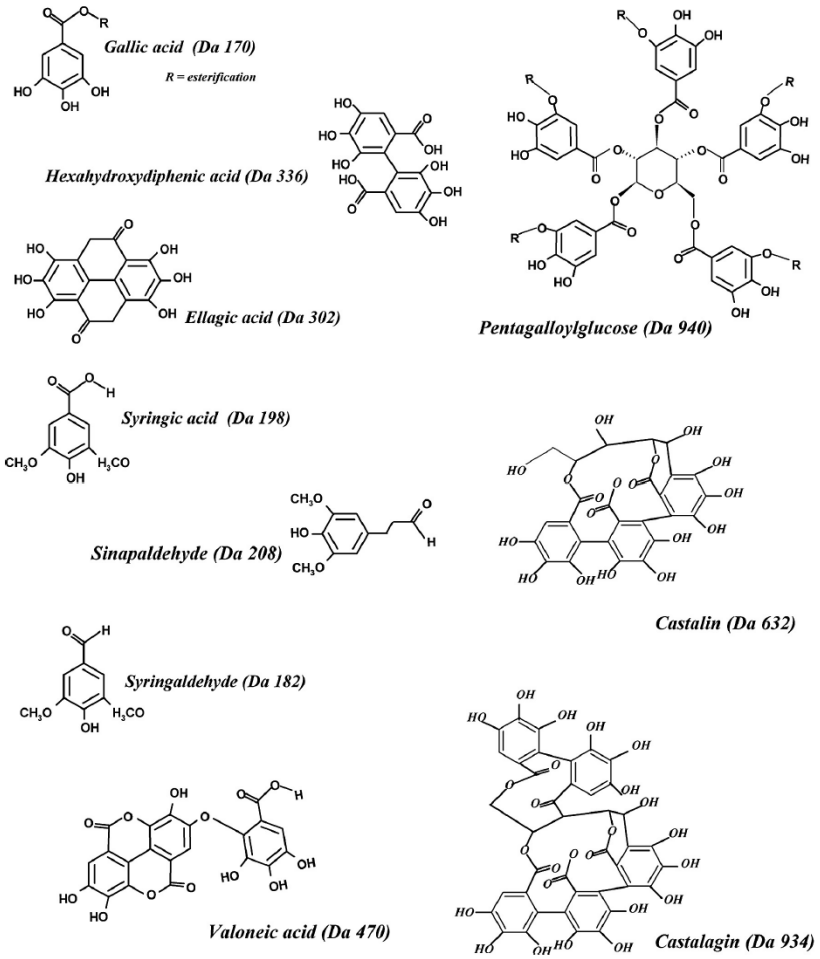

Fig. 1. Molecular structure of selected compounds identified in MALDI-TOF spectroscopy.

& Giovando, 2014). Purified water (18-Ohm), acetone (HPLC grade, 99.98%), 2,5-dihydroxybenzoic acid (99%) and phosphorus red reference standard (99.999%) for MALDI-TOF analyses were purchased by Acros (Acros Organics, NJ, US).

3. Results and discussion

3.1. ATR-FTIR spectroscopy

The ATR-FTIR spectrum of chestnut tannin, obtained using the average of 128 scans per samples, revealed the presence of several functional groups that are typical of hydrolysable tannins, mostly located in the middle-infrared spectral range (Table 1). Five bands were considered diagnostic of chestnut tannin, including 1600,

Table 1
ATR-FTIR vibrational peaks attribution in the middle-infrared range (4000–760 cm^{-1}) for the characterisation of commercial oenological tannin from *Castanea sativa Mill.* wood.

Peak (cm^{-1})	Attribution
3700–2900	OH stretching; aromatic CH stretching
2972; 2934	Aliphatic CH_3; CH_2 stretching
1736	Phenyl ester linkage vibration
1718	Carbonyl C=O stretching
1600	Aromatic C=C *sym* stretching
1509	Ring in-plane bending
1447	Aromatic C=C *antisym* stretching
1309	Saturated C–C chains stretching
1197	Aliphatic C–O *sym* stretching
1032	Aliphatic C–O *antisym* stretching
780	Phenolic OH wagging; aromatic out-of-plane bending
762	Glycosides ring breathing

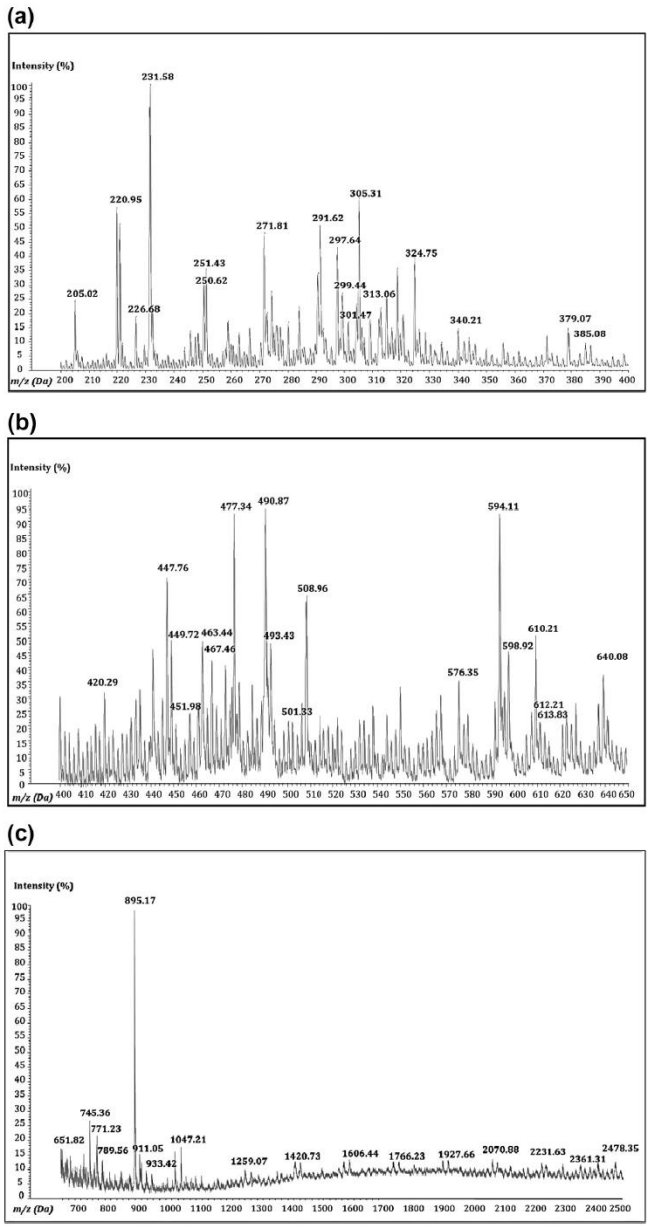

Fig. 2. MALDI-TOF spectra recorded in linear positive mode. (a) range 200–400 Da, ion gate off; (b) range 400–650 Da, ion gate off; (c) range 650–2500 Da, ion gate 400 Da. The most significant peaks are highlighted in the figures; a detailed description is provided in Table 2.

Table 2
MALDI-TOF fragmentation patterns and peak attribution for the characterisation of commercial oenological tannin from *Castanea sativa Mill.* wood.

Calculated m/z (Da)	Experimental m/z (Da)	Description
117 (94 + 23)	110; 114	Sodium adduct of phenolic rings
126 (170 − 44)	127	Protonated gallic acid fragment with loss in –COO function
132 (180 − 48)	131	Glucose_cleavage mechanism aromatic ring fragment
143 (180 − 60 + 23)	145	Sodium adduct of glucose fragment
152	151	Arabitol; deprotonated
154 (198 − 44)	155	Syringic acid fragment with loss in –COO function
167 (182 − 15)	166	Syringaldehyde fragment with loss in $–CH_3$ function and H proton
170	169	Gallic acid
a. 208; b. 205 (182 + 23); c. 207 (184 + 23)	205	a. Sinapaldehyde; b. protonated syringaldehyde; c. cationised methyl gallate
221 (198 + 23)	220	Sodium adduct of syringic acid
228 (180 + 48)	226	Glucose sugar + glucose residue
231 (208 + 23)	231	Sodium adduct of sinapaldehyde
	250; 251	Not assigned
268 (302 − 34)	271	Quercetin with loss in 2 –OH functions; protonated
291 (268 + 23)	291	Sodium adduct of quercetin with loss in 2 –OH functions
302	297; 299; 301	Ellagic acid; deprotonated
306	305	Gallocatechin
314 (788 − (158 × 3)	313	Tetragalloyl glucose fragment (with loss in three galloyl moieties)
328 (305 + 23)	324	Sodium adduct of ellagic acid with loss in protons
338	340	Protonated Hexahydroxydiphenic acid
376 ((158 × 2) + 60)	379	Gallic acid dimer + sugar fragment
390	385	Stilbenoid glucoside (piceid-like structure)
420 (448 − 28)	420	Ellagic acid deoxyhexose with loss in –CO function; deprotonated
448	447	Ellagic acid deoxyhexose
a. 449((470 − 44) + 23); b. 448	449	a. Sodium adduct of valoneic acid dilactone with loss in –COO) function; b. Protonated ellagic acid deoxyhexose
458	451	Possible flavogallonic acid fragment released after hydrolysis of ester linkages of ellagitannins
464	463	Ellagic acid hexoside or quercetin hexoside
466 (636 − 170)	467	Trigalloyl glucose fragment, protonated
493 (470 + 23)	490; 493	Sodium adduct of valoneic acid dilactone
a. 502; b. (636 − 158)	501	a. Nonahydroxytriphenoic acid_possibly released after hydrolysis of ester linkages; b. Trigalloylglucose with loss in galloyl function
507 ((636 − 152) + 23)	508	Sodium adduct of trigalloyl glucose fragment
552 (586 − 34)	550	Ellagic acid galloyl pentose conjugated with loss in two –OH moieties; deprotonated
576 (301 + 120 + 155)	576	Galloyl ellagic residue with stripped sugar fragment
586	584	Ellagic acid galloyl pentose conjugated; deprotonated
598 (632 − 34)	594	Castalin / vescalin with loss in two –OH moieties; deprotonated
598 (632 − 34)	598	Castalin / vescalin with loss in two –OH moieties
614 (934 − 301 − 18)	610; 612; 613	Castalin, vescalin fragments (castalagin mass 934 – ellagic – H_2O)
635 ((940 − 170 − 158)+23)	640	Sodium adduct of a pentagalloylglucose fragment with loss in gallic acid and a galloyl moiety
650	651	Protonated Galloyl-HHDP-glucoside (lagerstannin C)
750 (784 − 34)	745	Pedunculagin with loss in 2 –OH functions; deprotonated.
771 (784 − 13)	771	Pedunculagin with loss in –CH function
788	789	Protonated tetragalloyl glucose
895 (940 − 45)	895	Pentagalloyl glucose with loss in –COO function
915 (933 − 18)	911	Castalagin/vescalagin with loss in a water molecule; deprotonated
934	933	Castalagin/vescalagin; deprotonated
1047 (1065 − 18)	1047	Roburin E/Grandinin with loss in a water molecule
1261 (934 + 169 + 158)	1258	Tentatively attributed as castalagin linked with an additional gallic acid unit and esterified with a galloyl function; deprotonated
1422 (934 + 484)	1420	Castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid fragment through an ester bond
1612 (1422 + 190)	1606; 1612	Castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid fragment through an ester bond and an additional hexose sugar
1770 (1612 + 158)	1766	Castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid fragment through an ester bond, an additional hexose sugar and a galloyl function
1928 (1612 + (2 × 158))	1927	Castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid fragment through an ester bond, an additional hexose sugar and two galloyl functions
2069 (1612 + (3 × 158) − 17)	2070	Castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid fragment through an ester bond, an additional hexose sugar and three galloyl functions. Loss in –OH; protonated
2230 (2069 + 161)	2231	Castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid fragment through an ester bond, an additional hexose sugar, further sugar moiety and three galloyl functions. Loss in –OH; protonated
2361 (2230 + 120 − 12)	2361	Sodium adduct of castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid fragment through an ester bond, an additional hexose sugar, further sugar moiety and three galloyl functions. Loss in –OH; loss in carbon; protonated. An additional glucose fragment is obtained by glucose cleavage mechanism
2481 (2361 + 120)	2478	Sodium adduct of castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid fragment through an ester bond, an additional hexose sugar, further sugar moiety and three galloyl functions. Loss in –OH; loss in carbon; protonated. Two additional glucose fragments are obtained by glucose cleavage mechanism

1509, 1447, 1197 and 1032 cm^{-1}. The 1600 and 1447 cm^{-1} peaks were due to symmetric and antisymmetric stretching of C=C groups in the aromatic rings, respectively. The 1509 cm^{-1} medium to weak band was assigned to phenolic ring in-plane deformation

vibrations, as well as the 1077 cm^{-1} shoulder feature associated with C–H in-plane aromatic bending (Fernández & Agosin, 2007). The 1197 and 1032 cm^{-1} peaks were due to the symmetric and antisymmetric stretching vibrations of aliphatic C–O bonds, occurring in gallic and ellagic-based compounds (Ricci, Olejar, Parpinello, Kilmartin, & Versari, 2015).

3.2. MALDI-TOF spectroscopy

The MALDI spectra of the tannin showed the presence of monomeric fraction, composed of phenolic acids, aldehyde derivatives and sugar fragments (Fig. 1) that were likely formed by lignin degradation during the extraction process (Puech, 1981). Two main patterns were detected in the spectral profiles (Fig. 2a–c; Table 2); the first series was related to ellagic and valoneic acid-based tannin monomers; while the second pattern was predominant and composed of polygalloylglucose, i.e. gallic acid oligomers that often showed fragments of further sugar molecules. The presence of a consistent number of gallic acid and its derived structures is characteristic of chestnut tannins and accounts for its astringency and bitterness and for the protection against wine oxidation (Alañón et al., 2011).

Polygallic compounds in MALDI-TOF analysis showed typical mass increments around 152 Da, which coincide with galloyl repeat units, or the 161–163 Da mass increment related to glucose fragments contained in the esters. In this view, the series 1420-1612-1766-1927-2070-2231-2361-2478 Da was representative of castalagin esterified with polygalloylglucose structures. The MALDI-TOF technique also revealed the presence of glucose and other carbohydrates (Table 2), naturally present in the tannin as a result of the hydrolysis of ester linkages, that in winemaking could affect the solubility of tannin–protein complexes over a certain concentration (Harbertson, Yuan, Mireles, Hanlin, & Downey, 2013). The peak at 151 Da, tentatively attributed to arabitol, can be considered a marker for the chestnut tannin.

4. Conclusions

The combined use of ATR-FTIR and MALDI-TOF spectroscopies was an effective tools to analyse the phytochemical composition of commercial chestnut tannin used in winemaking, which fingerprint is based on the occurrence of phenolic and sugar monomers and oligomerics. Due to the elucidation of the occurrence of galloylation and glycosylation patterns that influence the chemical and technological properties during winemaking, this approach is recommended and will be further implemented to provide a tailored prevision of the impact of using exogenous tannins in wine.

Conflict of interest

On behalf of all Authors I declare that there is no conflict of interest.

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Chapter 7

General discussion and conclusions

Chapter 7: General discussion and conclusions

7.1 Brief overview

In this 3 year PhD study, large amounts of data on chemical and sensory composition, oxygen intake and antioxidant activity were collected in a selection of international wine varietals, both bottled and stored in cellar prior packaging, in the perspective of studying wine oxidation and the main factors enhancing or limiting its occurrence. Oenological tannins commercially available and currently used as exogenous additives in wines were also studied in view of their potential use to limit the use of sulfur dioxide, which was demonstrated to be potentially harmful for human's health.

The factors mainly affecting the occurrence of wine oxidation were determined through bibliographic review and ad-hoc experimental design, which provided supply chain and post-bottling management. This approach would be informative for wine producers, enabling them to plan suitable production and bottling strategies to maximize the shelf-life of their product. The study aimed to provide new insights into using the device currently available to monitor quality parameters involved in the potential development of oxidation in wine, to optimize their use and to introduce new approaches to maximize the information simplifying the analytical procedures.

The application of statistical design of experiment which allow a fast and reliable monitoring of the supply chain was accomplished by detailed insight on the analysis of wine components, to highlight the correlation between wine and oenological tannins composition and antioxidant/prooxidant activities. The main focus was on the phenolic fraction, which was characterised in terms of composition in monomers and polymers, and antioxidant capacity (reducing and redox power, radical scavenging activity). Transition metals involved in wine oxidation (Fe, Cu) and oenological treatments (K) were also quantified in relationship with parameters like oxygen and free sulfur dioxide consumption.

The multiple analytical approach was intended to cover the main aspects responsible to detrimental oxidative modifications in wine, to elucidate the complex problem of oxidation in wine and to provide practical information to maximize the shelf-life durability.

7.2 General discussion of findings according to original objectives

7.2.1 Objective I: Monitoring of the oxygen intake along the supply chain

Even though relatively large differences exist between production processes in cellars, the importance of oxygen management in determining the sensitivity of a wine to incorporate an excess of gas was highlighted in this research. Oxygen accumulated along the supply chain constitute a potential source of oxidative stress in bottled wines.

It was also highlighted the importance to use the most appropriate analytical tools and to collaborate with the cellar staff, in order to define an experimental plan *a priori*, maximizing the information and including the occasional contingencies that characterize the production processes.

The transfer of wine between storage tanks and the cold stabilisation showed to be the most critical points regarding an excess oxygen supply. On the other hand, when made aware of DO variations during production the winemaker can intervene by planning the addition of protective, which can neutralize this excess bringing the dissolved oxygen concentration at optimal levels and keeping constant the other oenological parameters.

The proposed experimental design also enabled the individuation of occasional oxygen sources (lack in inert gases streams, malfunctioning of pumps or delivery equipment).

In general, the fluorescent dot coupled with optical fibres offered the possibility of measuring the dissolved oxygen concentration with higher accuracy, thus limiting the atmospheric interferences which occasionally affected the electrochemical device in this experiment. Dots are generally inserted in specula at the basis of tanks, and they are poorly affected by the liquid/air interface effects. However, the results provided by the two equipment in preliminary experiments are aligned and therefore the choice of the most suitable must be evaluated in the specific case, on the basis of the structure of the cellar and of the position of the sampling points.

To conclude, results were promising for this preliminary trial, and general guidelines were proposed for an efficient monitoring, to be optimized in specific production contexts.

7.2.2 Objective II: Evaluate the impact of different packaging and closures in the shelf-life of wines

Shelf-life experiments in this study reproduced the use of difference packaging currently available to store wines; white wines, which are more likely to suffer

oxidative damages, were studied according with their physico-chemical and sensory evolution during an hypothetic commercial storage period. The requirements for a proper wine conservation were dependent on a complex number of factors, which can be grouped in technological, environmental, and compositional.

Technological factors included the O_2 permeation/migration during the storage period, induced by different packaging and closures. The electrochemical oxygen meter enabled to effectively monitor the dissolved oxygen consumption in bottled/packed wines, thanks to the specific design of the sampling chamber. Time-course of DO consumption was plotted as a logarithmic curve in all cases. The importance of oxygen supplied during the bottling process was also highlighted, setting the time zero of the experiment in this phase of the production chain.

Environmental factors, including temperature fluctuations, light exposure, humidity variations, were neglected assuming constant storage conditions: bottles upright in cardboard boxes, avoiding from light, at room temperature.

Compositional factors included all oenological parameters routinely analysed to assess the wine quality, according with the *International Organization of Vine and Wine* (OIV). The evolution of free sulfur dioxide and optical density at 420 nm (browning onset) in time, coupled with the DO consumption, showed to be the most informative parameters to monitor the occurrence of oxidation in wine.

Moreover, the measurement of the optical density at 420 nm in the real time monitoring and in accelerated aging tests have allowed a description of the thermodynamic and kinetic parameters of the browning phenomenon, which are informative on the susceptibility of wine to oxidation and therefore the most appropriate storage conditions.

The experiment was considered informative on the impact of packaging and closures in wine storage, although a further implementation is needed; considering the variability induced when combining all factors and when adding the further "wine varietal" variable, the experiment could be modelled to provide generalizable results only using a significant sampling.

7.2.3 Objective III: Determine the structure-activity relationship between polyphenols occurring in wine and their antioxidant capacity. Objective

IV: Proposal of innovative analytical strategies to determine TPC and AA parameters in wine and oenological products

Several methods were proposed to derive wine composition, taking into account the phenolic fraction (antioxidants) and the content in Cu and Fe metal ions (pro-oxidant).

The approach was therefore an attempt to define not only the Total Polyhenol Content (TPC), which is the parameter currently monitored for a fast wine quality assessment, but also to produce a more detailed information on the simple phenolic fraction (flavonoids, benzoic acids, anthocyanins); the aim was to demonstrate that every class of compound contribute to the antioxidant activity in a different extent. Experiments were performed assuming different winemaking conditions (skin contact, use of selected yeast strains for fermentation), and measuring total and individual phenolics. Qualitative and quantitative determination of the phenolic fractions were coupled with the measurement of the antioxidant activity (AA) in terms of radical scavenging ability (DPPH method) showing a generally good correlation. Simple phenolic fractions showed to contribute to AA in different extent, and this finding would be informative for the optimization of the extraction processes during vinification.

Based on previous work where fast analytical methods were developed or improved to measure the TPC and AA parameters in wine, a further experiment was set up to develop an innovative device based on a tandem diode-array-electrochemical detectors system. Oenological tannins were also added to the sample dataset, finding a good correlation between electrochemical response and DPPH radical scavenging; this property has been further exploited using cyclic voltammetry to characterize the phytochemistry and antioxidant activity of botanical extracts used in oenology.

The development of fast, reliable and easy to use analytical approached would allow to monitor the evolution of the phenolic fraction in the various phases of the supply chain and in bottled wines thus improving the effectiveness of monitoring; preliminary results presented in this dissertation would be further implemented in future experiments.

7.2.4 Objective V: Highlight the potentiality of oenological additives (tannin) to assist the sulphur dioxide action thanks to their expected antioxidant properties.

Spectroscopic methods, HPLC-ECD and CV provided a detailed information on botanical extracts commercially available to be added to wine. Extracts were

characterised by different degrees of purity and different phenolic monomers/polymers ratio, but they were generally recognized as effective antioxidant agents through the multiple analytical approach. Interesting correlation were found between analytical methods, and in particular CV was particularly suitable to qualitative and quantitative characterization of the extracts. Tannins were dissolved in model wine solution to reproduce the oenological conditions; this preliminary approach would be further implemented planning tannin additions in wines, and evaluating their synergistic action with wine polyphenols.

Since the composition of commercial products is only partially declared by suppliers and due to the high sensory impact that the addition of exogenous tannins would provide to wine, the study also highlighted the importance of the commercial tannin compositional profiling to avoid improper uses and possible adulterations. Results of spectroscopy studies (ATR-FTIR, MALDI-TOF) on the compositional characterisation of commercial tannins are presented in *Addendum*, in the form of published research papers.

7.3 Major findings: limitations, novelty value – implications. Future perspectives.

All results were presented in separate sections as an illustrative examples of how to program the experiment on the basis of different requirements and conditions. Obviously, taking into account the great variability of winemaking conditions, the extension of current finding to different context should reconsider the great complexity of wine oxidation under tailored condition.

The monitoring experiment along the supply chain allowed to provide assistance to producers in the specific winemaking process, and to increase knowledge on the production processes and on their influence in the quality of wine.

To effectively monitor the shelf-life of wine an increasing number of parameters would be added to the experimental design (i.e., simple phenolic fraction, reducing activity, redox activity), and the number of sampling would be implemented. More emphasis should be given to the sensory analysis, especially in comparative experiments that provide the use of different packaging solutions for the same wine.

The study of the technological performances of materials used for packaging would enable their tailored use. In this dissertation, gas permeation was indirectly evaluated

while measuring the DO content in wines; an experiment based on the transmission rates of oxygen through closures would provide additional information and implement the information available in literature (see **section 2.5.1- .2**).

The development of devices for fast analysis of chemical parameters of oenological interest would be improved designing portable devices to perform on-line analyses. To conclude, although experiments have demonstrated the effectiveness of oenological tannins as antioxidants to be added in wine, the potential impact that their use could induce in consumer's expectation impose the transition from model solutions to real wines and full consumers study. The assistance of a trained panel would correlate compositional data with sensory responses, enabling the definition of standard addition levels.

7.4 Final remarks

While studying the oxidation of wine on the basis on the main mechanisms and factors involved, an extensive in-depth study of the molecular 3D structures produced during oxidation is still needed. As the aim of the thesis was to prevent the occurrence of oxidative phenomena, a better understanding of the oxidation by-products in the wine it would be desirable to support the evaluation of unexpected color variation and the occurrence of undesirable aroma at different sensory levels.

With the availability and competitive cost of innovative packaging materials a further strategy would be the use of material with enhanced technological properties, able to limit the oxygen inlet (barrier packaging) or scavenge the ROS species inducing the oxidative damage (antioxidant package). Glass bottles are considered inert materials, and a wide selection of closures is currently available. The cardboard-based packaging (Tetra pack, Bag-in-Box) and in particular the inner plastic layers still remain the most sensitive to oxygen permeation; several experimental solutions were presented so far, and some of them are listed in **section 2.5**. A further experiment has been performed to produce a plastic film with enhanced antioxidant properties, which is described in detail in Addendum A (original research paper).

The Author of this PhD thesis partially contributed to and expects a further development of technologies to improve active packaging and systematic studies on their application; this would support the wine industry in using economic efficient and

environmental friendly solution to protect their wine increasing consumer's confidence. The most important remark is that such technologies must be economically competitive, to allow their widespread use beyond the research purposes.