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PROPOSAL FOR A NEW MODEL FOR EVALUATION OF WINE OXIDATION

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Declaration

I hereby declare, by submitting this thesis, that all the contents therein are authored by myself, in my own and original work, save to the extent explicitly otherwise stated. I declare that the work described here have not previously in its entirely or partially submitted it for obtaining any qualification.

Cesena, 23 March, 2017

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Preface

This Ph.D. thesis challenges the wine oxidation phenomenon through the study of selected parameters directly or indirectly related to its occurrence. It is organized in five chapters which overview aim is described along with a focus on the issue. Before the presentation of the scientific study performed during these three years, a preliminary literature review is presented regarding the topics of investigation. The format and writing are in accordance with the ABNT (*Associação Brasileira de Normas Técnicas* / Brazilian Association of Technical Standards).

In each chapter, the overview regarding the topic is followed by the main findings of research results along with discussion, surveyed by a conclusion of them.

NOMECLATURE

Abbreviations used:

mha: millions of hectares
kha: thousands of hectares
khl: thousands of hectolitres
mhl: millions of hectolitres
bn EUR: billion euros
vol: volume
BDN: biodynamic
BIO: organic
SPO: spontaneous fermentation
LSA: fermentation through selectes yeasts
DPPH: 2,2-diphenyl-1-picrylhydrazyl radical

I. INTRODUCTION

a) GRAPE AND WINE PRODUCTION WORLDWIDE

Grape production worldwide has as a total area, reached 7.5 mha in 2015 whereas European vineyards have slightly diminished, while in the other hand, in Asia and South America, plantations have slightly increased. In 2014, China became the country with the second biggest vineyard surface area (almost 800 kha), following Spain (AURAND, 2016).

World wine production in 2015 is estimated at 275.7 million of hectoliters (mhl), presenting an increase of 2% when comparison to the previous year, while in 2014, the world area under vines rose to 7573 kha.

In 2015 Italy has become the biggest wine producer in the world, by the production of 48.9 mhl. France follows Italy with the production of 47.4 mhl of wine production, which is followed by Spain (36.6 mhl) (AURAND, 2016).

In north America, United States achieved 22.1 mhl, while in South America, Argentina's production declined (13.4 mhl) while Chile (12.87 mhl) increased its production and Brazil stayed stable (2.7 mhl) increased its production. In Oceania, the production has been stable for almost three years, with the Production of 12 mhl liters of wine by Australia and 2.4 mhl by New Zeland. In African continent, South Africa has maintained its 2014 level at 11.3 mhl (AURAND, 2015).

b) INTERNATIONAL WINE & TRANSPORTATION

In America about 23% of shipments are in temperature-controlled containers, whereas in South Africa research indicates that the figure may be as low as 2%. Among this 274.4 mhl produced worldwide, 38% are transported, shipped and/or exported worldwide. The last data available regarding the wine global market states that wine commerce have a movement of 28.3 billion euro worldwide (AURAND, 2016). These makes it important knowing the circumstances and parameters involved to ensure its security regarding the final quality, and to ensure the wine which left the winery is the same which arrives the final consumer.

c) SANGIOVESE

Sangiovese's Origin

Despite the historical and economic importance of wine in Europe, little is known about the origin of the different grape cultivars (*Vitis vinifera*). The origin of Sangiovese wine is still discussed nowadays because since the beginning of the 19th century, 'Sangiovese' was mentioned several times in the literature with different nominations: Prugnolo (Toscana), Sangiovese piccolo, Brunello (Toscana), Prugnolo gentile, Sangiovese grosso, Uvetta, San Zoveto, Nielluccio (Corse, FR), Sangioveto, Sangiogheto, Sangioveto montanino, Morellino (Scansano, GR). It occurs even nowadays, when we find as an official synonym (reported in the Italian National Catalogue) the nomination Sangioveto (BOSELLI, 2001; BREVIGLIERI AND CASINI, 1964, SCALABRELLI et al., 2003).

Through time, 'Sangiovese' has been always considered as a good-quality cultivar for wine production. Its first source of information is dated from XVI century, when the document "*Delle coltivazioni delle viti e del frutto che se ne può ricavare*" of G. Soderini, published at Firenze in 1590, names the "Sangiogheto", defining it as "juicy and full of wine" (*sugoso e pienissimo di vino*). The first notarial deed which states the Sangiovese's cultivation dates from 1672, in the territory of Casola Valsenio (Province of Ravenna, Italy) (SANGIORGIO, ZINZANI, 2014).

However, it is believed that Sangiovese's grapes were cultivated 2000 years ago, by the etruscans (MAINARDI, 2001), in the north zone or Tevere river (**Figure 1**), which was then diffused at the Apennines, until Toscana and Romagna (CATTAROSSI, 2014).

Documents from several authors dated before 17th century mention characteristics of several denominations of places, which refers to Sangiovese, which contributes to the attempt of mapping its origin and dissemination.

Finally, at 1834, within the work of Giorgio Gallesio, *Pomona Italiana* (1817-1839), which includes descriptions and plates of about 150 fruit cultivars, an entire chapter was dedicated to Sangiovese's grapes, where it is described in details (MARINONI et al., 2009).

Figure 1. *Sangiovese's grapes were cultivated 2000 years ago, in the north of Tevere river*

Since then, with the development of new technologies, Sangiovese has been studied deeply and with some genetic studies and new achievements, there are more clearance on information found. Sangiovese is also considered of great economical value worldwide, especially in Italy, where it occupies 10.8% of total vines areas with 70.289 ha, and it constitutes the base of several internationally known denomination of origin wines (DOC and DOCG), as for example the “Chianti Classico” DOCG and “Chianti” DOCG, where it is presented within their composition from 75% to 100% of these wines, or in Brunello di Montalcino and “Vino Nobile di Montepulciano” DOCG, where Sangiovese is presented as composition of 70% to 80% of these wines (AIES, 2012).

Sangiovese vine is known by a by great genetic and morphological intracultivar heterogeneity. Researchers have described six different biotypes based on fruit, cluster, leaf, ripening and must characteristics (CALO et al., 1995). Within this research, two biotypes were individuated from central Tuscany, one from the Tuscan coast near Pisa (Peccioli di Pisa), one from the Emilia – Romagna near Predappio (Romagnolo), one cultivated along the Adriatic Sea coast (Marchigiano) and one from Corsica (Nielluccio).

Sangiovese Vine

The vine has a shoot top expanded or semi expanded, arachnoid, shiny green. Leaves have medium size; they are pentagonal with three or five lobes. Bunch of magnitude from medium-small to large, conical-pyramidal with one or two wings, relatively dense. Berry of medium size, sub-rounded, occasionally almost elliptical, regular in shape, purplish-black in color, not very thick. The budding and the flowering are medium. Sangiovese vine prefers hill areas, with soils with medium or low fertility, clay – calcareous soils also rich in gravels. The vine is sensible to *Oidium tuckeri, Stereum hirsutum* and mites, whereas it is medium sensible to *Plasmopora vitícola* (CALÒ et al., 2001).

Sangiovese Wine

Concerning the Sangiovese cultivar, there are more than two hundred DOC and DOCG wines which have this varietal in its composition, and among them (**in Appendix A**), are the famous traditional Tuscan wines, such as Chianti, Brunello di Montalcino and Nobile di Montepulciano:

One of the main characteristics distinguishing Sangiovese from other red wines is its delicate pigment profile.
According to Consorzio *Vini di Romagna*, Sangiovese wine has a characteristic color of purple ruby with purplish edges, and when compared to other two extreme *Vitis vinifera* varieties, it is practically a middle term between Syrah wine and Pinot Noir (**Figure 2**).

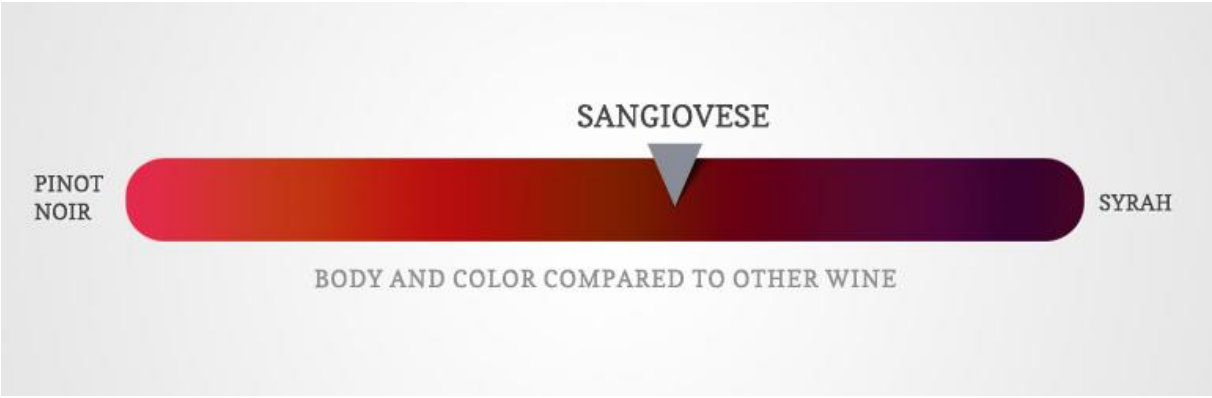


Figure 2. *Sangiovese wine color (Source: WineFolly).*

Sangiovese wines are also characterized by its tannic experience, as high acidity. Flavors and aromas of any particular Sangiovese wine can vary according to where

the grapes are grown and how the wine maker creates the wine, as it can be seen by the delicate floral strawberry aromas of Montefalco Rosso and the intensely dark and tannic wines of Brunello di Montalcino. Although it is prominently known to have odor of a delicate scent reminiscent of violet, as its general flavor is dry, harmonious, and slightly tannic with pleasantly bitter aftertaste. Some of other general characteristics of Sangiovese wine can be found in **Table 2**.

Table 2. *Sangiovese wine general characteristics.*

Characteristic	Note
Aroma	Fruit, strawberry, cherry, plum, floral, violet, nutty, fig
Flavor	Earthy, fruit, strawberry, cherry, plum, floral, oak, nutty
Character Profile	medium body, crisp acidity, dry, tannins
Acidity	High
Tannin	High

d) ORGANIC WINE PRODUCTION

Basically, organic Wine are derived from organic farming, which is a method that prohibits the use of additives or alterations to the natural seed, plant, or animal including, but not limited to: pesticides, chemicals, or genetic modification in its farming system.

It is important to notice the differences between organic production of grapes and organic wine. In Europe, organic wine is provided by grapes organically handled, i.e., it is allowed the addition of chemical substances in the wine; however, in United States, an organic wine is free of any chemicals, i.e., it is sulphite addition free. However, in US wine labelled "Made with organically grown grapes" may have sulphur dioxide.

e) BIODYNAMIC WINE PRODUCTION

Overview / Biodynamic Agriculture

Derived originally from ancient Greek, biodynamic literally means “like the power of life”. Biodynamic agriculture is somehow a branch of organic farming system, which focus the production in food quality and soil health, as it differs from organic farming by being associated with the spiritual science of anthroposophy founded by Rudolf Steiner (1861-1925), which initially emphasized the biodynamic agriculture not only in

farming practices, but in the balance between the physical and higher, non-physical realms, to acknowledge the influence of cosmic and to enrich the farm its products and its inhabitants with life energy (DIVER, 1999).

As mentioned, Biodynamic agriculture was first mentioned by the Austrian scientist and philosopher Rudolf Steiner, in the last year before his death, in 1924, when challenges regarding the direction and practice of contemporary agriculture first started to appear by the proliferation of chemical agriculture. Steiner proposed alternatives to the chemical agriculture, one in which was based in 'heal the Earth' in the agriculture course to farmers in Breslau, in which was based in eight lectures in the current city of Kobierzyce, Poland, in the year of 1924 (PAULL, 2011).

Steiner therefore articulated the first steps to the process in which developed to the publication of Biodynamic Farming and Gardening, wrote by Ehrenfried Pfeiffer in 1938. One of the basic ecological principles of biodynamic agriculture is to perceive the farm as an organism, a self-sufficient organization, as it has its own individuality. Emphasis is given to integration of crops and livestock, recycling nutrients, maintenance of soil and the health and wellbeing of crops and animals, as the farmer is also part of this mentioned organization (DIVER, 1999).

According to Steiner there are nine types of preparations for fertilizers that are allowed to use in Biodynamic agriculture, which are numbered from 500 through 508 (**Table 3**). Farmers in which apply Biodynamic agriculture in their field use these mineral, plant, animal manure extracted preparations to liven up the soil and stimulate plant growth in small quantities (CARPENTER-BOGGS, 1997).

Table 3. *Biodynamic compost preparations.*

NUMBER	PREPARATION
500	Horn-manure
501	Horn-silica
502	Yarrow blossoms (*Achillea millefolium*)
503	Chamomile blossoms (*Chamimilla officinalis*)
504	Stinging nettle (whole plant in full bloom) (*Urtica dioca*)
505	Oak bark (*Quercus robur*)
506	Dandelion flowers (*Taraxacum officinale*)
507	Valerian flowers (*Valeriana officinalis*)

508	Prepared from silica-rich horsetail plant (*Equisetum arvense*)

The preparation number 500 is a horn-manure made of cow manure, fermented in a cow horn that is buried in the soil for six months through autumn and winter and is used as soil spray to stimulate root growth and humus formation. The number 501 is a horn silica made from powdered quartz (packed inside a cow horn and buried in the soil for six months through spring and summer) and applied as foliar spray to stimulate and regulate growth (CARPENTER-BOGGS, 1997; DIVER, 1999).

Those preparations from number 502 to 507 are used to make compost. The number 508 is used as foliar spray to suppress fungal diseases in plants. (CARPENTER-BOGGS, 1997; DIVER, 1999).

Besides the above-mentioned preparations, planetary influence also plays a key role in the timing of biodynamic practices. Recognition of celestial influences on plant growth are part of biodynamic awareness that subtle energy forces affect biological systems. According to Diver (1999) there are still diverging points of view regarding which lunar, planetary and stellar influences should be followed.

According to Demeter International (2014), Italy is the second biggest producer in the world regarding Biodynamic Agriculture, with 9.003 hectares and 325 agricultural enterprises and 20 distributors. The first one is Germany with 68.193 ha and 1.431 companies and the third bigger producer of biodynamic agriculture is France (8.500 ha and 420 companies). Therefore, there is an increase in this Biodynamic reality, considering that in 2010 there were only 209 biodynamic companies in Italy.

Biodynamic wine

The biodynamic wine is obviously composed by biodynamic grapes, in which have the vine grown based on biodiversity and chemicals free. During the vinification, the fermentation comes spontaneously according to the protocol as it is not common the control of the temperature during it. The most is transformed by indigenous yeast presented in the grapes, which are specific and diverse from vine to vine. Therefore, it is usually forbidden the use of selected yeast manufactured industrially, as it is not allowed: acidification / de-acidification, sweetening, concentration methods such as reverse osmosis or freezing (JOLY, 2008).

Moreover, no sterile filtration is allowed (filters below 2 microns); the use of sulfur dioxide is a crucial issue: some manufacturers use it in small quantities, under adverse

conditions for winemaking, to manage the process; others, however, exert the absence of sulfites, which is more a convention and a matter of principle, a hallmark that distinguishes biodynamic production from the conventional.

f) WINE CONSTITUENTS

Wine is a hydrochloric beverage, derived from *Vitis vinifera* pressing and fermented by yeasts. It is constituted of water (80% to 85%) and alcohols (in which ethanol is the one present in major quantity), and several other constituents in minor quantity (JACKSON, 2008). Within these constituents presented in minor quantity, there are organic acids, sugars, phenolic compounds, enzymes, vitamins, lipids, inorganic anions and cations and innumerous volatile compounds.

The most important organic acids presented in wine are: tartaric acid, malic acid, citric acid and acetic acid, and their measurements serve as part of quality control during winemaking or storage quality control. It is due to the fact that, for example, tartaric acid and its salts give rise to wine total and the titratable acidity whereas acetic acid is mostly responsible for wine's volatile acidity (AMERINE & OUGH, 1974).

Moreover, organic acids and phenolic compounds are intrinsically related to the wine quality. It is due to their contribution to wine's astringency, colour and antioxidant activity (DE BEER et al., 2004).

g) PHENOLIC COMPOUNDS IN WINE

Phenolic compounds are responsible for wine's antioxidant activity, color and bitter/astringency characteristics. The protection against wine oxidation characterizes an essential feature to preserve its initial characteristic during the winemaking and to rise the shelf-life.

Phenolic compounds have their structures derived from a hydroxy-substituted benzene ring (**Figure 3**).

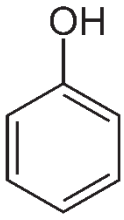


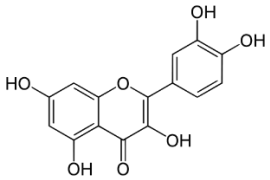
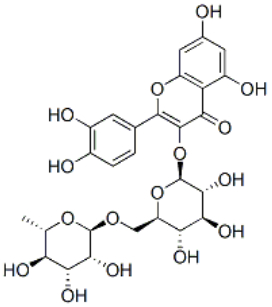
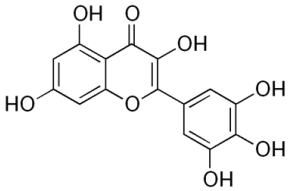
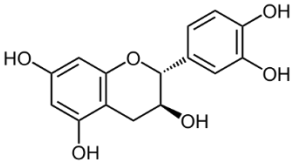
Figure 3. *Phenolic basic structure.*

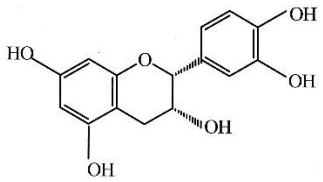
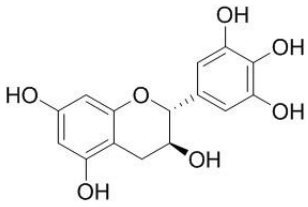
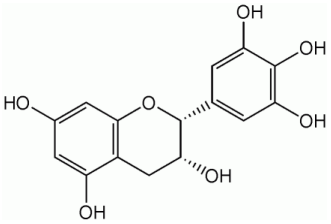
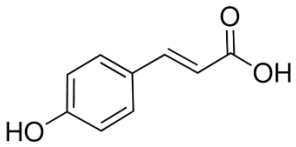
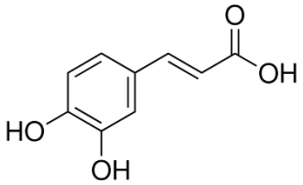
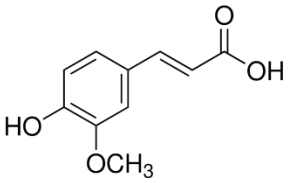
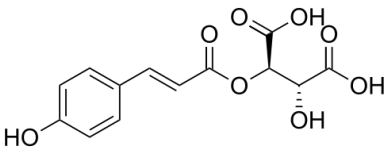
Derived from this basic structure (Figure 3), there are several phenolic compounds in which have being studied and identified in grapes and wine, as their origin by grape

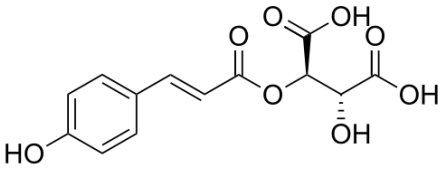
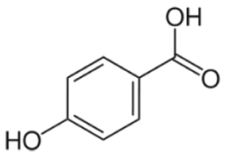
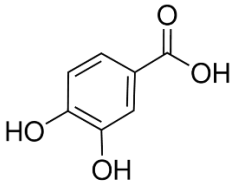
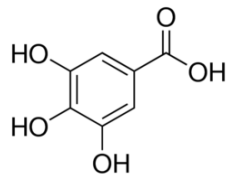
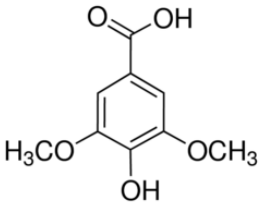
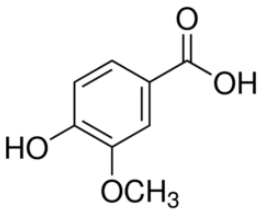
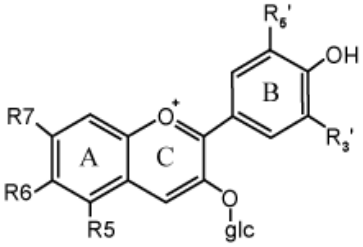
metabolism (HARBORNE 1967, HRAZDINA 1992, WINKEL-SHIRLEY 2001, DIXON et al. 2005).

Phenolic compounds can be divided in 2 groups, according to their chemical structure: flavonoids and non-flavonoids (**Table 4**). In the first group, there are the flavanols (catechin, epicatechin and epigalocatechin), flavonols (quercetin, etc) and anthocyanins, and in the second group, the phenolic acids, hydroxibenzoic and hidroxicinamic acids (CABRITA et al., 2003).

Table 4. *Chemical division and structure of the main flavonoid and and flavonoid compounds*

Chemical group	Compound	Chemical structure
FLAVONOID COMPUNDS		
Flavonoids	Quercetin	
	Rutin	
	Myricetin	
Flavan-3-ols	(+) - catechin	

	(-) - epicatechin	
	(+) - galocatechin	
	(-) - epigalocatechin	
NON FLAVONOID COMPOUNDS		
Hydroxycinnamic Acids	p-Coumaric Acid	
	Caffeic Acid	
	Ferulic Acid	
	Coutaric Acid	

	Caftaric Acid	
Hidroxybenzoic Acids	p-Hidroxybenzoic Acid	
	Protocatechuic Acid	
	Gallic Acid	
	Syringic Acid	
	Vanillic Acid	
Anthocyanins		

h) FLAVONOIDS

The phenolic group of flavonoids in grapes can be chemically divided in flavonols (catechin, epicatechin and epigalocatechin), flavan-3-ols (rutin and quercetin) and anthocyanins (**Table 4**).

According to Rice-Evans (1996), the position of methyl and hydroxyl radicals of B ring are directly linked to flavonoid compounds stability. Generally, when there is a dihidroxy structure in orto position in B ring, there is a higher antioxidant activity, since these compounds are efficient hydrogen donors. Still according to these Authors, the antioxidant activity of quercetin and miricetin are not related to the reductant power of the orto-dihidroxy groupment at B ring of these two compounds (**Table 4**), since it diminished its antioxidant activity in vitro. Moreover, studies in vitro have shown that flavonoids glycosylation also alters antioxidant activity of this compounds (RICE-EVANS et al., 1996; WANG et al., 1997).

The other phenolic group of flavonoids, flavan-3-ol group (o-dihidroxy substitution in B ring), have a high capacity on free radicals scavenging, in which it is believed that the degree of polymerization of these compounds also alters the antioxidant activity of these compounds, since studies have shown that monomers and dimers of flavan-3-ols were more efficient in preventing LDL oxidation than its trimers or tetramers (PLUMB et al., 1998).

i) ANTHOCYANINS

Anthocyanins are a complex chemical group which are responsible for red purple and blue colors in several fruits, vegetables, flowers. In grapes, these compounds are mainly present in the skin, occurring sometimes in the pulp.

Anthocyanins (**Figure 4**) are chemically composed by two parts: a glycoside bounded to an aglycon (anthocyanidin) and a sugar residue. Anthocyanins are formed when the basic anthocyanidins are coupled to sugars.

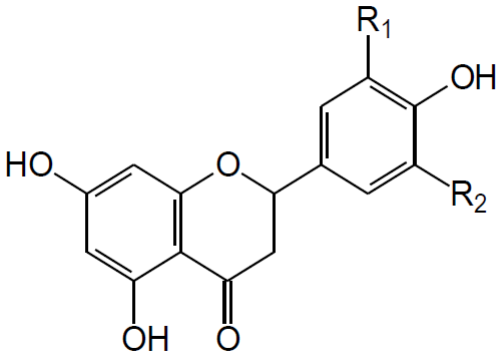


Figure 4. *Basic anthocyanidin chemical structure*.

Anthocyanins present in wines and identified in *Vitis vinifera* grapes are 3-O-monoglucosides and the 3-O-acylated monoglucosides of five main anthocyanidins – delphinidin, cyanidin, petunidin, peonidin and malvidin (**Figure 5**). What differentiate these five anthocyanidins are the position and number of -OH and (hydroxyl) and CH_3O- (methoxyl) groups attached to the B ring in the molecule (MONAGAS AND BARTOLOMÉ, 2009).

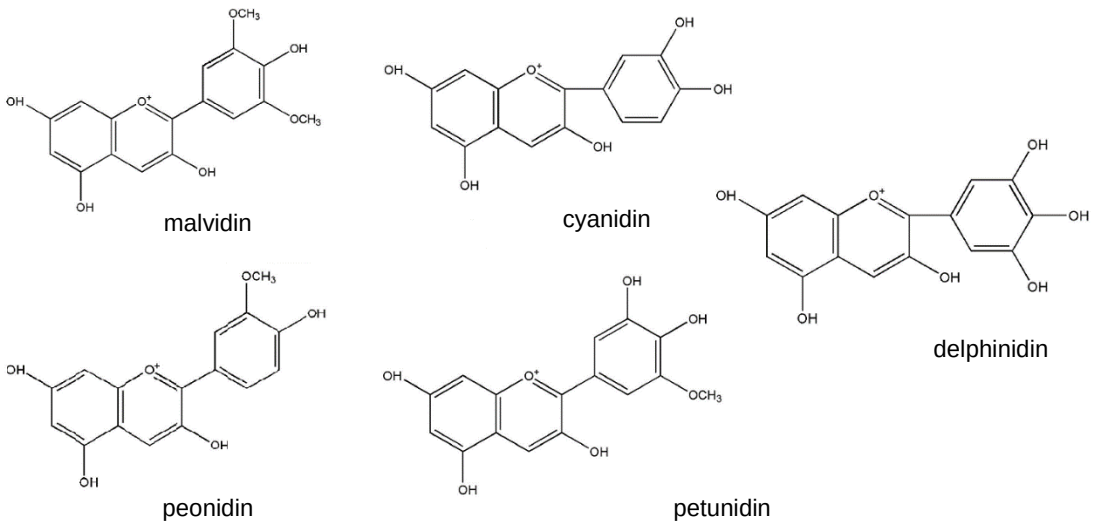


Figura 5. *Five anthocyanins which occur in wine and grapes*

In wine, anthocyanins are present partially as free molecules and partially associated with other phenolic compounds (BOULTON, 1996) and its content reaches a maximum primary in fermentation (NAGEL and WULF 1979). Anthocyanins have a great importance in wine as they play a role in its color and in its antioxidant activity. The free molecules of anthocyanins are more susceptible to oxidation, while the bounded

fraction is generally more stable, and it also plays an important role in the chromatic characteristics.

During wine maturation and aging, phenolic compounds are subjected to chemical reactions due to oxidation-reduction, condensation, polymerization and complexation with other wine compounds, specially to phenolic compounds, due to their reactivity (HASLAM, 1980; SOMERS and EVANS, 1986; RICARDO-DA-SILVA et al., 1991). Anthocyanins are highly reactive, since they are gradually transformed in oligomeric and polymeric pigments, which are more stable. Thus, they contribute to color transformation of wine over time (ROBICHAUD and NOBLE, 1990; MONAGAS et al., 2005; LOPES et al., 2006).

The concentration of anthocyanins, co-pigments, acetaldehyde and other yeast metabolites, as the pH, temperature, light, SO_2 and oxygen are factor in which alter the chemical reaction of wine over time (DALLAS et al., 1995; SOMERS and EVANS, 1986; ROMERO and BAKKER, 1999; ROMERO and BAKKER, 2000).

More information regarding the chemistry of anthocyanins in wine can be found in **Chapter 5** of the present thesis.

Researches indicate that grape cultivar affects the amount of total color and the co-pigmentation of wine (BOULTON et al., 1999; MAZZA et al., 1999; VERSARI et al., 2004; VERSARI, 2007).

One of the Sangiovese's particularities is its sensible anthocyanin profile and pigment (MANGANI et al., 2001; MATTIVI et al., 1990). Due to the importance of color in the evaluation of wine quality, researches focuses on protect and enhancing the color of Sangiovese wine (MANGANI et al., 2001; MATTIVI et al., 1990; BOSELLI et al., 2004; CASTELLARI et al., 2001; MATTIVI et al., 2006). According to some studies (MATTIVI et al., 1990; MATTIVI et al., 2006), Sangiovese grapes are poorer in anthocyanins when compared to other grape varieties, as it contains more quantity of unstable pigments: cyanidin 3-glucoside, delphinidin 3-glucoside, and petunidin 3-glucoside, which are dihydroxy pigments, i.e., those in which have the content diminished during winemaking, instead of methoxylated anthocyanins (peonidin 3- glucoside and malvidin 3-glucoside), which are more stable. Moreover, Sangiovese grapes are not rich in acylated anthocyanins (BALDI and ROMANI, 1992; MANGANI et al., 2011).

Versari et al. (2007) evaluated the anthocyanins content in 128 commercial wines, including Sangiovese, Cabernet Sauvignon, Nero d'Avola, Merlot, Marzemino,

Negroamaro, Aglianico, Cannonau and Rossese di dolceacqua. In their findings, Marzemino and Aglianico wines showed the greatest colour content, followed by Cabernet Sauvignon, and then the other wines, whereas the level of copigmentation was lowest in Sangiovese among the wines tested. Moreover, regarding SO_2-resistant pigments content in Sangiovese wine was found as in intermediate level when compared to the other wines. It is due to the lack of acylated pigments mentioned above.

j) NON-FLAVONOIDS

Non-flavonoid compounds correspond to the chemically simpler phenolic compounds, such as the following phenolic acids: hydroxybenzoic acids (C6-Cl), hydroxycinnamic acids (C6-C3) and its derivatives, besides other phenolic derivatives of great importance such as stilbenes (SOMERS et al., 1987; MONAGAS et al., 2005; VITRAC et al., 2005).

Hydroxycinnamic acids are present in the skin and pulp of grapes, as in its vacuoles, being one of the most important phenolic acids in it, as they can also be found as tartaric esters compounds (RIBÉREAU-GAYON,1965). Additionally, due to hydrolysis, tartaric hydroxycinnamic derivatives re also found in the free form (caffeic acid, ferulic acid, *p*-coumaric acid) (CARTONI et al., 1991; VRHOVSEK, 1998. Since they present a phenolic structure, these compounds play an important role in the antioxidant properties of most and wine (SINGLETON, 1987). Moreover, these phenolic compounds also influence indirectly aromatic properties of wines, since hydroxycinnamic acids are involved in the arising of volatile phenols (CARTONI et al., 1991; VRHOVSEK, 1998).

The most important derivatives of hydroxybenzoic acid in grapes and wines are: vanilic acid and syringic acid, which appear attached to cell wall in grapes and gallic acid, which is found in the ester form of flavan-3-ol and is one of the most abundant monomeric components in red wine (FRANKEL et al., 1995; SILVA ET AL., 2005).

Some compounds appear in minor quantities, such as protocatechuic and p-hydroxybenzoic acids, which are found in grapes esterified and It not only originates from the grape itself but is also formed by hydrolysis of hydrolysable and condensed tannins, as the gallic acid esters of flavan-3-ols (RIBERÉAU-GAYON,1965; SINGLETON, 1987).

Antioxidant activity of phenolic acids and their esters, as in flavonoids, also depend on the number and position of hydroxyl groupments in the molecule (GROOTVELD and HALLIWELL, 1986; RICE-EVANS et al., 1996). Theoretically the hydroxylated hydroxycinnamic acids are more effective electron captors than their respective benzoates, due to the negative influence of carboxylate groups in hydroxybenzoic acids in donating hydrogen by benzoate groups (GROOTVELD E HALLIWELL, 1986). Moreover, according to Rice-Evans et al. (1996), in vitro analysis demonstrated that hydroxybenzoic acid derivatives have their antioxidant property influenced by the position of hydroxyl groups in B ring, as it was also demonstrated that the esterification of carboxylic group of gallic acid reduced the antioxidant activity of this compound.

As the previous mentioned phenolic compounds, hydroxycinnamic acids' antioxidant activity is also influenced by the position of their substituents in the molecule (GROOTVELD and HALLIWELL, 1986). Diphenolic hydroxycinnamic acids, such as caffeic acid, have a higher capacity of neutralizing free radicals than monophenols, such as p-coumaric acid, in which is in accordance with the chemical criteria applied to diphenolic flavonoids (BORS et al., 1990). The methoxylation of the hydroxyl group in ortho position of diphenolic compounds, as in ferulic acid, results in a decrease in the ability to capture radicals, while hydroxylation, as in caffeic acid, instead of methoxylation is substantially more effective (RICE-EVANS et al. 1996).

k) TANNINS

The term "tannin", as it is classified as a high number of polyphenolic compounds in nature, can interact with proteins to form stable complexes (BATE-SMITH, 1973; HASLAM e LILLEY, 1988). The vegetable tannins can be divided into two major groups: hydrolysable tannins and proanthocyanidins (condensed tannins).

Only the family of proanthocyanidyins are present in *Vitis vinifera* specie, therefore the presence of hydrolyzable tannins in wine is of exogenous origin, result of certain practices such as the use of barrels, since wood is rich in hydrolysable tannins.

Proanthocyanidins present in wines in large quantities come from the skins and pips of grapes during the maceration and fermentation (BOURZEIX et al., 1986; RICARDO DA SILVA and ROSEC, 1992). In general, the levels of 3-flavanols (monomers, oligomers and polymers) of pips are superior to skins (BOURZEIX et al., 1986; DE FREITAS e GLORIES, 1999; SUN et al., 1999).

l) CHEMISTRY OF WINE OXIDATION

Oxidation is a well-known problem in the wine industry, and several researches have been done with the aim to properly identify the parameters involved in this phenomenon, to properly control it.

Therefore the oxidation occurring in wine can be basically divided in the control of two parameters: i) reaction occurring in wine, which are caused by oxidative stress ii) external parameters affecting the chemical oxidation of wine constituents, such as gas permeation during its storage, temperature and other technological practices which may favor and speed up such reactions (BRADSHAW at al., 2003; DANILEWICZ, 2007; WATERHOUSE and LAURIE, 2006; WILDENRDADT and SINGLETON, 1974; KARBOWIAK et al., 2009; TAO et al., 2014; WATERS et al., 1996).

Regarding the facts mentioned above, the right control of the parameters involved in wine storage can provide information to properly predict the chemical oxidation followed vinification, as the impacts of external parameters can influence wine oxidation, with the aim to control it.

Mechanisms of wine oxidation

Wine oxidation can be divided in two chemical mechanisms: enzymatic oxidation and chemical oxidation (DANILEWICZ; SECCOMBE; WHELAN, 2008; OLIVEIRA et al., 2011).

The process of wine oxidation is favored by the presence of polyphenols in which contain in their molecules moieties of o-dihydroxybenzene (which is also known as catechol ring, **Figure 6**), or 1,2,3-trihydroxybenzene (galloyl group: (+)-catechin, (-)-epicatechin, gallocatechin, gallic acid (and its esters), caffeic acid), which can be considered the most oxidable constituents of wine (DANILEWICZ, 2003; KILMARTIN; ZOU; WATERHOUSE, 2001; LI; GUO; WANG, 2008; SINGLETON, 1987).

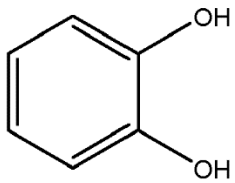


Figure 6. *Catechol ring*

The chemical process of non-enzimatic wine oxidation in known to be like a cascade, in which these catechol phenolic substrates play the main role in the first step of these reactions. Once these catechol substrates are oxidized, semiquinone radicals are formed, which are chemically unstable. Therefore, semiquinones are leading to the formation of quinones subsequently, while oxygen is reduced to hydrogen peroxide (H_2O_2), which can be seen in **Figure 7**, preconized by Singleton using catechin as example:

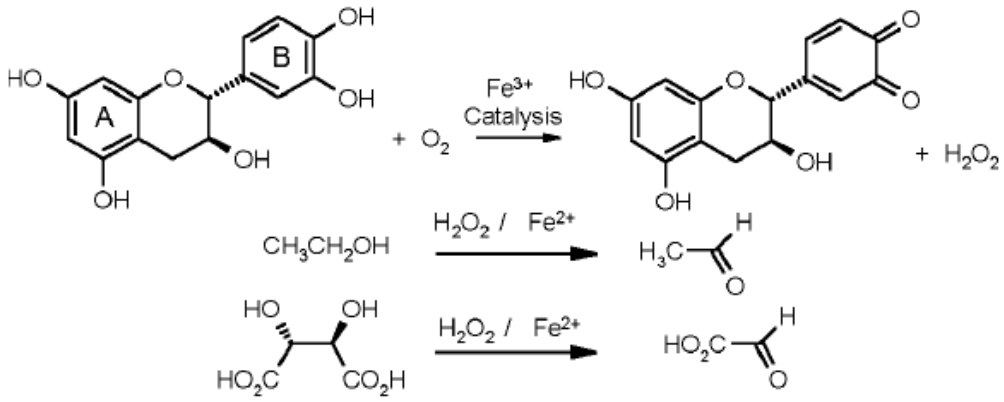


Figure 7 *Oxidation of (+)-catechin, ethanol, and L-tartaric acid in wine (Source: Danilewicz 2003).*

Posteriorly H_2O_2 with the presence of ferrous ions will succeed in oxidize other saturated hydroxy compounds, ethanol and tartaric acid and other compounds, being those with large concentration in wine, the first, leading to the formation of other products, such as acetaldehyde, glyoxylic acid (as seen in example of **Figure 7**) and other compounds, depending on the subsequent molecule to be oxidized (DANILEWICZ, 2003; ES-SAFI et al., 1999).

Wine oxidation is not occurring by the acts of oxygen per se, but this process is mediated by metals, as it can be seen in **Figure 7**. Iron and copper are normally presented in wine in concentration range of 2.8 – 16 mg L^{-1} and 0.11 – 3.6 mg L^{-1} respectively (OUGH and AMERINE, 1988) and on these two elements lay the main role in wine oxidation. In wine oxidation process, the O_2 can be reduced to H_2O in a cascade of electron transfer, in which result in intermediate products of reactive oxygen species (ROS). Danilewicz (2010) demonstrated that catechol oxidation depends on metal catalysis, once previous studies have had already cleared that oxygen cannot combine directly with reducing substances present in wine (PEYNAUD, 1984). The demonstration was made with a model wine solution added with 4-methylcatechol and

saturated with oxygen, where no oxidation occurred after five days of sealed environment, whereas the same solution in addition of Fe and Cu presents oxidation (DANILEWICZ 2010).

The metal catalytic effect on oxidation of polyphenols in wine is due to the fact that oxygen is a diradical. In other words, the direct oxidation of these organic compounds is not kinetically possible because it is spin prohibited, for the reason that polyphenolic compounds are presented in singlet state ($^1O_2$), whereas O_2 in the triplet state ($^3O_2$). Thus, oxygen molecules need an additional energy source for its triplet state to be converted in singlet electronic state, in order to be able to be reactive. Consequently, the role of Fe is to act as catalytic of these oxidation reactions, due to its ability to donate and accept electrons (MILLER et al., 1990), turning molecules of oxygen in a reactive oxygen species (ROS), which are presented in the singlet state, and therefore highly reactive. Thus, oxygen reactive species are formed by reduced transition metal ions in the stepwise addition of a single electron to triplet oxygen (WATERHOUSE AND LAURIE, 2006; DANILEWICZ 2003).

The cascade (**Figure 8**) initiates with the allocation of an electron of Fe ions, which triggers the formation of superoxide radical anion ($O_2^{\bullet -}$) which due to low wine pH, is presented in its protonated form: hydroperoxyl radical ($HOO^{\bullet -}$). Once a second electron is transferred, anion peroxide (O_2^{2-}) is formed, which in wine pH is presented in its protonated form: hydrogen peroxide (H_2O_2). The next electron transfer leads to a more unstable radical: hydroxyl radical ($HO^{\bullet}$). This radical can take one hydrogen atom from organic compounds, such as polyphenolic compounds, producing water, where oxygen reduction is the final product.

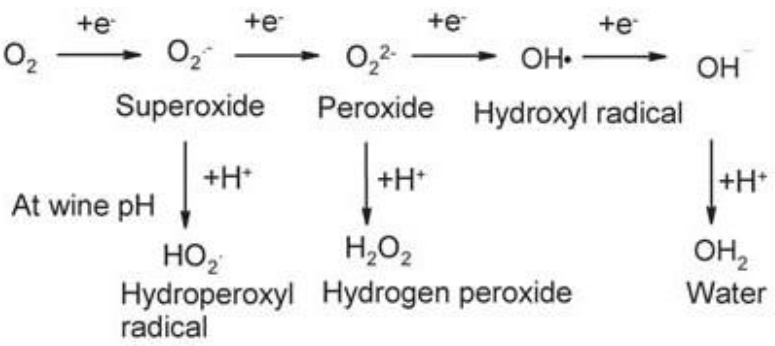


Figure 8. *Scheme of oxygen reduction, proposed by Waterhouse and Laurie (2006).*

Hydrogen peroxide in association with ferrous ions generates hydroxyl radical ($HO^{\cdot}$) which is called Fenton Reaction:

$Fe^{2+} + H_2O \rightarrow HO^{\cdot} + HO^{-}$

Hydroxyl radical is a product of reduction of oxygen, which can oxidize all organic molecules in wine (WATERHOUSE; LAURIE, 2006). It is important to notice that these reactions are not selective, i.e., it can lead to the reaction with other molecules in wine depending on their concentration (DANILEWICZ, 2003, 2007; LI; GUO; WANG, 2008).

Thereafter polyphenols containing a catechol group or a galloyl group are oxidized to semiquinone radicals and benzoquinones whereas oxygen is reduced to hydrogen peroxide, as the entire process is dependent on the redox cycle of Fe^{3+} / Fe^{2+} and Cu^{2+}/Cu^{+} **(Figure 9**)(DANILEWICZ; SECCOMBE; WHELAN, 2008). These reactions are not selective, i.e., it can lead to the reaction with other molecules in wine depending on their concentration (DANILEWICZ, 2003, 2007; LI; GUO; WANG, 2008).

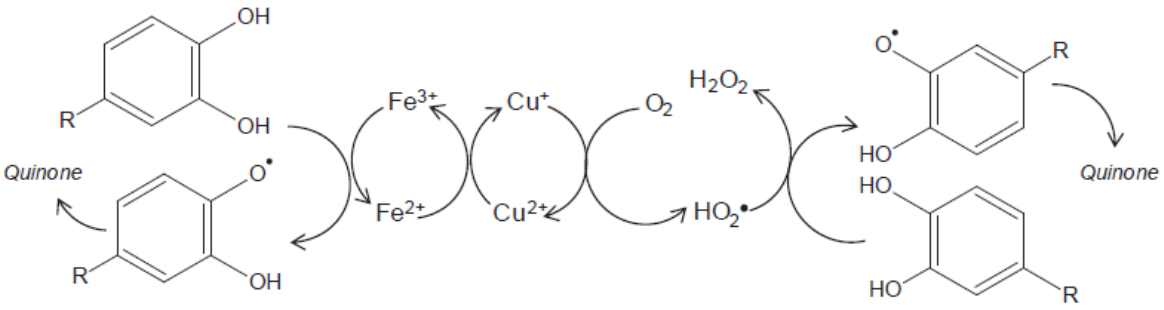


Figure 9. *Scheme of successive monovalent reactions of iron and copper ions in the polyphenolic oxidation to produce quinones and hydrogen peroxide (Source: DANILEWICZ et al., 2008).*

Therefore, substances containing a catechol moiety are oxidized to quinones in a sequential transfer of two hydrogen atoms and the speed of these reactions with reactive species of oxygen (ROS) depends on the ability of the quinones to be stable as a final product. As previously reported, the galloyl and catechol moieties are more easily oxidized due to the resultant radical be stable with a second atom of oxygen (DANILEWICZ, 2003).

The quinones which are formed from polyphenolic oxidation are unstable and possess an electrophilic character, thus they continue reacting. They can spontaneously combine with nucleophilic compounds (LI et al., 2008)

Sulphur Dioxide

Sulphur dioxide is extensively added into wine during winemaking process, as potassium bisulphite salt ($KHSO_3$), in order to protect it against oxidation and acting for microbiological control of yeasts and bacterial growth. The legal limit of this substance in European Union is 150 mg L^{-1} for red wines, while 200 mg L^{-1} white wines (*Council Regulation (EC) N° 479/2009 of April, 29, 2008 on the common organization of the wine market; Commission Implementing Regulation (EU) N° 203/2012).*

As an antioxidant, the bisulphite ion HSO_3^-, which is the form in which the bisulphite salt turns while in wine pH solution, it acts by limiting the oxidation towards reacting with H_2O_2 radicals and other oxygen radicals. Moreover, Sulphur dioxide limits aldehyde formation, once it competes to reduce H_2O_2 (ELIAS and WATERHOUSE, 2010). Additionally, it increases the rate of reverse conversion of quinones derived from polyphenols to their hydroxylated form **(Figure 10)** (DANILEWICZ, SECCOMBE and WHELAN, 2008).

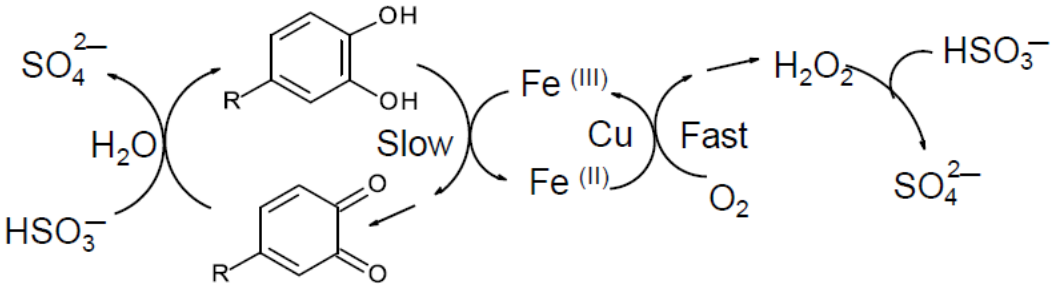


Figure 10. *Proposed mechanism of catechol oxidation in wine: Fe(III)/Fe(II) redox cycling and involvement of SO_2 by Danilewicz (2016).*

Sulphur dioxide in excess can cause wine to develop sulphur aroma, but in normal quantities, it is avoided due to its binding with carbonyl compounds, acetaldehyde, anthocyanins, glutaric and pyruvic acids, glucose and phenolic compounds. Once these compounds are formed, they help wine stabilization, and for this purposes, sulphur dioxide is chemically divided in wine between its active form, the free Sulphur dioxide (HSO_3^- and SO_2) and the total Sulphur dioxide content, in which considers the free SO_2 mentioned plus the adducts formed (NIKOLANTONAKI et al., 2010).

Quinones

As mentioned, wines are rich in phenolic compounds which play several roles in wine, being antioxidant and therefore responsible for wine aging, they're responsible for the red color of wine, moreover it affects the wine taste and bitterness.

As good antioxidants, polyphenols are oxidized to an intermediate product, the quinones, which are intermediate product of phenolic oxidation, responsible for wine oxidation. Currently, theory regarding wine oxidation is O_2 independent, i.e., it has as major responsible for triggering its reaction metal ions of Fe and Cu.

Quinones are consequently key reactive electrophilic oxidation intermediates in wine in which can participate in several different reactions with wine nucleophiles, as sulfur dioxide, ascorbic acid, thiols, amino acids, etc, and also lead to the loss of aromatic compounds (NIKOLANTONAKI & WATERHOUSE, 2012; MAKHOTKINA, 2011; WATERHOUSE & NIKOLANTONAKI, 2015) or development of off-flavors (Bittner, 2006). These reactions are very important in wine aging because they mediate oxygen consumption during both production and bottle aging phases.

Effect of temperature on Wine Oxidation

The temperature in the entire process of wine production needs to be controlled. By this means, it is important since prior the fermentation, because the grapes cannot have high temperatures while processed into must, as the fermentation temperature needs to be properly controlled to have the yeasts working properly, as to avoid undesirable aroma, and finally, once the wine is bottled, the temperature of transport and storage needs to be controlled to maintain the wine chemically stable and to avoid the acceleration of chemical oxidations that may occur.

The ideal temperature for wine storage can be considered between 4-18ºC (BEER et al., 2005). The reaction in which can occur if this temperature is fixed to below or above this temperature will depend on the kind of reaction, as it can be explained in this section.

Once the wine is bottled, even if it is not damaged in high temperatures, it will change its aging characteristics, such as the premature release of glucose-bound flavor precursors, as the fruity–floral terpenes found in Muscat-type varietals (FRANCIS, SEFTON, and WILLIAMS, 1994), which can lead to a faster decline in free

sulfur dioxide content (OUGH, 1985), browning (BERG and AKIYOSHI, 1956) and other destructive oxidative reactions (RIBEREAU-GAYON, 1963).

This chemical and sensory changes of a wine are consequences of complex chemical reactions, which can be elucidated by physical chemical principles. These are mainly regulated by two key characteristics: equilibrium and kinetics. According to Smith et al. (1996), the equilibrium guides the maximum extend in which a reaction can occur, as the kinetics describe the speed in which it can occur, and there two keys are affected by media composition and temperature.

Arrhenius was the first scientist to recognize the dependency of the speed constant and temperature. To illustrate it, it can be proposed that a reaction is ruled by the following equation:

$$aA + bB \leftrightarrow cC + dD \qquad (a)$$

It can be assumed that, either by the reaction being exothermic or endothermic, the constant value (k) can be modified according to the temperature. Regarding the kinetics, the reaction rate of the component A above (-rA) can be described with a different constant (b), which is temperature dependent (FOGLER 1999, LEVENSPIEL 2004) as it was exemplified in the reaction (a).

$$-rA = -\frac{dCA}{dT} = K\,(T)\;\; C_A^{\alpha} C_B^{\beta} \qquad (b)$$

According to Laidler (1984), the rate constant is temperature-dependent and it can be modelled with Arrhenius equation (c):

$$K = A\, e^{-Ea/RT}$$

Where:

k is the speed constant

A is the known “Arrhenius constant” or “pre-exponential factor”;

Ea corresponds to the “energy of activation”

R is the gas constant (8.314 J K^{-1} mol^{-1});

T is the absolute temperature.

Ea and A are known as the Arrhenius parameters, both of which can usually be treated as being independent of temperature over the small temperature range (RICHARDSON and PEACOCK 1994) likely to be relevant in most wine reactions (SCRIMGEOUR et al., 2015).

Once the energy of activation of a reaction is known, it is simple to predict the constant speed value (K2), in a certain temperature (T2), from a known value of K1 in T1 temperature (d):

$$\ln\frac{k2}{k1} = \frac{Ea}{R}\left(\frac{1}{T1} - \frac{1}{T2}\right)$$

Nevertheless, the reactions in wine are complex and several steps are involved, with different chemical reactions and parallel pathways which are not well known and defined. Even though, reaction rate expression rates can still be determined, as it can be seen in several studies in which the Arrhenius equation have had been applied to wine and its process kinetics, as oxidative browning (BERG and AKIYOSHI 1956; OUGH 1985; CILLIERS and SINGLETON 1989; BOULTON et al. 1996, SERRA-CAYUELA et al. 2014), volatile ester hydrolysis and formation (RAMEY and OUGH 1980), co-pigmentation (BARANOWSKI and NAGEL 1983, KUNSÁGI-MÁTÉ et al. 2009), ethyl carbamate formation (OUGH et al. 1988), sotolon formation (FERREIRA et al. 2005) and wine protein unfolding (FALCONER ET AL. 2010). Scrimgeour et al. (2015) states the if reaction rates are determined by the rate limiting step, a rate expression is requiring in order to directly or indirectly approximates a certain step instead of necessarily describe the entire chain of reactions involved.

Practically the ideal temperature proven to be more efficient to prevent wine to suffer from deleterious chemical modifications mentioned is due to several compounds. The main effects of a wine undergoing different temperatures than the practical ideal can be seen in **Table 5.**

Table 5. *Indicative effects of wines kept under different temperatures.*

Higher Temperatures (above 18°C)	**Lower Temperatures (below 4°C)**
Visible protein hazes are occasionally precipitated	Tartaric salts precipitation
excessive extraction of odors from the bottle closures	Lack of fully developed aroma and flavor
increased scavenging and permeation-based loss of protective sulfur dioxide or certain wine aromas	Cork rupture due to coldness
Microbiological instability	
Accelerated formation of ethyl carbamate	

Thus, it has been proven that some wines which were exposed to heat during transport have had the same chemical resemblance as the wines aged between 1 and 18 months when related with conventional and regular cellar storage (BUTZKE, 2012).
Moreover, as a practical approach of Arrhenius equation, the rate constant of a chemical reaction is exponentially associated to the temperature of the system. Studies have been made to summarize (BOULTON et al., 1996) researches (RIBÉREAU-GAYON 1933, OUGH 1985) to reveal the relative rates of oxygen uptake, browning, and total SO_2 decrease in red wine would increase approximately 270, 21, and 5 or 2 times faster in white wines at 40°C when comparing the rates at 10°C.

Besides the temperature, which plays a main role in the wine storage, the light exposure after bottling may also affect constituents of alcoholic beverages, particularly between 350 nm–500 nm wavelengths. Light, breaks down the complex molecules that create some of the special flavours in properly aged WINES (D'AURIA *et al*. 2003).
Dark glass bottles can protect wines from the environmental exposure to light, although in order to not be affected by light degradation low-level lighting is advised.

II. WINE PACKAGING

In the past years, the scientific knowledge of the wine in all productive levels had evolved in a way that nowadays there are a lot of choices in winemaking process and bottling materials.
The wine chemistry changes during storage time followed by sensorial changes that may affect the final consumer. This section englobes the external parameters affecting wine oxidation, and it is divided in closures and packages section.

a) CLOSURES

Among the wine packing materials, one of the main parameters affecting the wine preservation in which should be considered is the transfer of gases through packing material to which it is exposed at and after packaging and to its resistance to oxidation, which is detailed in Chapter 1 of the present thesis. Shelf-life of a table wine is directly related to the oxygen content. Wine closures are used with the objective of diminishing the extensive contact of the wine with the external oxygen, which can lead to wine's oxidation and further deterioration (ROBINSON, 2006). Cork is historically

the primary closure type due to the fact other materials were not able to seal the bottle in a way to avoid the wine from turning to vinegar. Natural corks has been used since ancient times, when Greek and Romans started using it for closing amphoras (MARIN et al., 2007).

Natural Cork
- The role of cork and enclosure permeability

Natural corks (**Figure 11)** are derived from a suberized cellular tissue, which is continuously produced by the phellogen of the cork oak tree (*Quercus suber* L.) (**Figure 12**).

Figure 11. *Natural Corks*

Figure 12. *Quercus suber* tree

These corks can be commercially classified according to the homogeneity of the external surface, which leads to cork porosity. This porosity allows gas exchange between the environment and the wine inside the bottle, which can alter the wine

aroma. Besides the gas permeability, natural corks can change the wine also by having some of its components migrating into wine, which can have either a positive or negative impact on wine color, bitterness and astringency (PEREIRA, 1988; VAREA et al., 2001; ROCHA et al., 2005).

Polyphenols susceptible to migrate from cork stoppers to wine. Most of the migrating components are phenolic compounds, which can participate on several chemical reactions, which, according to Azevedo et al. (2014) can vary according to the porosity of the cork. Their work confirmed previous studies (SEFTON, 2005) which states that, among the closures tested, the two natural cork stoppers used were the ones with the higher phenolic components extracted, which can be explained by their internal porosity, which is higher.

Although some studies have been demonstrating that natural corks are the best when concerning gas permeability, it has some negative issues, such as cork taint, random oxidation and leakage (STELZER, T., 2003; CHATONNET et al., 2004).

Therefore, besides the low permeability to gas, natural cork presents an average of 2 to 5% of economic loss of all bottled wines, due to contamination of trichnoroanisole (TCA), which is believed to be caused by microorganism growth metabolites or by chemicals installed during natural cork processing (PEREIRA ET AL, 2000). Thus, the wine closure industry has developed alternatives since the decade of 1990. They are the screw caps, synthetic, co-extruded synthetic, technical corks (SKURRAY et al., 2000; ROBINSON, 2006; CAPONE et al., 2002; LOPES et al., 2007).

TECHNICAL CORK

As the natural corks, the technical (**Figure 15**) corks are also made by *Quercus suber*, although with a relatively inferior quality, since they are made of leftover cork parts (PHILLIPS, 2007, ROBINSON, 2006). Similarly to the natural corks, technical corks can also have problems linked to TCA presence.

Figure 15. *Technical cork*

Technical corks are grounded small pieces of cork which pass throughout a extruding process or a molding, until it has the shape of a cork (PHILLIPS, 2007).

SCREW CAPS

Screw cap closures **(Figure 13)** were introduced in the wine industry aiming to solve the problem of “cork taint”, which occurs when wine a cork is contaminated, causing from 2% to 15% of wine bottles worldwide that use natural cork closures to have a musty flavor (SOGG, 2005).

Figure 13. *Screw Caps*

Thus, Screw cap closures have solved the problem of cork taint and have recently become a popular alternative for Australian and New Zealand. Though this closures have been successful in proving their capacity of preserving wine quality, wine producers faced resistance from their consumers.

Nowadays, however, in Australia and New Zealand the screw caps have overcame the natural cork as choice of closure acceptance by wine consumers'. This behavior was not adopted worldwide, since it faces resistance still in America (specially US) and European countries (apart from UK) (SOGG, 2007).

Besides the cork taint factor, which makes the use of screw cap more favorable to wine industry, there are some pros and cons regarding its usage. One of the problems is that, economically, their use presents the same cost to the wine producer, since it requires special wine bottles to make it properly fit in the glass bottle (**Figure 14**).

Figure 14. *Screwcap in a wine bottle.*

In addition, the main problem of screw caps is the increased presence of wine reduction, which causes the wine to develop sulfur aroma, due to this closure's inexistent or low permeability to oxygen. Moreover, due to the reductive environment, red wines can also develop aromas which can lead to to rubber, stuck flint, vegetal, cabbage, or mercaptan-smelling odors, which are highly undesirable (TUDOR, 2005; GOODE, 2007). Nevertheless, it is likely that the development of reduced characters depends on wine composition at bottling as well as closure type.

SYNTHETIC CLOSURES

Alternative closures, made with synthetic polymers have also been used, representing 17% of the global stoppers Market (CORK, 2011). Synthetic closures **(Figure 16a** and **16b**) are another alternative to avoid cork taint in bottled wines. They are made by plastic material and can be manufactured in three ways: co-extrusion, injection

molding, and these two processes combined (NEOCORK, 2007; NOMACORC, 2007; VINOVA, 2007).

Figure 16 (a) and (b). *Synthetic Cork / synthetic cork cut in a half.*

The co-extrusion process arises from a low-density polymer (polyethylene) in the core and a polyethylene-bases thermoplastic elastomer surface coating (NEOCORK, 2007).

Instead, the in synthetic closure produced by injection molding technology arises by a polymer injection, ie, the polyethylene is molten then injected into a mold, under pressure, which is kept until it becomes solid (ZOECKLEIN, 2004).

The main issue regarding the use of synthetic corks are the rate transfer of oxygen into the bottle. Although, in this case the problem is the excess amount of oxygen permeation during the wine bottle storage (when compared to natural corks, for example), which can lead to wine oxidation (LOPES et al., 2006) and its consequences, such as change in wine color, oxidative aroma.

Due to this problem, wineries usually use this kind of closures for wines in which are not aged, with a consume time of a year, or a year and a half after bottling (PHILLIPS, 2007).

In addition, another benefit of synthetic corks is its uniformity in form parameters: size, appearance, weight and overall quality (ZOECKLEIN, 2005).

COMPARISON AMONG WINE CLOSURES

Therefore, every wine closure has their particularities, in which can be assumed as pros and cons (**Table 6)** whenever there is a choice of the which closure to be used.

Table 6. *Comparison of positive and negative characteristics regarding closures when applied to wine bottles.*

CLOSURE	POSITIVE	NEGATIVE
Natural cork	- Natural Renewable Resource - Historically Preferred - Long-term Aging Proven	- Variable cost - 1-3% Affected by TCA 'Cork' Taint - Limited Natural Resource - Variable Quality - Natural Corks have variable rates of gas exchange with the exterior media
Technical cork	- Most of the same pros and cons as the natural cork, with the added benefit of sturdy construction - Cork taint reduction when compared to natural cork	- Can be susceptible to leaks - Composite is prone to crumbling or breaking upon extraction. - There is a risk that glue and other unnatural materials can leech into the wine and may have unhealthful consequences long term.
Synthetic cork	- Inexpensive - Unlikely to crumble - Not as prone to drying out and expanding or contracting as its natural counterparts.	- Don't seal as natural cork, enabling unwanted oxidation - They can be tricky to get out of the bottle. - Synthetic corks are made from non-natural materials that may affect the wine and have long-term health effects.
Screw cap	- Tight seal, avoiding the wine to deteriorate - Prevent oxidation caused by too much air entering the wine bottle - Long-term aging of wine - No cork taint problem - Resalable	- Can the wine to "reductive" problems - Not ideal for long-term aging or for wines that need a little oxidation

It is important to adapt it according to the wine to be bottled, in order to promote the best possible development of flavors and color, as to avoid wine spoilage. As wine becomes consumed on a daily, some alternatives to cork become more important. There are wines produced in which aren't meant to age for more than a year, in which could be sealed with other material than the classic natural cork, for example. As there are traditional wines in which are produced to age for years, in which would not benefit of new closure alternatives.

b) PACKAGES

The role of packaging at food and beverages industry is to protect its content in order to keep it consumable and unmodified as long as possible, until the human

consumption. Thus, considering this relation between the product and the packing, it is necessary, within the wine industry, to develop wine packing in which quality attributes might be conserved. Therefore, it is necessary a complete knowledge about the influencers of change in packaging, as the contributor factor in which are enabling these changes.

Wine storage is an ancient activity which has been improved since thousands of years ago. By 1500 BC until approximately 500 AD, a large ceramic vessel, named amphora, was used to ship wine throughout the Mediterranean region in ancient Greek and Roman empires (TWEDE, 2002). A lot have changed since that period and nowadays, the material and shape of packages are not the same as it used to be in ancient times. Since that period, wine packing has developed, as there several are options available in the market nowadays. Glass manufacture has been the most traditional way for storing wine for many years, and yet is the preferable material, because of the reduced gas exchange with the environment, since the oxygen is one of the more important factors in wine deterioration.

However, in the decade of 1990, the global bottles sales accounted for over 90% in the wine industry, however it represents less than 60% nowadays, allowing a crescent trend in the market worldwide considering wine packaging such as Bag in Box®, glass, and plastic bottles (PET).

The main function of, either the packaging material or the closure, is to guarantee a good seal, in order to avoid organoleptic deterioration of the wine during the storage. A strict control of the packaging material is fundamental, since the mass transfer of small molecules of gases, ie, oxygen, into the package can vary, being the knowledge of this gas permeability fundamental to ensure the wine will not be deteriorated until it gets to the final consumer (GODDEN et al., 2005; FU et al., 2009; MENTANA et al., 2009; GHIDOSSI et al, 2012).

PET

One of the alternative materials of wine-preservation technology is the polyethylene terephthalate, a combination of ethylene glycol and terephthalic acid, which forms a polymer chain commonly named PET, has been developed for wine industry in recent years. PET is a packaging material widely used for foods and beverages as for carbonated beverages, also an environment friendly and inexpensive alternative solution for wine bottling as option to glass. (DEL NOBILE et al., 2003; ROS-

CHUMILLAS et al., 2007; GHIDOSSI et al., 2012). According to Van Lune et al. (1997) there are several benefits of utilizing PET as packing material: transparence, excellent mechanical properties, low price, light in weight, and good oxygen barrier properties. Dombre et al. (2014) have studied the differences regarding virgin PET, recycled PET and PET (all 750mL bottles) containing an oxygen scavenger within the three specific compounds (aroma and oxidation markers) of a rosé Cinsault wine from south of France, as their thermal properties and gas permeability. They found out that the structural and thermal properties showed a small difference between the PETs, proving that the recycled PET was a less effective barrier to aroma loss than the other two types used in the study, and that the PET containing scavengers seem to provoke slightly improvement on protecting oxygen sensitive aroma compounds. Another study compared a Rosé wine bottled in two 750 mL PETs bottle (recycled and no recycled) to this same wine bottled in glass bottles using the same closure, storing it at 20 °C in both light and dark conditions for 372 days. It was observed that CO_2 and SO_2 levels diminished, as O_2 concentration increased in PET bottles after 6 months of bottling, which may be due considerable gas permeability of monolayer PET. The impact of PET could also have seen in different parameters, as the wine bottled in a monolayer PET bottle aged faster than in a glass bottle. It was seen that after 162 days of storage, free SO_2 in the PET bottle reached the critical value of 10 mg/L, whereas wine in a glass bottle was still with reasonable values after a year of storage. Oxygen levels (dissolved and headspace) in PET bottles, increased after three months, which led to the conclusion that O_2 consumption became slower than O_2 ingress. Thus, in practical terms, the study indicates that wines aging with monolayer PET need to be consumed within 5 months after bottling (TOUSSAINT et al., 2014).

Tetra Pak®

Tetra Pak® (**Figure 18)** is the largest producer of beverage packaging system in the world, as it provides ways for these products to be easily transported and kept without the necessity of low temperatures.

Figure 18. *TetraPack package.*

It is made basically by paperboard (**Figure 19**), which according to the manufacturer it provides stability, strength and smoothness to the printing surface. In the intermediate layer there is an aluminum foil (**Figure 19**) coating, which protects the wine against oxygen and light, which was made in order to make the package to resist in ambient temperatures. The external part is recovered with polyethylene (**Figure 19**), which protects against outside moisture and enables the paperboard to stick to the aluminum foil.

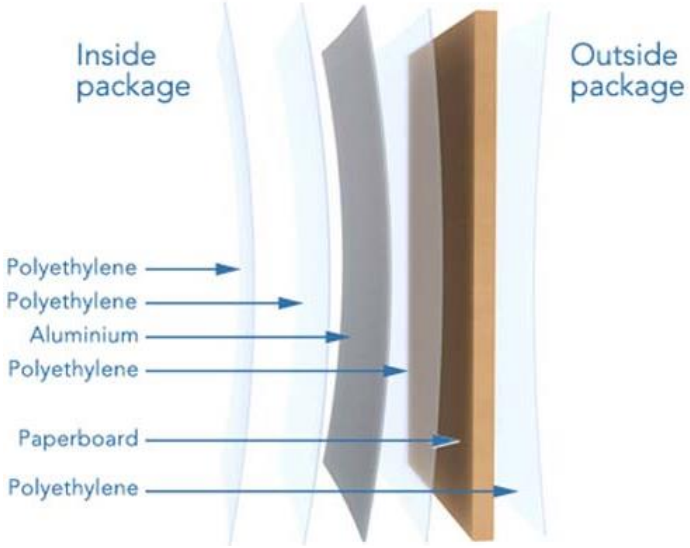


Figure 19. *Layers of TetraPAck package. (Source: http://www.tetrapak.com/packaging/materials)*

Pickering et al. (2009) evaluated changes in 3-alkyl-2-methoxypyrazines concentrations (an important class of odor-active compounds associated with wine quality during bottle aging) varied with closure/packaging option, with the greatest decrease evident in Tetrapak® cartons, which showed that wines which had as package the Tetrapak® had the lowest concentration of acetate esters (Riesling wine) and the highest concentration of ethyl esters after 12 months.

Bag-in-Box

Bag-in-Box (BIB) (**Figure 20**) packing allows the wine to be sealed in a bag covered by one or more layers of flexible films, which is externally covered by paperboard carton. Attached to it there is a valve fitment which allows the wine to be dispensed (MOREIRA, 2016).

Figure 20. *BIB package*

It is important to notice that due to BIB's large volume capacity, wine is usually consumed over a prolonged time after the package is opened, which makes wine's secondary shelf life to be affected by oxygen ingress through the dispensing fitment, or temperature variation (FU et al., 2009; LEE et al., 2011; REVI et al., 2014). These factors may diminish the secondary shelf life by accelerating the deterioration of freshness and fruity qualities, as well as browning reactions, which are signals of decline of young white wines (PÉREZ-COELLO et al., 1999; ORTEGA et al., 2001).
Therefore, studies on wine quality parameters are important in order to understand how this package is affected and how are the conditions to be avoided, in order to not have wine deterioration.
Revi et al. (2014) evaluated the enological parameters and volatile compounds of a white wine stored in dark with three kind of packages: colored glass and two

commercial bag-in-box (BIB) pouches (low density polyethylene – LDPE and ethylene vinyl acetate – EVA lined) for a period of 6 months at 20°C. It was seen that the glass bottle was better in retaining mots of aromatic compounds, when compared to the two plastic, and that between these, the LDPE lined pouch showed a considerably higher aroma sorption as compared to EVA. They concluded throughout a sensory evaluation that the wine packed with glass had accepted quality for twice the time of those who were packed with glasses.

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CHAPTER 1

CHAPTER 1

THE SHIPPING CASE STUDY

ABSTRACT

Since the beginning of 21st century, wine trade has been changing significantly as it grows, as its structure has been experiencing changes. These changes are shaping the way that the wine market is presenting itself, such as the development of new packaging, as a greener way to pack the product, or a cheaper way to achieve distant consumers. The aim of this study is to predict the effect of shipping condition on the composition of wine by modeling the temperature change with time. Temperature was monitored during real wine shipping from Italy to Brazil, then simulated condition were replicated in laboratory on white wines with different packaging (dark colored glass with natural cork as closure, transparent glass closed with screw cap, tetrapak and bag-in-box, BIB). Samples were analyzed for selected parameters, including titratable and volatile acidity, pH, total and free SO_2, color, volatile compounds and sensory attributes. Wine packaging and shipping/transportation conditions can affect wine composition to large extent, especially due to temperature variation. In our conditions, the packaging used did not significantly affect basic wines' chemical parameters, whereas some differences were found in electronic nose analysis, indicating that the volatile fraction of wines was most stressed by the temperature shift. By modeling the time-intensity approach, new parameters were proposed as index for evaluating the thermal stress of wine during transportation.

1.1 INTRODUCTION

According to the International Organization of Vine and Wine (OIV), the wine exports continued its raising trend in the past few years globally with continued volumetric increase (104 mhl, +3% compared with 2013) (AURAND, 2015). In this view, the imported wine consumed from 2005 to 2015 raised from 27% to 43% of the total wine consumption worldwide. Within this context, it is crucial to ensure the wine quality during its transportation, and its shelf life.

The shelf life of a food product is defined “as the period in which it remains safe, retaining the sensory, chemical, physical and microbiological attributes, and it complies with any label declaration of nutritional data” (FRANKS, 1993).

Wine is considered one of the most valuable and complex products within the global food and beverage industries as long-term exposure of it to heat can adversely affect its sensory properties as well as its physical and chemical stability (BUTZKE; VOGT; CHACÓN-RODRÍGUEZ, 2012; PRESA-OWENS; NOBLE, 1997). In this context, preserving the quality and preventing the shelf life depletion relies heavily on the wine making practices, such as the lower pH, use of sulfites, good hygiene practices and sterile filtration prior to bottling limiting microbiological growth in packaged wines. Thus, wine is a complex product towards shelf life prediction, as its chemical composition varies depending on the initial conditions of the product processing. Moreover, after the wine making practices, the packaging and storage can affect directly the shelf life of the product until it reaches the final consumer, as it influences important and undesirable physic-chemical changes in the wine (ROBINSON et al., 2010).

Besides being an influencer in consumer’s product acceptance, wine packaging also involves art, science and technology with the aim to protect the product in the best way possible during the entire chain of distribution, shipping and storage until consumption. Most of the wine that is sold worldwide is presented in glass package, which has been a traditional medium and fulfills the requirements to preserve wine quality over time. However, with the current global awareness of climatic changes allied by the fact that the increase in greenhouse gases is linked to many industries, including wine industry, the consumers now-a-days seek to purchase more environmentally friendly products. Some studies proposed that alternative packaging, when compared to bottles, can be less harmful to the environment, with, for example, PET bottles generating around seven times less CO_2 when compared to traditional glass wine bottles. Moreover, Tetra Pak is estimated to use 70% of recycled material in its whole production. Also, companies that have shifted from the traditional glass bottles to the Bag-In-Box (BIB) estimated that there is a minimum of 50% reduction in carbon emissions during the manufacture of these packages.

In view of this new trend, it is important to ensure that the wine within these innovative packaging systems are properly sealed and present no possibility of leaking, by

estimating gas exchange (O_2 and CO_2) with the environment during the supply process.

Furthermore, unwanted exposure to temperature variation can result in leaking and pushed corks observed in bottles, and oxidative damages, which can occur in all kinds of packaging. Few data are available in literature providing concrete evidences regarding the quantification of the effects of wine during long-term exposure as such during wine transport to their destination, especially with regard to new and alternative packaging systems (BUTZKE; VOGT; CHACÓN-RODRÍGUEZ, 2012). The study presented in this chapter makes note on the selected literature on wine shipping along with the work carried out during the PhD.

1.1.2 Wine Shipping

As mentioned in the preceding paragraphs, among the external parameters affecting wine oxidation, temperature and light, can be directly associated with the wine storage and its transportation until it reaches the final consumer. Few studies are available regarding the effects of transportation (import/exports) of wine around the globe on its quality from storage till its destination.

According to a study on effects of temperature on wines (WEISKIRCHER, 2008), exposing a wine either to temperatures over 25°C for long durations or at 40°C for short periods can adversely affects the wine quality.

Among the studies performed, Ospack and Fosters (2007) tested the shipping from Adelaide (Australia) to the Napa Valley in California (USA) of six containers filled with wine and temperature loggers. It was found that wine temperatures fluctuate more while on land than at sea, except with containers placed on deck of the ship in full sunlight exhibiting the same temperature differences as on land. Meanwhile the center box of the container with the OsPack liner did not exceed 30°C even if the temperature above the liner exceeded 50°C. While on land, the roof temperature of containers sheltered by other containers was at or below ambient temperatures while it peaked up to 70°C for unsheltered containers due to solar radiation, while wine temperature peaked 10 to 15°C lower. An important observation that can be drawn is that in a hypothetical situation, if a container is not exposed to direct sunlight at sea, the wine temperature with or without liners is similar and acceptable. Therefore, it is necessary to understand the questions of how the temperature fluctuations affect wine

commercially and what is the extent of damage with respect to expansion and contraction of the wine at high temperatures.

To address the questions related to influence of the temperature on wine quality, several indices based on temperature measurements during wine transportation could potentially serve as a valuable tool:

1. Excessive thermal accumulation (ETA) – literature information regarding storage temperature for the wine vary among Authors, however in most of the cases optimal temperature range falls within 10° and 15°C (ROBINSON; HARDING, 2015; STEVENSON, 2005). Wine transportation under temperature condition higher than optimal could induce detrimental effects on wine quality, while the magnitude of outcome will depend on time and temperate (WEISKIRCHER, 2008). Thus, linking these variables (temperature and time) by calculating excessive "thermal accumulation" of the wine during transportation could potentially reveal correlation of temperature and quality of wine.

2. Total number of hours in certain temperature range (TNH) – excessive thermal accumulation presented above, could have limitation to predict influence of the temperature fluctuations on wine quality during the transportation. This due to fact that equal summary value of the excessive thermal accumulation could be derived from transport conditions where daily temperature amplitudes are relatively high (land transportation) or low (water transportation) (OSPACK and FOSTERS 2007). Thus, calculating the total number of hours within certain ranges could provide additional information related to influence of the temperature fluctuations on wine quality.

The study by Du Toit et al. (2007) tried to answer these questions by simulating shipping of Chenin blanc and Sauvignon blanc wines from South Africa to Europe (MEYER et al., 2002; BERMEJO et al., 2007). The study was divided in four trials as it is shown at **Table 1.1**.

It was concluded in the Study 1 that the variation of temperatures is less harmful to wine aroma when comparing it to wines left at a constant high temperature. Robinson et al. (2010), were the first to investigate the potential sensory changes in wines under conditions that are expected to be experienced by wines during transit.

Table 1.1. *Scheme of two studies aiming to evaluate the effect of shipping or transporting wines by their simulation. Study 1 by Du Toit et al., 2007, and Study 2 by Robinson et al., 2010.*

TRIALS	STUDY 1 *DU TOIT ET AL., 2007*		STUDY 2 *ROBINSON ET AL., 2010*	
	Temperature	**Duration**	**Temperature**	**Duration**
TRIAL 1	-4°C	Constant	20°C	Constant
TRIAL 2	30, 37 and 20°C	Every 8 hours during the first week	40°C	Constant
	15°C	30 days	-	-
	-4°C, 4°C and 8°C	Every 8 hours during the last week	-	-
TRIAL 3	15°C	Constant	20°C	Every 12 hours
			40°C	
TRIAL 4	37°C	Constant	Unmonitored	*

**Wines were stored in the trunk of a private car to simulate wine shipment with movement (20 days during the month of December in northern hemisphere)*

In study 2, the effect of simulated shipping conditions on sensory attributes and volatile composition of six varieties of wines was assessed. Both the studies as in **Table 1.1** comprised of four treatments used to identify the prospective effects of storage and transit temperatures.

Gas chromatography and sensory analysis of wines stored at high temperatures showed significant differences from the control stored at lower temperatures, as indicated by differences of concentration of several compounds, including higher concentrations of vitispirane 1 and 2, norisoprenoid 1,1,6-trimethyl-l,2-dihydronaphthalene, and p-cymene and reductions in several esters and acetates, which are characteristic of aged wines, among the wines tested.

Among several published studies (LAFFER, 2004) as in the literature regarding wine transport, some conclusions that are of interest can be drawn as follows:

- Excessive heat in a part or in all the supply chain from producer to consumer is responsible for 90% of quality faults;

- The location of the container during wine shipping has a major impact on the temperature variation;
- Storage of wines in docks is often more detrimental to wine quality than the shipping;
- Wine tasting (or volatile fraction analysis of wine) is a better tool for evaluation of wine quality rather than standalone chemical analysis.

1.1.2.1 Influence of Packaging

The wine quality is in fact not only affected by the external packaging but also by the package which is in direct contact with the product itself. Nowadays, as mentioned, there are several alternatives of packages for wine available in the market.

Glass bottles are known to be the traditional choice as they are inert and present “clearness”, nevertheless polyethylene terephthalate (PET) bottles, multilayer Tetrabrik type containers and bag-in-box type containers (ROBERTSON, 2006) are of rising popularity, especially among new world wine countries. It can be exemplified by data from Australia Bureau of Statistics (2008), which demonstrates that in Australia more than half of the wine consumed is packaged in bag-in-box containers (AUSTRALIA, 2008). Hence it is of importance to understand and elaborate the effects of varied packaging systems in elevated temperatures on the final quality of wine.

In an attempt to understand Hopfer et al. (2013) evaluated the influence of different packages among different constant temperatures in a red wine, devoid of real or simulated shipping conditions. In this simulation, volatile composition of wines was investigated of wines packed with glass bottles with natural cork, synthetic cork, and screw cap closure, as well as two Bag-in-Box. It was seen that the packaging effect became more pronounced in the highest temperature evaluated (40°C), resulting in the largest changes in the Bag-in-Box wines. The highest storage temperature facilitated the oxidation in wines irrespective of the packaging type although the levels of oxidation varied. The study is a reliable source for information as it provided chemical information allied with sensory tests, but cannot be used for prediction as the shipping conditions were not truly simulated.

Ghiossi et al. (2012) also performed an elaborate study in which the evolution of wine quality in different packages during 18 months was evaluated. The sensory and chemical parameters were evaluated in both red and white wines packaged in glass bottles, Bag-in-Box and PET bottles. There were no significant differences observed

among the red wines packed in different systems, whereas white wines exhibited significant difference after a mere six months of study when packed with PET bottles. Though most of these studies present immense information, some details regarding interference of different packages on wine quality during a real shipping for wine exports is unanswered. The present study attempts to understand the effects of different packages on the final wine quality under real-time export conditions, which in our knowledge, as described was not adequately investigated.

The Case Study

The aim of this PhD study is to improve the understand whether the package material is crucial to the final quality of a wine during a real time transcontinental shipping, wherein the country of destination was Brazil.

Brazil in the recent years is transforming into the largest wine import market from Latin America. According to the Brazilian Institute of Wine (IBRAVIN) Index, wine imports in Brazil increased from 50.9 millions liters to 81.6 millions liters (60%) between 2006 and 2016. This market increment makes this business to count more than 300 importers, 30,000 labels from 32 countries available today for the Brazilian consumers. Besides Brazil also acts as portal for the wine imports that are further distributed in entire Latin America.

According to UVIBRA (Brazilian Union of Vitiviniculture), Italy occupies the fourth place in export ranking of wines in Brazil with 8.566.756 liters of Italian wines being exported to Brazil last year, which corresponds to 12.1% of total wine consumed in Brazil, out of which 33.30% by transport "*free on board*", in which the buyer assumes all the risks regarding transportation and the final quality of product since the wine is delivered for shipping. Together, Portugal, Italy, France and Spain contribute to 34.8% of the imported wine in Brazil, which corresponds to almost 25 million liters of wine shipped in 2014. With the growing market and increasing consumer demands the quality control of bottled wines is emerging as a crucial step to ensure good product delivery. Thus, it is important to study the conditions in which the wine is transported and the wine quality, by evaluating the product both at the departure and the destination.

Within this context, the aim of this study was to evaluate the effect of shipping from Europe (Italy) to South America (Brazil) on the physic-chemical characteristics of wine as evaluated by oxidative stress approach.

1.2.1 Materials and Methods

1.2.1.1 Samples

A white wine vintage 2015 (blend of Trebbiano and Chardonnay) was bottled in five different packages represented in F**igure 1.1** as follows: 750 mL bottle and screw cap, 1000 mL bottle and screw cap, 750 mL bottle and natural cork, TetraPak, Bag in Box (BIB).

Figure 1.1 *Wine bottled in: (1) 750 mL bottle and screw cap; (2) 1000 mL bottle and screw cap; (3) 750 mL bottle and natural cork; (4) TetraPak; (5) Bag in Box (BIB); respectively.*

1.2.1.2 Wine shipping simulation trial

The temperature protocol was performed in a thermostat chamber in following the real shipping conditions, during which a portable temperature recorder device (EL USB-1-PRO, high temperature data logger, Lascar Electronics, Wiltshire, UK) was put in the wine box for continuous recording of the temperature during wine transportation from Italy to Brazil. The time-temperature profile was then simulated in laboratory accordingly (**Figure 1.2**):

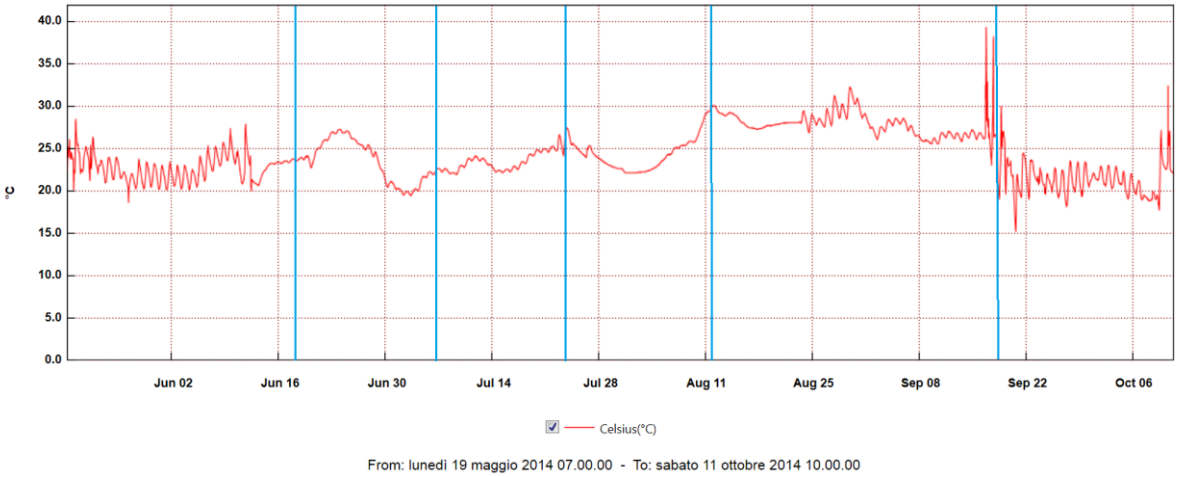

Figure 1.2. *Temperature profile during simulated transcontinental wine transportation from Italy to Brazil.*

The simulation performed is applicable to most of the wines shipped from Europe to Latin America as the wines departed from Italy (A, Figure 3) (day 1), till Hamburg Port (B, Figure 3, Germany, day 22), from where it continued the transit on day 36, arriving in Valencia on day 56 (Spain)(Figure 3, C) and reached the destination on the 92nd day, the northeast of Brazil (green mark, **Figure 1.3**) where it was docked for three days until the delivery. The period of the year in which the shipping was performed was June, hence did not have mild temperatures in neither of the hemispheres. The distance travelled by the wines between the winery and Port of destination is approximately 10.000 km.

1.2.1.3 Packaging

Transparent glass bottles with 750 ml and 1500 ml capacity adapted to screw caps and one green bottle adapted to agglomerate natural cork of 750 ml capacity were used. Brik (multilayer: 4 layers of polyethylene, paperboard and aluminum) and Bag in Box (polyethylene and ethylene vinyl alcohol copolymer) were also used, totalizing 5 different wine packages.

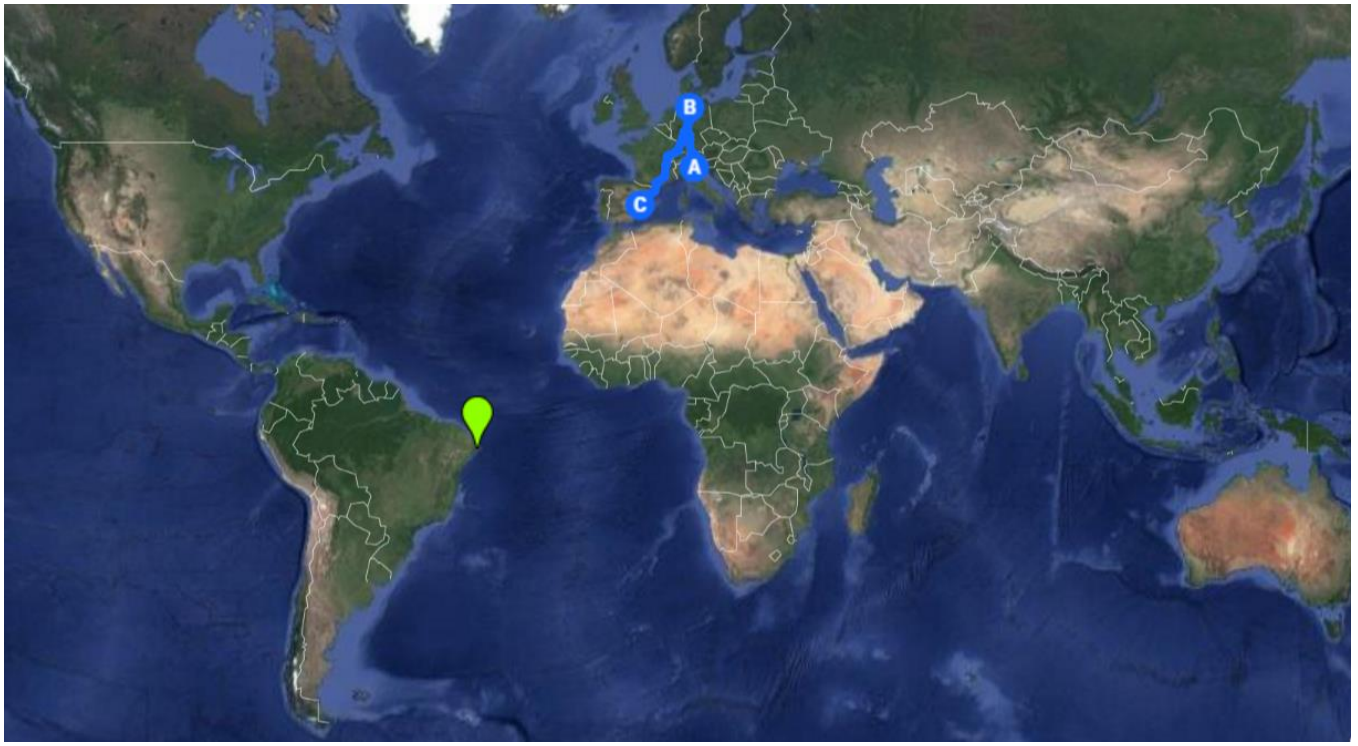

Figure 1.3. *World map with indication of the route travelled by the wine exported from Italy to Brazil. A: departure local, Italy. B: Hamburg Port, Germany. C: Valencia, Spain. Green Target: wines' destination: Brazil.*

1.2.1.4 Wine analysis

Wines were analyzed for the following parameters that are considered among the most appropriate to monitor the evolution of white wine under oxidative conditions: total and free SO_2, alcohol content, pH, total and volatile acidity were measured according to standardized methods of the "Office International de la Vigne et du Vin" (AOAC® Official MethodsSM) (OIV, 2014).

Spectrophotometric analysis

Total polyphenols were measured according to Riberéau-Gayon (1970), where the optical density of each wine was diluted in distilled water (1:100 fold) measured in a spectrophotometer at λ_{280nm} on a 10 mm quartz cuvette. The polyphenol index (TPI) was measured as the following formula: TPI = absorbance x dilution.

Browning was measured by the increase of optical density at $\lambda_{420\ nm}$ using a 10 mm quartz cuvette (SUDRAUD, 1958)

Antioxidant Activity

The wine's antioxidant activity was determined by its capacity of neutralizing the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS (2,2′-azinobis (3-

ethylbenzothiazoline6-sulfonate)). The DPPH scavenging ability was performed according to the procedure described by Brand-Williams et al. (1995). Briefly, 200 µL of wine was added to 2.8 mL of a methanol solution of the radical DPPH with concentration of 25 mg/L, and measured at λ_{515nm} after 1 h storage in the dark. Antioxidant activity was expressed as percentage (%) of scavenging activity, where 100% was considered the "blank" (methanol was used in the place of the sample). For ABTS scavenging activity the method used was as described by Re (1999), where 100 microliters of sample were added to 2.9 mL of ABTS radical solution, left for incubation for 300 minutes and measured at $\lambda_{734\ nm}$ on plastic cuvettes.

1.2.1.5 Electronic Nose Analysis

Once the wine packages were opened, 10mL of wine sample were poured in a 40mL vial and left at room temperature for equilibration to approximately thirty minutes. Then, the headspace was analyzed with a commercial portable electronic nose (PEN2 Airsense Analytics, Milano, Italy) composed of an array of 10 temperature-moderated metal-oxide sensors (MOS), a sampling system, a data acquisition device and a data processing system (**Figure 1.4**). Signal output was measured each second in the intervals of 60 seconds, which is time to most of the sensors to reach the steady state.

Figure 1.4 *Eletronic nose used in analysis (PEN 2 Airsense Analytics, Milano, Italy)*

1.2.1.6 Thermal indices

1. *Excessive thermal accumulation (ETA)*

$$ETA = \sum_{End}^{Start} Tn - 15\ °C$$

ETA: excessive thermal accumulation of the wine during transportation process [°C];
Tn – mean daily temperature of storage room / wine [°C];
ETA is based only on wine temperature accumulation above 15°C, which represents upper temperature limit for optimal wine storage (ROBINSON; HARDING, 2015; STEVENSON, 2005). Therefore, days when ETA was below 0°C shouldn't be taken into consideration.

2. *Total number of hours in certain temperature range (TNH)*

TNH 25 ≤ x < 30°C: total number of transporting hours with temperature of storage room/wine in range *25 ≤ x < 30°C*;
TNH 30 ≤ x < 35°C: total number of transporting hours with temperature of storage room/wine in range *30 ≤ x < 35°C;*
TNH 35 ≤ x <40°C: total number of transporting hours with temperature of storage room/wine in range *35 ≤ x <40°C*;
TNH ≥ 40°C: total number of transporting hours with temperature of storage room/wine ≥ 40°C.

1.2.1.7 Statistical Analysis

XLStat-PRO v. 18.07 software (Addinsoft, Ney York, NY) was used for data analysis and elaboration.

1.3 Results and Discussion

Regarding the basic enological parameters there was no different among the wines packed with different packages after the shipping temperature simulation (**Table 1.2).** Alcohol content was the same for all wines (11.7-11.8% v/v), as volatile acidity (approx. 0.25 g L^{-1} of acetic acid eq.) and total acidity (from 5.9 to 6.3 g L^{-1} tartaric acid) after shipping. In wines, which underwent oxidation, volatile acidity content were observed to be higher, which in this case did not change, indicating wines were not oxidized within the time and condition of the study.

Table 1.2. *Oenological parameters of white wines stored on different packages and submitted to shipping simulation from Italy to Brazil.*

sample number	Description	Alcohol (% v/v)	Volatile Acidity (g L^{-1})	Total Acidity (g L^{-1})
1	Screw Cap 0.75 l	11.7	0.25	5.9
2	Screw Cap 1.5 l	11.8	0.25	6.0
3	Tappo Agglomerato 0.75 l	11.8	0.25	6.3
4	Brick	11.7	0.25	5.9
5	Bag in Box 3 l	11.7	0.24	6.0

Several parameters like acidity, browning index, pH, free and total SO_2 and antioxidant activity (ABTS and DPPH scavenging activity) were measured to evaluate wine's shelf life quality. DPPH scavenging activity values as represented in **Figure 1.5,** were maximum both before and after the shipping (57 and 60%, respectively), in screw capped wine (bottle of 750mL).

Wines on TetraPak showed higher values of free and total SO_2 before shipping, when compared to other packages as can be observed from **Figure 1.6**. This tendency was similar in free SO_2 content post temperature shipping simulation. The lowest values before (114 mg L^{-1} and 22 mg L^{-1} of total and free SO_2, respectively), and after shipping simulation (116 mg L^{-1} and 23 mg L^{-1} of total and free SO_2, respectively), were found in wines packed in glass bottles and with natural corks.

As a general deduction, no significant changes in parameters which are indicative for wine oxidation were observed.

Free SO_2 in either ship or control group when comparing TetraPack and natural cork was almost half of the values respectively, indicating an important fact that it did not change due to the shipping but was rather an internal change.

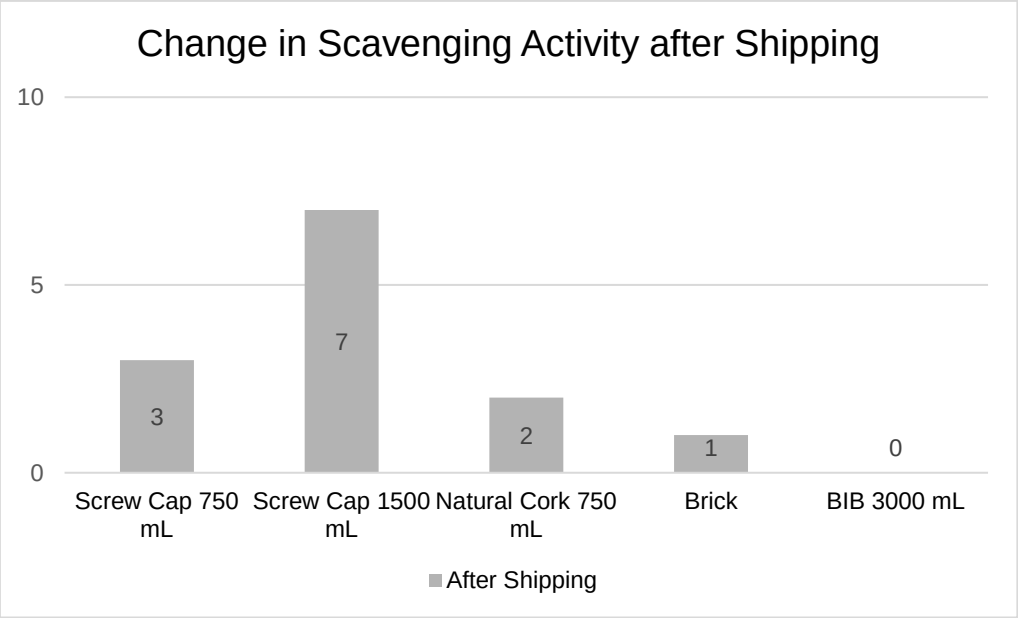

Figure 1.5. *Change in DPPH scavenging activity of wines packed in different packages after shipping simulation from Italy to Brazil in comparison to before the transport (Control) and after a shipping simulation (After Shipping) from Italy to Brazil.*

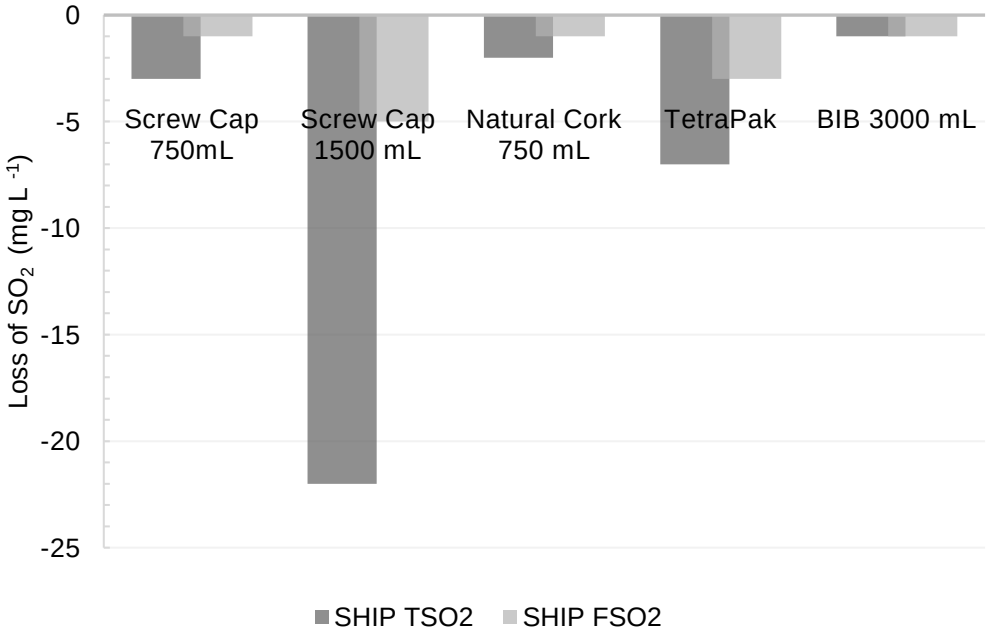

Figure 1.6. *Free and Total SO_2 content (mg L^{-1}) of wines packed in different packages before (Control) and after a shipping simulation (SHIP) from Italy to Brazil. Abbreviations: FSO_2: free SO2; TSO_2: Total SO_2; CTRL: "control wines", i.e., wines before shipping simulation; SHIP: wines submitted to shipping temperature simulation; BIB: Bag in Box.*

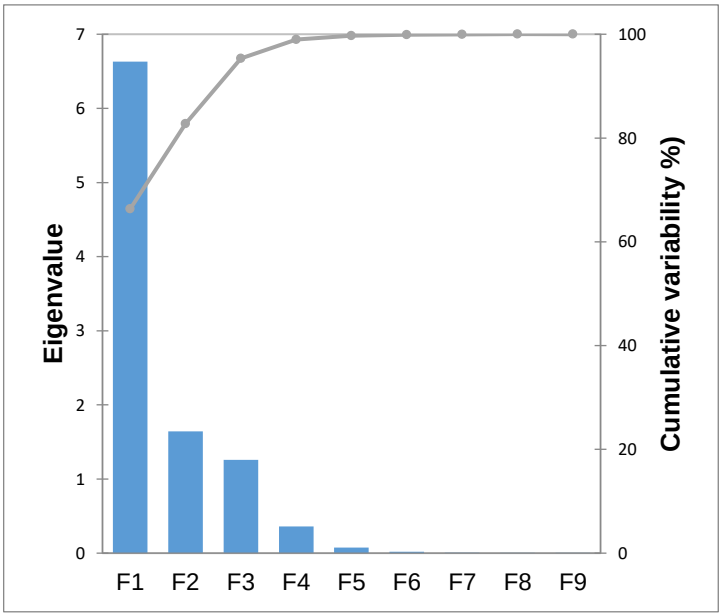

Figure 1.7. *Scree plot of Eigenvalue and cumulative variability of 9 first factors (F) obtained in principal component analysis performed of electronic nose analysis of wines in trial.*

Regarding the sensory properties, the electronic nose analysis followed by principal component analysis revealed that the first four principal component analysis explains 98.9% of the variables (**Figure 1.7** and **Table 1.3**).

Table 1.3. *Variability and cumulative values of first six principal components evaluated in the electronic nose analysis followe4d by principal component analysis of wines analyzed.*

	PC1	PC2	PC3	PC4	PC5	PC6
Variability (%)	66.3	16.4	12.6	3.6	0.8	0.2
Cumulative %	66.3	82.7	95.3	98.9	99.7	99.9

The projection of the samples along the directions identified by the first two principal components, i.e., a plot of the scores (PC_1 vs PC_2), along with the sensors in which are responsible for their plot **(Figure 1.8)**. It is apparent that the samples are grouped according to their shipping (CTRL and SHIP) when the analyses throughout the PC_1, in a way that reflects their storage in shipping temperatures, that is, samples that have lower scores are those which suffered the shipping stress, while those which have remained in constant low temperature of storage for the same time, present higher scores, except for the screw cap wine 750 ml control wine, in which can be due to the fact that screw caps allow practically no oxygen permeability through the wine.

Wines bottled in natural cork glass bottle were in opposite quadrants, indicating wines that suffered more change in volatile during shipping, and as it can be seen in the graphic, two sensors (8 and 9) were able to capture this change. Besides that, as mentioned previously, wines packed in bottle and natural cork were those presenting the lowest SO_2 values, indicating less protection against oxidative damage.

This comparison of scores can probably shows us that PC_1 is responsible for the compounds in which concentrations decrease more rapidly with storage time, although further studies are necessary to properly identify them, notwithstanding electronic nose analysis can give indicative results.

It is noteworthy that the different packaging grouped into two groups according to the vector of change (i.e. PC): (i) screwcap and Tetrapack mainly moved along the PC_1, whereas (ii) BIB and cork mostly moved along the PC_2. This most probably imply a different evolution of the wines according to the packaging conditions.

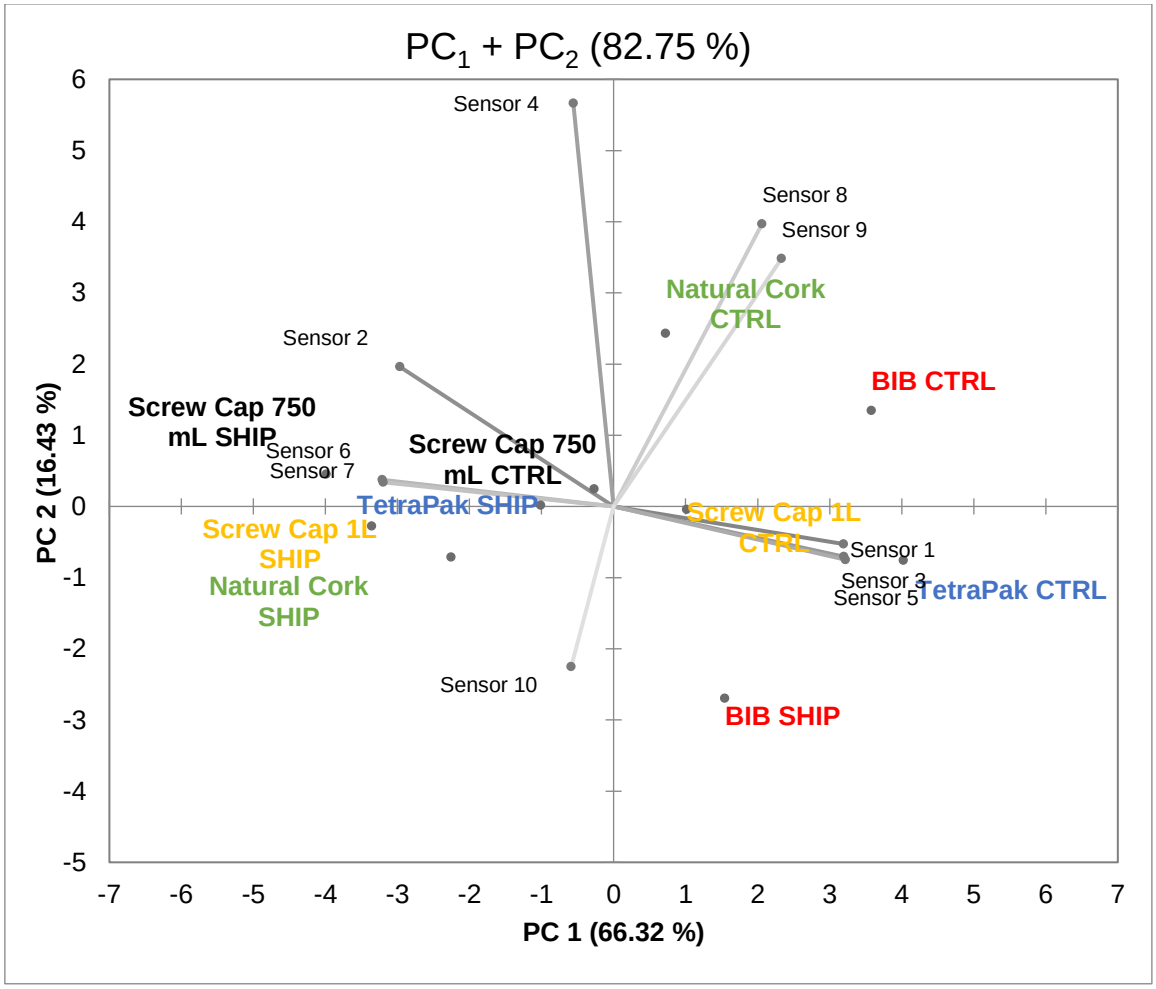

Figure 1.8. *Scores biplot of the wines using the two first principal components obtained by PCA.*

It is important to notice that only the electronic nose could detect such changes, since the parameters evaluated regarding oxidative damage to wine did not reveal changes indicative of oxidative stress.

During simulation of wine shipping from Italy to Brazil, wines were exposed to total excessive heat accumulation of 1339.4°C, with maximum, mean and minimum wine temperatures at 39.3°, 24.3° and 15.3°C, respectively (**Table 1.4; Figure 1.2**). The optimal temperature for wine storage is noticeably lower comparing to wine temperatures during simulated transporting conditions, thus it is possible that excessive heat had an influence on wine sensory characteristic analyzed by electronic nose in presented study. This is consistent with other studies which reported a significant influence of excessive heat accumulation on wine sensory when in the magnitude of ~520°C (ROBINSON; HARDING, 2015) and ~1000°C (DU TOIT; PIQUET, 2014). Even though is evident from the literature that excessive heat accumulation has significant impact on wine quality during transportation or storage, it still remains unclear which is the minimum excessive heat condition which could cause the detrimental influence on wine quality. Thus, it is necessary to conduct a study which includes continuous (*e.g.* on daily basis) control of thermo-sensitive components relevant for wine sensory with appropriate equipment (*e.g.* electronic nose).

Table 1.4. *Thermal indices during wine shipping simulation from Italy to Brazil.*

Thermal index	Temp max [°C]	Temp Mean [°C]	Temp min [°C]	ETA [°C]	TNH 25 ≤ x < 30 [h]	TNH 30 ≤ x < 35 [h]	TNH 35 ≤ x < 40 [h]	TNH x ≥ 40 [h]
	39.3	24.3	15.3	1339.4	1280	66	3	0

With presented design of the experiment, wines were mostly exposed to temperature fluctuations from 25° to 30°C (1280 hours or 53.3 days) and for a short period to temperature > 30°C (69 hours or 2.9 days) (**Table 1.4**). Therefore, it could be possible that sufficiently long (1280 hours) temperature fluctuation in the range from 25° to 30°C may alter the sensory characteristics of wines. However, other studies reported that during the relatively shorter period (~ 500h) of temperature fluctuations in the range from 25° to 35°C didn't significantly change the wine sensory characteristics (ROBINSON; HARDING, 2015). Other study conducted wine shipping simulation from

Southern Africa to Europe with temperature fluctuations in the total duration of ~1100 h, whereas wines were exposed to temperatures higher than optimal for a short period (20°, 30° and 37°C for 56 h each). Authors reported that during wine shipping trial wine sensory characteristics didn't significantly change, with the conclusion that average temperature comparing to temperature fluctuations, has a greater impact on wine sensory modifications during transportation (DU TOIT; PIQUET, 2014). Even though several studies reported the low impact of temperature fluctuations on wine sensory modifications during relatively short-term transportation (DU TOIT; PIQUET, 2014; ROBINSON et al., 2010) it is necessary to conduct more studies in order to better understand the possible impact of temperature fluctuations during relatively long-term transportations (*e.g.* Italy-Brazil). The study would need to include trials with constant temperature (*e.g.* 25°C) and trials with temperature fluctuations that have an equal average temperature (25°C). Thermo-sensitive components relevant for wine sensory should be controlled continuously with appropriate equipment (*e.g.* electronic nose). Trials would also need to include several levels of constant temperature (*e.g.* 25°, 35°, 40 °C) with following trials of temperature fluctuations.

Suggested indexes (ETA and TNH), are only based on wine temperature measurements, while other variables relevant for the potential wine quality modifications (*e.g.* light, package, variety, free SO_2, etc.) during the transportation are excluded. Thus, additional studies could be conducted in order to improve suggested indices, whereas relevant variables (*e.g.* light, package, variety, free SO_2, etc.) for wine sensory modifications during transportation should be included.

Moreover, the findings in this study can be corroborated by previous studies, as previously seen by Leinberger (2006) and Paffard & Dean (2002) that temperature changes at sea are gradual, occurring over days rather than hours, consequently the temperature affecting mostly wines are in land, since daily temperature differences can be extreme on land. Also, according to their study, temperatures in containers on the same ship are usually identical unless one of them is above deck. But even for above deck containers, the temperature variations are smaller than on land.

1.4. CONCLUSIONS

In conclusion, this study highlighted the performance of the selected packages tested, which are currently a trend in the market, by providing an effective and distinctive

protection against thermal stress during simulate shipping, which could further affect the wine quality under more severe conditions. Obviously, the packaging strategy of each winery depends on this scientific evidence, but also on marketing and budget available. Although the white wine tested was ‘designed’ for export – which usually includes medium-long transport, therefore a little to any variation in composition was expected – some changes on the volatile composition of wines were detected by the electronic nose sensors.

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CHAPTER 2

CHAPTER 2

THE PERMEABILITY CLOSURES STUDY

The use of synthetic closures with different permeability and their effect in wine oxidation

Abstract

With the increase of worldwide transport of wines, along with the awareness of consumers regarding wine quality, wine industry has become more concerned with the effects of corks or synthetic closures on final wine quality. The use of synthetic closures with specific oxygen permeability have become an option for winemakers to control the evolution of wine after bottling, but there are few data presented in the literature regarding evolution of wines under these features, and so far, none concerning biodynamic or organic wines. The aim of this study was to verify the hypothesis that closures with different permeability could significantly affect the composition of wines. In this view, the evolution with time of organic and biodynamic Sangiovese red wines –from two different vintages and fermented with two conditions, then bottled with three synthetic closures with different permeability – was monitored. It was found that, the antioxidant activity of wines increased with time, along with polymeric fraction. Among wines tested, the biodynamic agriculture derived wine, of year 2013 with the closure with permeability 700 presented sensory differences in comparison to others, suggesting being more susceptible to oxidation than those organic wines from the same vintage.

2.1. Introduction

As previously mentioned in the general introduction, oxidation of commercial bottled wines have been reported to have raised in the last few years, especially during storage conditions (WATERS et al., 1996). Although the increase of this phenomena has been recently studied, this problem has probably always occurred.

Oxidation phenomena of wines after bottling is generally irreversible and commercially detrimental since the final quality of wine is reduced. Apart from obvious failure of wine closures, oxidation usually occur at bottling due to dissolved oxygen in the wine and oxygen in the headspace (CASEY, 1992).

Synthetic closures have been used to seal wine as an alternative to avoid cork taint, and other microbiological issues in wine after they are bottled as to enable oxygen management, since they have their porosity controlled during their manufacture. Moreover, these synthetic materials can allow the control of some parameters, such as the oxygen transfer rate (OTR) in the bottle and to adapt to wines necessity.

Italian authorities have approved the use of synthetic closure and screwcaps on higher quality DOC and DOCG wines for the first time in the year of 2012 opening a new front in the highly-charged wine industry debate on closures. Regardless its approval, scientific community has started studying the effect of synthetic closures to seal wine since the 90's (BURNS, 1999; MURRAY; LOCKSHIN, 1997; NOEL; LAUER, 1999), and there have been studies indicating that synthetic closures may, or not, substitute natural cork in wine bottling, comparing the wine shelf life in both cases.

Studies revealed that the consumer of wine were not able to ascertain in sensory blind test the differences among two wines bottled with natural cork, synthetic closure or other closures type (MARIN; DURHAM, 2007). Moreover, when these consumers had the knowledge that these two wines (one white and one red) were bottled with different closures, their perception did not change when evaluating the white wine, but when tasting the red wine having the information regarding the closures, the scores tended to be lower in the wine closed with synthetic closure, as the wine closed with screw cap had the lowest rating and quality scores when compared to the wine sealed with natural cork.

Thus, it can be affirmed that the sensory perception of wines sealed with synthetic closures cannot be differed from natural cork when the assayer does not know the closure in which the wine was bottled, whereas the type of closure has more

psychological impact than in the wine quality per se.

On the other hand, another study (SKOUROUMOUNIS et al., 2005) which evaluated the development of a Riesling and a wooded Chardonnay wine over five years bottled with natural cork, synthetic closure and screw cap concluded that the wines closed with synthetic closures had a relatively oxidized aroma, as their chemical parameters pointed it, since they developed browning and low content of sulfur dioxide, when comparing these wines to those wines sealed with closures. Only the wines sealed with natural cork, presented negligible reduced characters.

Based on these contradictory information, it is important to highlight that independently on the aroma, the primary compounds intrinsically tangled in wine oxidation process are: oxygen, polyphenolic compounds and metal ions (Fe^{2+}, Cu^{2+}, Mn^{2+}). Briefly, oxygen starts the process which is catalyzed by the metal ions above mentioned, followed by the polyphenolic compounds, which are the main oxidable substrates (as the precursor of the browning occurrence). Externally temperature and light also affect the process (MACÍAS; PINA; PÉREZ RODRÍGUEZ, 2001; SILVA FERREIRA; HOGG; GUEDES DE PINHO, 2003).

Oxygen is therefore the triggering factor and its content is mainly responsible for the levels of dissolved oxygen in bottling wines, which is under control of the wine producer, as the oxygen levels which ingress in the bottle, in which is directly affected by the closure, as the oxygen ingress in the bottle, the levels of this element in the headspace increase and consequently the dissolved oxygen content (CALOGHIRIS; WATERS; WILLIAMS, 1997; GODDEN et al., 2001; WATERS et al., 1996; WATERS; WILLIAMS, 1997). Therefore, the choice of closure type has an important impact on the extent of wine oxidation.

Moreover, most of wines sold in the market nowadays are intended to be consumed within two years after bottling, and thus as for the reasons mentioned above, as a part of this PhD thesis, we aimed to evaluate the content of oxygen in which pass through synthetic closures with different permeability in order to compare with the manufactory's information, as to evaluate the consequences of it during 650 days after bottling.

2.2. The Study

As part of this PhD project, we tried to understand the effect of oxidation on Sangiovese biodynamic red wine during a long-term study focusing on elucidation of chemical reactions leading to wine modification by oxidation by-products and impact of oxygen permeation through synthetic closures with different permeability.

As the claim of sustainable production have been increasing exponentially in the last decades around the world (GREENE, 2000; HAMM; MICHELSEN, 2000), the trials was focused on organic and biodynamic wines.

For that, the study was conducted according to **Figure 2.1.** Eight Sangiovese red wines, two biodynamic (BDN) and two organic (BIO), in which each one of them underwent through spontaneous fermentation (SPO) or were fermented with selected yeast (LSA) over the years of 2012 and 2013. Samples were bottled in glass using synthetic closures with selected permeability.

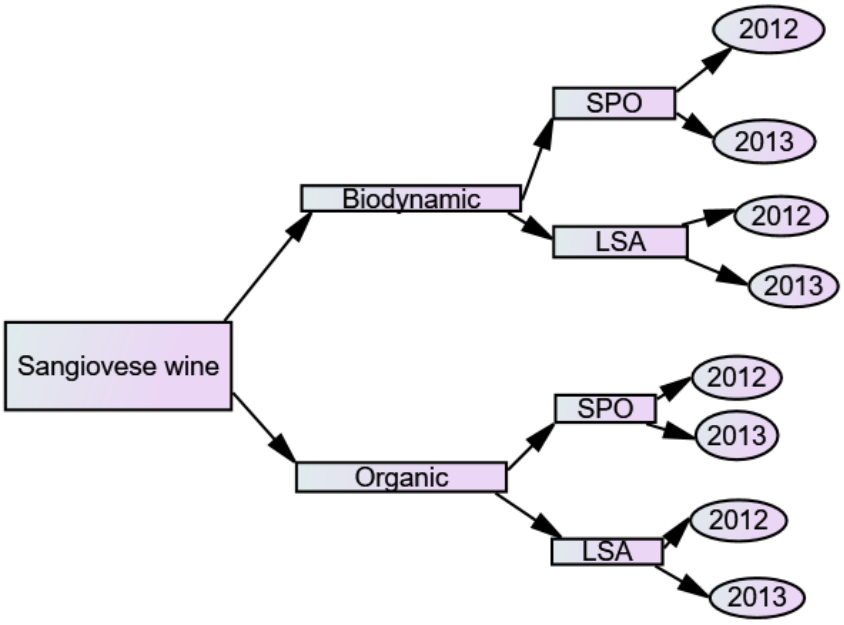

Figure 2.1. *Experimental design for the evaluation of the post-bottling oxidation of Organic and Biodynamic red wines sealed with synthetic closures with selected oxygen permeability. Abbreviations: SPO (spontaneous fermentation); LSA (wine fermented by selected yeasts).*

2.3. Materials and Methods

2.3.1 Wines

The eight red wines above mentioned were produced from grapes (ca. 200 kg each trials) harvested, processed (**Figure 2.2**) and bottled (**Figure 2.3**) at the Technological Pole of Tebano (Faenza, RA, Emilia Romagna region, Italy).

Figure 2.2. *Two of the tanks used for Microvinification of wines used in this study*

Figure 2.3 *Bottling process of wines of the study*

Figure 2.4. Insertion of PreSens Pst3 oxygen sensors

2.3.2 Closures

Four synthetic commercial closures with different oxygen permeability were used, according to the manufactory (Nomacorc), as it is stated in **Table 2.1**:

Table 2.1. *Manufactory's information regarding gas permeability and code of synthetic closures used in the present study.*

CODE	100	300 – 300B	700
Diameter	24 mm	24 mm	23 mm
Length	38, 44, 47 mm	38, 44, 47 mm	38, 44, 47 mm
Foam Density	0.261 $cm^{3\ -1}$	0.261 $cm^{3\ -1}$	0.306 g $cm^{3\ -1}$
Total Density	0.328 $cm^{3\ -1}$	0.328 $cm^{3\ -1}$	0.357 $cm^{3\ -1}$
Oxygen Ingress (mg Per Bottle)	0.37 mg of O_2 after 3 months 0.64 mg of O_2 after 6 months 1.2 mg of O_2 after 12 months 1.1 mg of O_2 yearly after the first year	1.35 mg of O_2 after 3 months 1.79 mg di O_2 after 6 months 2.4 mg of O_2 after 2 months 1.1 mg of O_2 yearly after the first year	1.72 mg of O_2 after 3 months 2.29 mg of O_2 after 6 months 3,4 mg of O_2 after 2 months 2.1 mg of O_2 yearly after the first year

2.3.3 Oxygen Ingress Measurement

Three wines were closed with different closures and have had their oxygen content (headspace and dissolved oxygen) controlled during the entire period of the study with Presens Fibox 4 with a sensor type PSt3a (PreSens GmbH, Regensburg, Germany). For the measurements of dissolved and headspace oxygen content, before the bottles was filled with wine, two PreSens Pst3 oxygen sensors (Presens, Regensburg, Germany), were inserted, one in the top and other in the middle of the bottle, according to demonstrated in the **Figure 2.4.**

These same bottles were used to monitor dissolved oxygen during storage of the wines under the different experimental conditions.

2.3.4 Chemicals and reagents

The following chemicals and reagents were from commercial source: methanol, acetonitrile, sodium disulfide and acetaldehyde (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).

Bovine serum albumin (BSA, fraction V, lyophilized powder), sodium dodecyl sulphate (SDS; lauryl sulphate, sodium salt, 95%), triethanolamine (TEA, 98%), $FeCl_3.6H_2O$ (98%) used for the Adams Harbertson's assay were purchased from Sigma (Sigma-Aldrich, Saint Louis, MO).

2.3.5 Antioxidant Activity

The wine's antioxidant activity was determined by its capacity of scavenging the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and it was performed according to the procedure described by Brand-Williams *et al*. (BRAND-WILLIAMS; CUVELIER; BERSET, 1995) (1995). Briefly, 200 µL of wine was added to 3.0 mL of a methanol solution of the radical DPPH with concentration of 25 mg/L, and measured at λ_{515nm} after 1 h storage in the dark. Antioxidant activity was expressed as % of scavenging activity, where 100% was considered the "blank" (methanol was used in the place of the sample). Measurements were performed in a Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan) and expressed as percentage of inhibition (DUDONNÉ et al., 2009) as it follows:

% DPPH scavenging activity = [(AbsDPPH – Abstannin) / AbsDPPH)] x 100

Where:

AbsDPPH is the absorbance measurement of DPPH solution (2.9 mL) with 0.1 mL of methanol;
Abstannin is the absorbance measurement of DPPH solution (2.9 mL) with 0.1 mL of tannin solution above mentioned.

2.3.6 Total Polyphenols Index

Polyphenolic Index was calculated according to previously described (PARDO et al., 1999), where 1 mL of wine was added to 99 mL of distilled water and the absorbance was measured at λ_{280nm} in a 10 mm quartz cuvette at Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan). The index was calculated by multiplying the absorbance per 100 (dilution).

2.3.7 Free and Total SO_2; Alcohol content; pH; Total and Volatile acidity

Oenological parameters were measured according to standardized methods of the "Office International de la Vigne et du Vin" (AOAC® Official MethodsSM) (OIV, 2014).

2.3.8 Total color; Copigmentation; SO_2 resistant pigments; Total anthocyanins

Before analysis, each wine had the pH adjusted to 3.6. Than total color, copigmentation, SO_2 resistant pigment and total anthocyanins were all measured according to Boulton et al. 1999

2.3.9 HPLC Analysis

A 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 DX600, Milano, Italy) was used for identification and quantification of 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. Knauer C18 polar endcapped column (length 150 x 3 mm with precolumn) (~ 50% hydrophilic endcaping; Eurospher II, Berlin, Germany) was used using the following mobile phases: solvent A (CH_3COOH: H_2O 1:20 v/v) and solvent B (CH_3CN: H_2O, 4:1, v/v), at flow rate of 0.5 $mLmin^{-1}$.

Each chromatographic run had 90 minutes and the proportions between the eluents are presented in **Table 2.2**.

Table 2.2. Eluents composition during the HPLC analysis

Retention time (minutes)	Eluent A (%)	Eluent B (%)
0	100	0
15	100	0
30	95	5
65	90	10
70	90	10
77	70	30
80	100	0
85	100	0

Protocatecuic acid, p-hydroxybenzoic and vanillic acid were quantified at 256 nm, gallic acid, syringic acid, (+)-catechin and (-)-epicatechin at 280 nm, whereas p-coumaric acid and coutaric acid at 308 nm, and caftaric acid and caffeic acid at 324 nm and rutin and quercetin at 365 nm.

2.3.10 Electronic nose

Once wines were open, 10 mL of wine sample were poured versed in a 40 mL vial and left at room there in environmental temperature for equilibration for approx. thirty minutes. Then, the headspace was analyzed with a commercial portable electronic nose PEN2 (Airsense Analytics, Milano, Italy) composed of an array of 10 temperature-moderated metal-oxide sensors (MOS), a sampling system, a data acquisition device and a data processing system. Signal output was measured each second in the intervals of 60 seconds, which is time to most of the sensors to reach the steady state.

2.3.11 Flash Gas Chromatography

For volatile fraction analysis, Flash Gas Chromatography Electronic Nose (Heracles, Alpha MOS®) was used. Two milliliters of each wine samples were pipetted in vials and immediately closed. They were then placed in the refrigerator (4-6°C) until analysis time. Before the analysis, samples were incubated for 20 minutes at 40°C in an agitator at 500 rpm.

The injection volume used was 1000 µl by 100 µl per second, with the injection temperature of 200°C for 15 seconds and 10 kPa of pressure. Initial trapping temperature was 40°C, split mode (10mL / second) for 60 seconds, with 60 kPa of pressure. The programmed temperature and pressure was isotherm mode of 240°C per 93 seconds, 80 kPa of pressure and 10mL per min of split mode. Valve temperature was set to 250°C and the initial oven temperature was 50°C (2 seconds), than temperature was set to increase by the rate of 2°C per second until 120°C, than 5°C per second, until 280°C. The chromatograph was equipped with two columns: MXT®-5 Columns and MXT®-1701 Columns (RESTEK®, Bellefonte, PA, USA). Each column have one Flame Ionization Detectors (FID), both at 280°C.

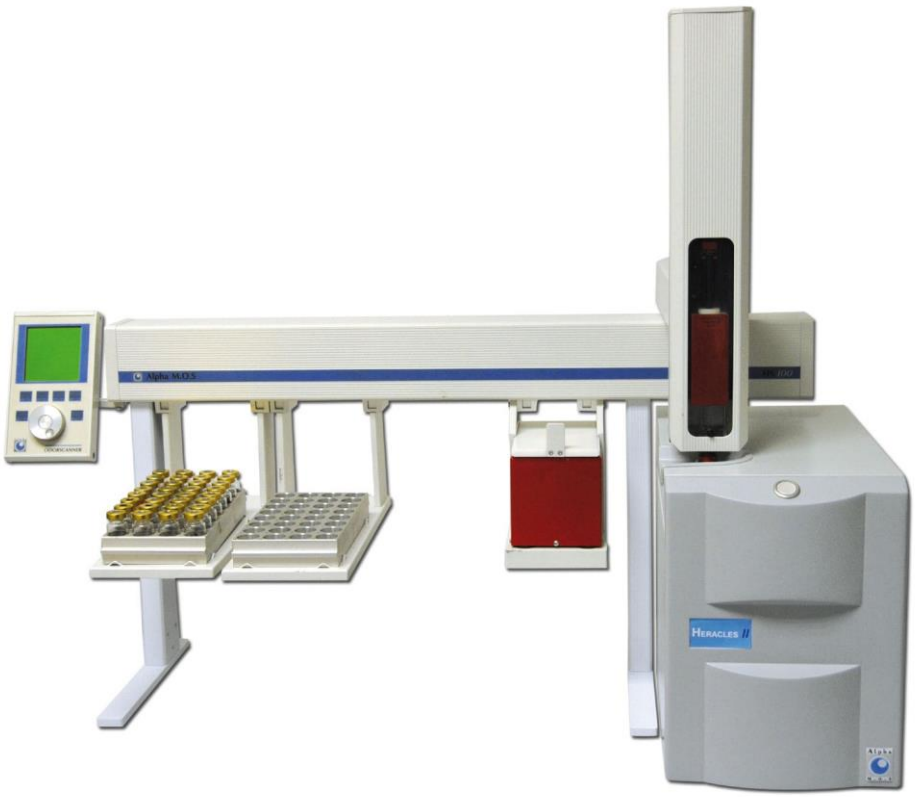

Figure 2.5 *Alpha MOS HERACLES Flash Gas Chromatography Electronic Nose*

2.3.12 Statistical Analysis

Statistical treatments and data management were performed using the XLSTAT Software, Version 2017.1 and Statistica version 8 (STATSOFT).

2.3.12.1 Modelling of Kinetic data

a) Sulphur Dioxide and Oxygen

The data for sulphur dioxide and oxygen losses recorded were subsequently fitted into zero order and first order reactions respectively, in order to derive their reaction rate (*k*). The equations presented below are the arbitrary formulae used for the proposed purpose;

Zero order reaction: $[A]=-kt+[A]_0$

First order reaction: $A = A_0 + kt$

Where;

A= concentration at time t

A_0= initial concentration

K= rate constant

t= time

b) Oxygen

For modelling the data on dissolved and head space oxygen, an effort has been made to utilize Peleg's model, primarily proposed for predicting water sorption kinetics in foods. The main advantage of using Peleg's model is its capacity to predict using short time experimental data, without any set criteria for selecting the initial and final data sets. However, this model is found to be adaptable to different parameters in studying food processing and shelf life (CHECMAREV; CASALES; YEANNES, 2013; CORZO; BRACHO, 2006; MIŠLJENOVIĆ et al., 2011), an attempt has been made herewith to understand its suitability and ability to predict the rate of loss of oxygen in bottled wines during a storage period of 120 days for both dissolved and headspace oxygen levels. This non-exponential model makes use of the differential concentration between the initial time and a given time of experiment under consideration to calculate the two parameters: k_1 which is inversely proportional to the initial rate of reaction and k_2 which relates to the minimum attainable level of oxygen at equilibrium or infinite point of time.

$$x_t - x_0 = -t/(k_1 + k_2 * t)$$

Where,

x_t = concentration at time 't'

x_0 = concentration at time '0'

k_1 = Peleg's rate constant

k_2 = Peleg's capacity constant

2.4. Results and Discussion

Figure 2.6 represents graphically the data obtained of oxygen ingress into the wines with diverse synthetic closure of different permeability during storage of 650 days in vertical position.

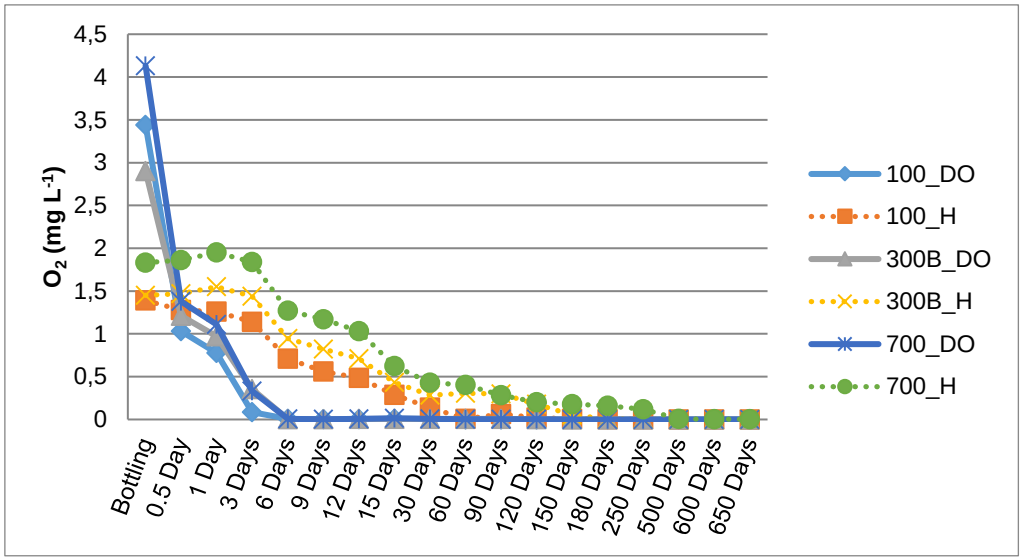

Figure 2.6. *Sangiovese red wine: storage trials. Oxygen measurements with time for Sangiovese red wine bottled with different synthetic closures permeability. Abbrev.: _DO: dissolved oxygen; _H: headspace oxygen content.*

As it can be observed, the dissolved oxygen content had a sharp decrease in the values irrespective of cork type during the first six days after bottling. The levels of head space oxygen, whereas, had a gradual reduction for the whole storage period, but more evident during the initial 150 days. However, it is of interest to note that the initial levels of both dissolved and head space oxygen varied between different closures, with 700 having the highest levels followed by 100 and 300.

This information suggests that the initial dissolved oxygen was consumed by the wines in three days. It can also be seen that before stabilizing, the oxygen permeation inside the bottles through the closures followed the manufacturer's information, where 100<300<700.

Kinetic models are widely utilized to evaluate the effects of time and temperature on the wine component concentrations, particularly for oxidative reactions which affects the phenolic composition of wines (MARTINS; MONFORTE; SILVA FERREIRA, 2013; OLIVEIRA et al., 2015). However, most of the literature as cited describes the kinetic of compound depletion as a zero order reaction ($c=c_0 + kt$). If a reaction is outwardly independent of the initial reactant concentration, the zero order may be applicable. However, when the kinetic of reaction depends on the initial concentration of a certain compound, it is assumed to be a first order reaction [$c=c_0 \exp(kt)$], where there is a dependence of the rate constant k upon the time (t) or temperature T, or further by a second order ($1/c = 1/c_0 + kt$) reaction model.

Therefore, following what was already approached in the literature, an attempt to apply the first-order kinetic reaction was made within dissolved and headspace oxygen, aiming to evaluate the different closures permeability (**Table 2.3**).

Table 2.3. *Regression equation, constant (k) and correlation index of headspace and dissolved oxygen contents according time within three different cork permeability.*

CORK PERMEABILITY	REGRESSION EQUATION (HS)	*k*	R^2	REGRESSION EQUATION (DO)	*k*	R^2
100	y= -0.0164x – 0.091	-0.016	0.7304	y = -0,0206x - 1,1973	-0.021	0,4046
300	y= -0.0072x + 0.0175	-0.007	0.7273	y = -0,0155x - 1,1104	-0.015	0,2641
700	y= -0.0081x + 0.1546	-0.008	0.8385	y = -0,0167x - 1,0344	-0.017	0,2998

Data was initially presented as a differential equation. Instead of using this representation, we used integrational representation. Therefore, to apply first order reaction kinetics, data was transformed into logarithm for fitting into the first order reaction equation using linear regression.

The rate constant (*k*), is a proportionality constant for a given reaction and the reaction rate is dependent on the concentration of the reactants as well as the rate constant. **Table 2.3**, represents the *k* values of the analyzed wines for which the R^2 values, in general, ranges from -0.007 to -0.016 for headspace oxygen indicating a good fit of data. Whereas, these R^2 values are much lower from -0.015 to -0.021 for dissolved oxygen indicating that the chosen model does not completely explain the behavior of DO during storage.

The head space oxygen increases initially with the time, whereas the dissolved oxygen in contrast remains relatively stable. However, after a prolonged storage time the dissolved oxygen content increases due to the dissolution of the headspace oxygen in the wine. Furthermore, it can be observed from the values that the HS_O_2 values are relatively stable with an increase in the DO values.

Given the figures **Figure 2.7, Figure 2.8, Figure 2.9,** plotted as time versus log values of concentrations of O_2, a correlation between the levels of headspace and the dissolved oxygen can be observed. This behavior can be explained by the fact that once the oxygen pass through the closure into the bottle, in which will depend on its gas permeability, it is going to remain in the headspace between the wine and bottle, and thus slowly get into the wine. It can be noticed from **Table 2.3** that this diffusion is

not linear, particularly because it relies mostly in the capacity of wine to react with the dissolved oxygen, which is linked to its metals (iron and copper), polyphenolic content, and the sulphur dioxide content as well.

As a general conclusion for the first-order kinetic reactions, for *k* values of dissolved oxygen, it can be observed that closure '100' had the highest rate constant, followed by closure *700*, and then *300*. It indicates that the rate of O_2 ingress was lower in closure *100* as observed for headspace oxygen content as well, followed by closure *700* and *300*.

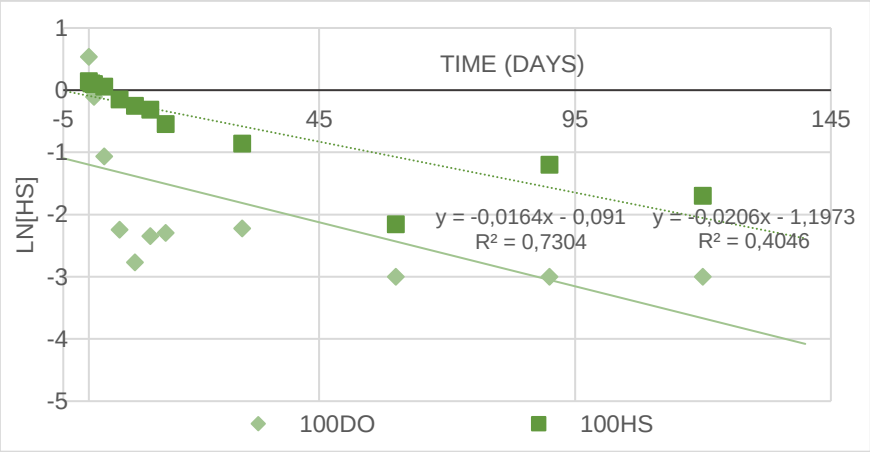

Figure 2.7. *Logarithm of K per time of dissolved and headspace oxygen one closure with 100 permeability.*

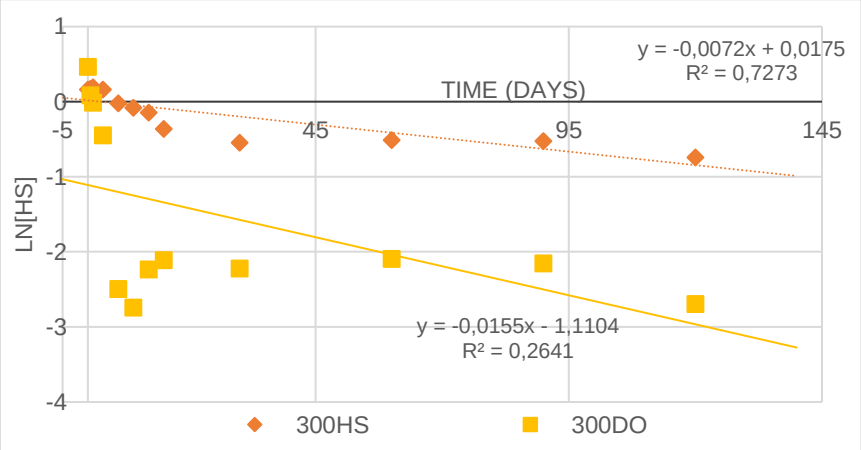

Figure 2.8. *Logarithm of k per time of dissolved and headspace oxygen one closure with 300 permeability.*

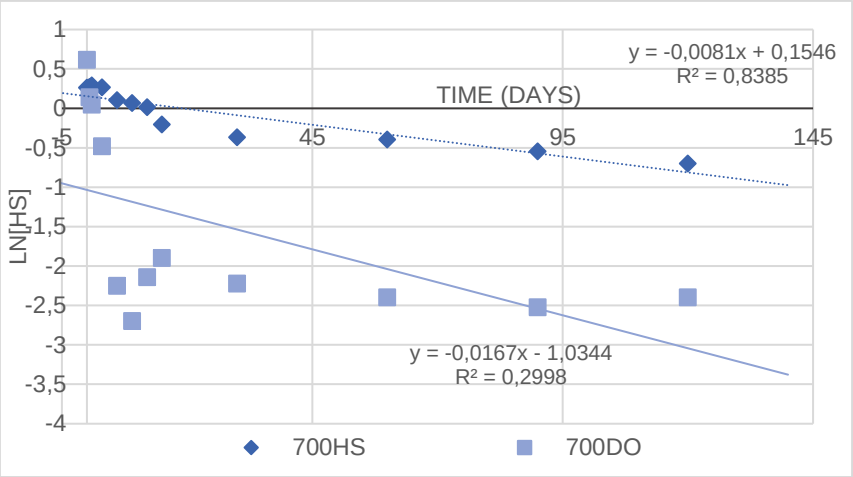

Figure 2.9. *Logarithm of k per time of dissolved and headspace oxygen one closure with 700 permeability.*

Also, *k* values for headspace oxygen is higher for closure coded 100, indicating that the rate of ingress of oxygen is higher in comparison to the closure 300, with value of -0.0022 and closure 700, with a value of -0.0081. These results are in contrast to what was expected form the manufacturer's information, closure 300 had the highest rate of oxygen dissipation from the headspace.

It is of interest to note that although the absolute values of rate of dissolved oxygen constants are not significant, the values are concurrent to those obtained for headspace oxygen. Moreover, from the model, it can be stated that the rate constant is inversely proportional to the oxygen content.

Furthermore, still considering this model, it can be observed from **Figures 2.7, 2.8** and **2.9** that the headspace oxygen content linearly decreased over time, whereas the dissolved oxygen content had no particular trend, due to wines' antioxidant capacity. Therefore, the dissolved oxygen, at a given point of time, is influenced by the availability and rate of headspace oxygen migration into wine.

Since the first-order kinetic reaction did not properly fir our data, another attempt was made for modelling wine oxidation within different closures permeability, by the application of Peleg's model. This model have been used in scientific studies approaching dehydration (CHECMAREV; CASALES; YEANNES, 2013; CORZO; BRACHO, 2006; MIŠLJENOVIĆ et al., 2011), water absorption (TURHAN; SAYAR; GUNASEKARAN, 2002) in food matrix, but so far, there are not studies in the literature applying this model to wine.

The results of headspace and dissolved oxygen kinetic along time, following Peleg's model, is presented in **Table 2.4.**

Table 2.4. *Rate and capacity constants derived by Peleg's model, for dissolved and headspace oxygen for wines bottled with closures with different permeability*.

closure	100	300B	700
HEADSPACE			
k_1	5.173*	7.970*	7.912*
k_2	0.660*	0.707*	0.536*
r^2	0.99	0.96	0.97
DISSOLVED OXYGEN			
k_1	0.069*	0.144*	0.071*
k_2	0.286*	0.334*	0.239*
r^2	0.99	0.99	0.99

**p<0.05 indicates significance.*

The r^2 (> 0.96) value indicates that the model exhibits a good fit with the experimental data for both headspace and dissolved oxygen. From table 1 it can be observed that the k_1 values for headspace oxygen follow the order 100<700≤300B and for k_2 follow an order 700<100<300B. As k_1 is inversely proportional to the initial rate of loss of oxygen whereas k_2 relates to the minimum attainable oxygen at infinite time, in this case at 120 days. Considering the values, it can be proposed that the initial rate of oxygen ingress is relatively similar for the closures 300B and 700, whereas the highest values are observed in the closure 100. However, towards the end of storage period, oxygen ingress rate is highest in 700 closure, followed by closure 100, with the lowest values being recorded in 300B closure (**Figure 2.10**).

Moreover, the values of constants for dissolved oxygen levels can be observed from **Table 2.4**, in which k_1 values follow the order 100≤700<300B and for k_2 follow an order 700<100<300B. Considering the constants obtained for dissolved oxygen, it can be observed that the initial rate of oxygen consumption is similar in closures 100 and 700, whereas this value is approximately two times lower in 300B closure. Towards the end of storage time it can be noticed that the dissolved oxygen levels are more depleted in 700 closure followed by 100. A higher k_2 in 300B indicates that the final oxygen content is the lowest in comparison to other closures (**Figure 2.11**).

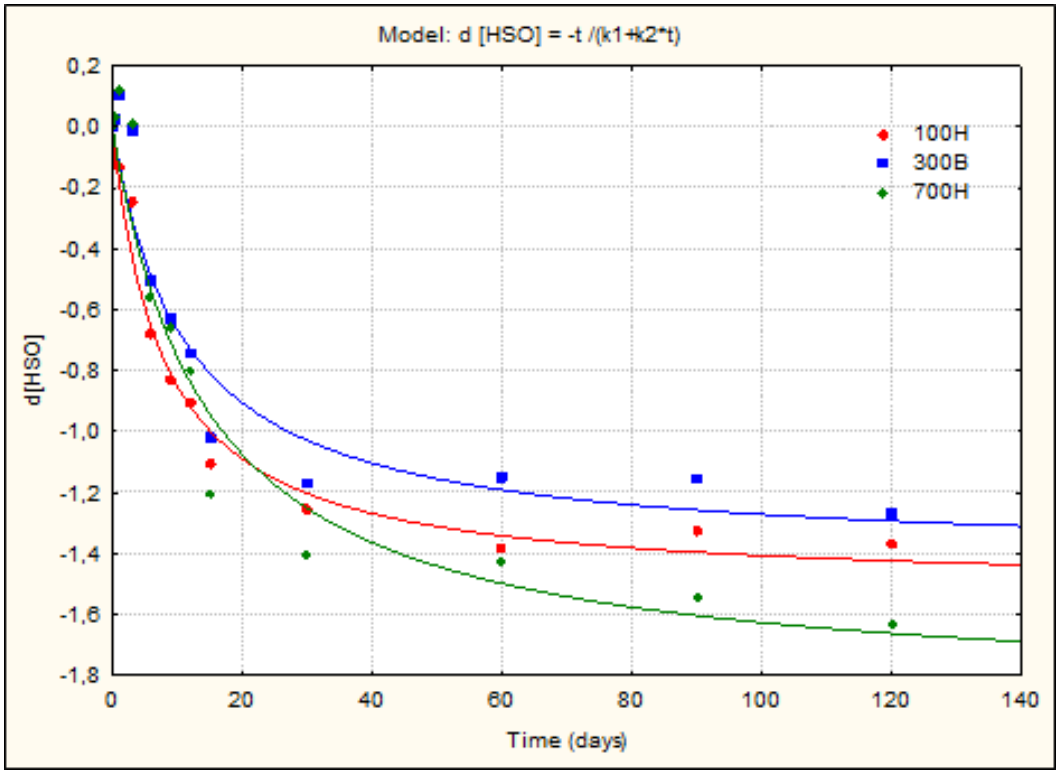

Figure 2.10. *Headspace oxygen content (Δx) of bottled wines with different closures in relation to storage time as fitted by Peleg's Model.*

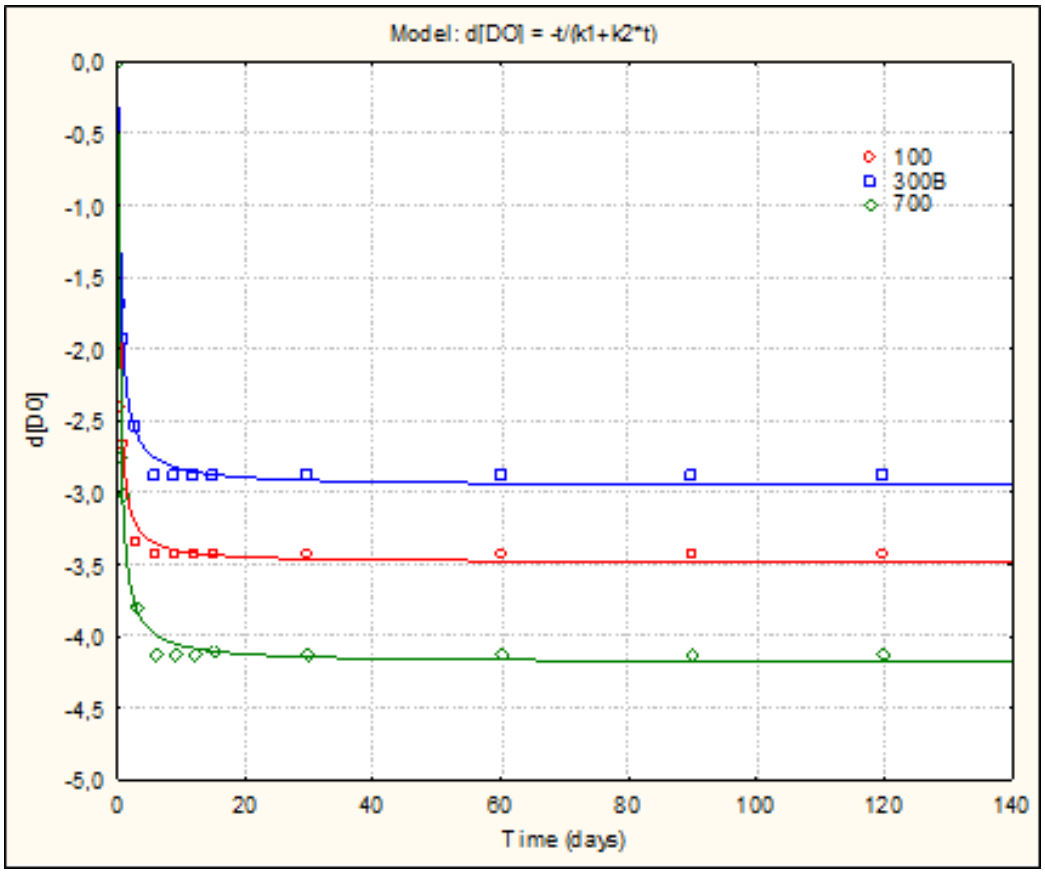

Figure 2.11. *Dissolved oxygen content (Δx) of bottled wines with different closures in relation to storage time as fitted by Peleg's Model.*

When comparing the first-order kinetic reaction previously described in the literature for this topic and the application of Peleg's model to dissolved and headspace oxygen, it was shown that the trends found were similar, however Peleg's model had successfully explained the oxygen evolution of oxygen availability towards time.

One of the first parameters to be affected by this "oxygenation" phenomena is free sulphur dioxide levels. The free sulfur dioxide content gradually decreases as wine ages, due to the degenerative reactions with H_2O_2 and further complex binding with other wine components. Therefore, we aimed to investigate whether the kinetics of oxygen permeation is followed by the free sulphur dioxide content in which is consume. **Table 2.5** shows the results of linear regression derivatives *k* and their respective regression coefficients.

Table 2.5. *Constant of reaction (k) applying the zero-order kinetic model for free sulphur dioxide consumption in wines evaluated with different synthetic closures permeability.*

	BIO LSA								BIO SPO							
	100		300		300B		700		100		300		300B		700	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
k	-0.025	-0.025	-0.027	-0.017	-0.024	-0.023	-0.027	-0.022	-0.027	-0.018	-0.027	-0.019	-0.021	-0.024	-0.026	-0.024
R_2	0.91	0.91	0.90	0.81	0.83	0.81	0.92	0.77	0.94	0.90	0.86	0.83	0.68	0.87	0.77	0.85
	BDN LSA								BDN SPO							
	100		300		300B		700		100		300		300B		700	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
k	-0.024	-0.016	-0.022	-0.022	-0.027	-0.023	-0.022	-0.027	-0.022	-0.023	-0.023	-0.025	-0.016	-0.023	-0.025	-0.031
R_2	0.74	0.65	0.82	0.63	0.74	0.77	0.73	0.84	0.64	0.75	0.61	0.51	0.53	0.70	0.78	0.83

Abbrev.: BIO: organic wine; BDN: biodynamic wine; SPO: spontaneous fermentation; LSA: fermentation with selected yeast. Closure permeability according to the manufacturer: 100<300=300B<700. Cold colors indicate smaller values, while warm colors indicate larger values, within the same wine typology.

It can be seen in **Table 2.5** that the trend proposed for the organic wines (BIO) with spontaneous fermentation have a much lower rate of reaction, particularly those of 2013, when comparing it to BIO LSA, except with the closure of 300B, which demonstrated an inverse trend.

BIO LSA wines fit properly the model, although similarities can be seen in closures 300 and 700, which may be due to the ability of wines interact with different amounts of oxygen ingress between these two closures similarly.

Biodynamic wines don't properly fit the model, when analyzing their R^2, because the variability among years, as it can be seen in organic wines. BDN LSA wines, have, in

general, within the year of 2012, a higher rate of sulphur dioxide reacting, and this rate of loss can be seen in all synthetic closure studies, except in 700 permeability closure, whereas in BDN SPO 2013 the rate if loss is higher irrespectively of the closure.

Therefore, based on the oxygen measurement and sulphur dioxide consumption, it can be assumed that the oxygen from the headspace was flatly diffused into the wine due to the difference in concentration, which is a dissolution process. Once dissolved in wine, the molecular oxygen could chemically interact with wine compounds, being consumed, and thus, diminishing its concentration in different rates. This rate was different among the different synthetic closures as their oxygen permeability was different. Subsequently to the oxygen consumption, the dissolved oxygen content in wine is decreasing to values near to zero. In the other hand, the sulphur dioxide content was not always closure permeability dependent for all wines, and it may be due to the interaction with other phenolic substances, which followed practically the same trend, except for vanillic acid in which disappeared from the second to third year of bottling, and epicatechin, which did not have trend associated with year of wine, closure, agriculture type nor fermentation.

Table 2.6. *Evolution of gallic, cutaric and caftaric acids along time after bottling in biodynamic, organic wines with different closure permeability.*

BIO LSA								
	100		**300**		**300B**		**700**	
	2012	2013	2012	2013	2012	2013	2012	2013
				Gallic acid (%)				
7	100	100	100	100	100	100	100	100
21	79	81	81	111	95	84	86	80
29	81	84	83	112	96	87	88	83
45	85	90	87	112	99	92	92	88
120	95	102	98	102	126	103	105	105
352	122	171	117	160	120	163	129	151
650	253	317	242	101	237	291	243	312
				Coutaric acid (%)				
7	100	100	100	100	100	100	100	100
21	92	106	90	101	90	95	91	96
29	90	105	89	101	89	94	90	95
45	88	103	87	99	88	93	88	93
120	88	100	87	99	89	101	86	100
352	0	72	15	61	16	27	21	20
650	3	24	25	41	32	47	27	33

Caftaric acid (%)

7	100	100	100	100	100	100	100	100
21	5	98	66	100	86	83	96	87
29	5	98	66	99	86	85	96	88
45	5	97	67	98	87	87	96	89
120	83	93	38	91	87	89	100	93
352	83	84	59	77	67	105	83	80
650	113	106	104	49	117	188	106	143

BIO SPO

	100		**300**		**300B**		**700**	
	2012	2013	2012	2013	2012	2013	2012	2013

Gallic acid (%)

7	100	100	100	100	100	100	100	100
21	102	91	84	81	87	75	89	80
29	102	93	86	83	89	77	91	83
45	102	97	89	88	92	82	94	87
120	94	126	97	103	111	100	116	107
352	114	128	125	134	115	114	115	132
650	84	272	227	278	239	288	237	294

Coutaric acid (%)

7	100	100	100	100	100	100	100	100
21	91	90	91	99	90	92	90	89
29	90	89	90	99	90	91	89	88
45	88	88	89	98	88	89	87	87
120	90	87	87	89	87	87	86	86
352	29	33	32	86	31	33	30	35
650	49	54	44	56	47	37	48	56

Caftaric acid (%)

7	100	100	100	100	100	100	100	100
21	96	84	83	88	92	84	91	92
29	96	85	84	89	93	84	91	93
45	96	87	86	91	94	86	92	94
120	90	84	85	91	100	83	97	101
352	95	98	95	100	96	95	88	101
650	154	159	163	154	148	157	143	159

BDN LSA

	100		**300**		**300B**		**700**	
	2012	2013	2012	2013	2012	2013	2012	2013

Gallic acid (%)

7	100	100	100	100	100	100	100	100
21	81	104	95	97	90	111	89	109
29	83	106	96	99	92	112	91	111
45	87	110	100	103	95	116	94	114
120	100	157	125	131	120	158	114	159
352	127	142	129	139	119	157	124	144
650	263	289	243	257	248	262	247	262

Coutaric acid (%)

7	100	100	100	100	100	100	100	100
21	92	93	93	93	93	95	101	92
29	91	92	92	92	92	94	100	91
45	89	90	91	90	90	92	98	90
120	91	95	93	91	92	97	110	93
352	25	24	26	27	26	27	30	24
650	42	45	37	39	36	38	40	43
				Caftaric acid (%)				
7	100	100	100	100	100	100	100	100
21	85	83	85	93	84	87	82	87
29	86	84	86	95	85	88	83	88
45	88	87	88	97	87	90	85	90
120	87	95	93	112	91	98	89	96
352	100	103	98	109	102	110	96	108
650	162	207	180	190	184	198	187	185

BDN SPO

	100		**300**		**300B**		**700**	
	2012	2013	2012	2013	2012	2013	2012	2013
				Gallic acid (%)				
7	100	100	100	100	100	100	100	100
21	88	80	91	84	91	81	87	87
29	90	82	93	85	93	83	89	88
45	93	84	96	87	96	86	92	91
120	112	94	118	96	120	99	111	107
352	121	99	116	101	118	94	123	99
650	243	216	231	203	239	218	250	207
				Coutaric acid (%)				
7	100	100	100	100	100	100	100	100
21	93	96	92	91	93	92	92	99
29	92	95	91	90	92	91	91	98
45	90	94	90	89	91	90	90	96
120	91	102	91	88	93	90	90	109
352	34	32	34	31	32	34	33	34
650	47	52	53	47	44	46	47	55
				Caftaric acid (%)				
7	100	100	100	100	100	100	100	100
21	88	89	89	85	83	85	86	91
29	89	90	89	86	84	86	87	92
45	90	92	89	88	85	88	88	94
120	97	105	83	88	83	93	98	106
352	93	98	90	99	93	102	82	97
650	166	189	118	165	160	185	166	175

The other abundant phenolic compounds are shown in **Table 2.6.** It was demonstrated that cutaric acid diminished along the time for all wines, such trend was also found by Lecce et al. (DI LECCE et al., 2013), when studying Verdicchio wine after six months

of storage, as the same was also reported by other study which evaluated three different wines: Tempranillo, Graciano and Cabernet Sauvignon for the period of 26 months (MONAGAS; BARTOLOMÉ; GÓMEZ-CORDOVÉS, 2005).

Gallic acid, instead, started having its concentration increasing between the days 45 and 120 after bottling for all wines. Within the wine aging, it is reported that gallic acid transforms into ellagic acid under oxidative conditions (TULYATHAN; BOULTON; SINGLETON, 1989), whereas flavonol glycosides and tartaric esters of hydroxycinnamic acids are being hydrolyzed into their corresponding free forms (SCHWARZ; WABNITZ; WINTERHALTER, 2003; ZAFRILLA et al., 2003).

Caftaric and cutaric acid were reported (GÓMEZ-PLAZA et al., 2000) to have similar trends as seen in our study, as a large decrease was seen for both caftaric and cutaric acids during 12 months of bottle-aging at temperatures between 10 and 35°C. Instead, the values of caftaric acid were higher in the second-year analysis, which may be due to depolymerization of polyphenolic adducts.

Whereas the content of individual phenolic compounds changed in wines during storage, the total polyphenolic index remained almost the same for all wines.

The increase with time in antioxidant activity (DPPH), is most likely due to the polymeric phenolic compounds formed in wine along time. Additionally, the total anthocyanin content behaved the same for all wines, in which they have had diminished with time, until the first year of storage, and then increased again, in the last measurement taken, as it can be seen in the scatter plot (**Figure 2.12**).

The first analysis (one week after bottling) is highlighted with a red extended circle, whereas the last anthocyanins content for all wines are emphasized in a red square. Instead, anthocyanin levels in 21, 29, 45 and 120 days are stressed in a square with circular borders whereas anthocyanin content after one year of bottling is within the red triangle in **Figure 2.12.**

This second order polynomial trend is probably due to the polymerization and copigmentation of anthocyanins (*data not shown*). In the beginning of wine storage period, anthocyanin molecules were binding to other phenolic compounds of wine and among themselves, whereas, by the end of experiment period, they may have been oxidized and or become heavy and precipitated in the bottom of the bottle, which was indeed observed in most of wines after two years' period in the bottle. This same trend

was also observed in another study, where Vranec *Vitis vinifera* wines were evaluated for 16 months (IVANOVA; VOJNOSKI; STEFOVA, 2012).

Additionally, the total color of wines followed the same trend, with no exception, and in two years this parameter diminished from around 20 to 30%. The wine which lost less color was the BIO SPO 300B 2012, in which had 97% of it after the 650 days in bottle, and it was followed by BIO SPO 100 2012 (90%), BDN SPO 300 2012 (93%), BDN SPO 300 B 2012 (91%), BSN SPO 700 2012 (90%), BDN SPO 100 2012 (92%). These results demonstrate that the loss of color is more related to the agricultural type than the closure permeability.

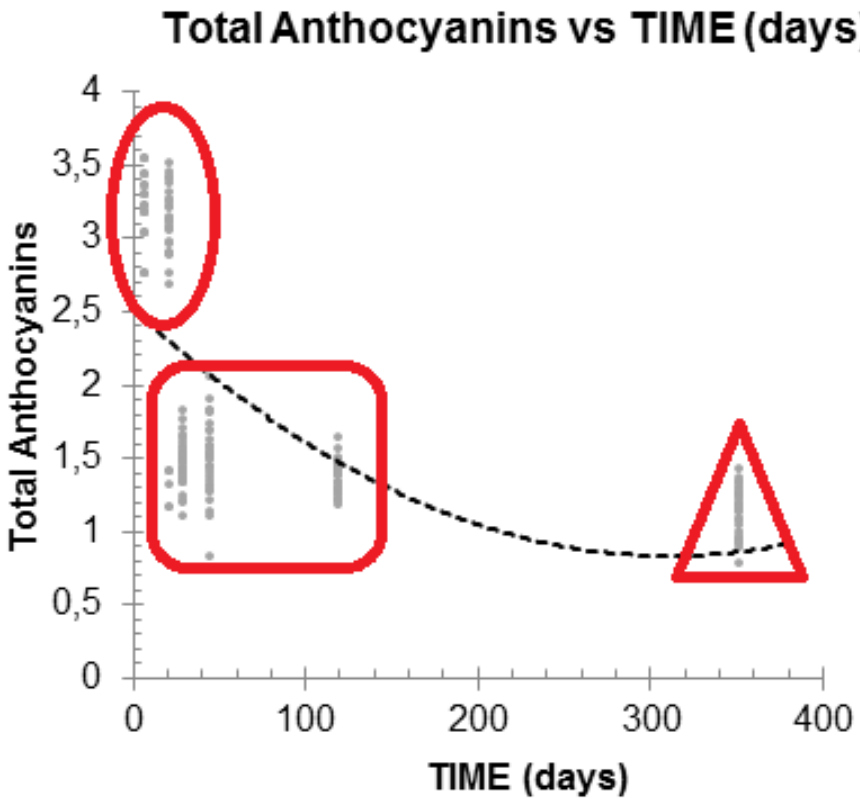

Figure 2.12. Scatter plot of total anthocyanins versus time for all 32 times evaluated according to time.

Regarding the sensory evaluation of the wines studied, electronic nose was not capable to distinguish differences among the samples, not even in the beginning of the study, when the wines were under practically the same parameter measurements. When principal component analysis was applied, BIO, BDN, LSA, SPO, 2012 and 2013

were randomly distributed among the four quadrants. Therefore, the data was not used in order to evaluate the effect of closure permeability.

In contrary, when these wines were analyzed by flash gas chromatography, in their 650 days of bottle, it can be seen by principal component analysis of data (**Figure 2.13**) some division among wines per year, as in the first and second quadrants, where BDN LSA 2013 and 2012, respectively are presented, and therefore it can be stated that, for this wine and time, the different permeability among closures did not affect wine volatile compounds. The only exception for this trend was found for wines produced by biodynamic agriculture of year 2013 with the closure with permeability 700, which may be due to the higher oxygen diffusion into wine allowed by the synthetic closure.

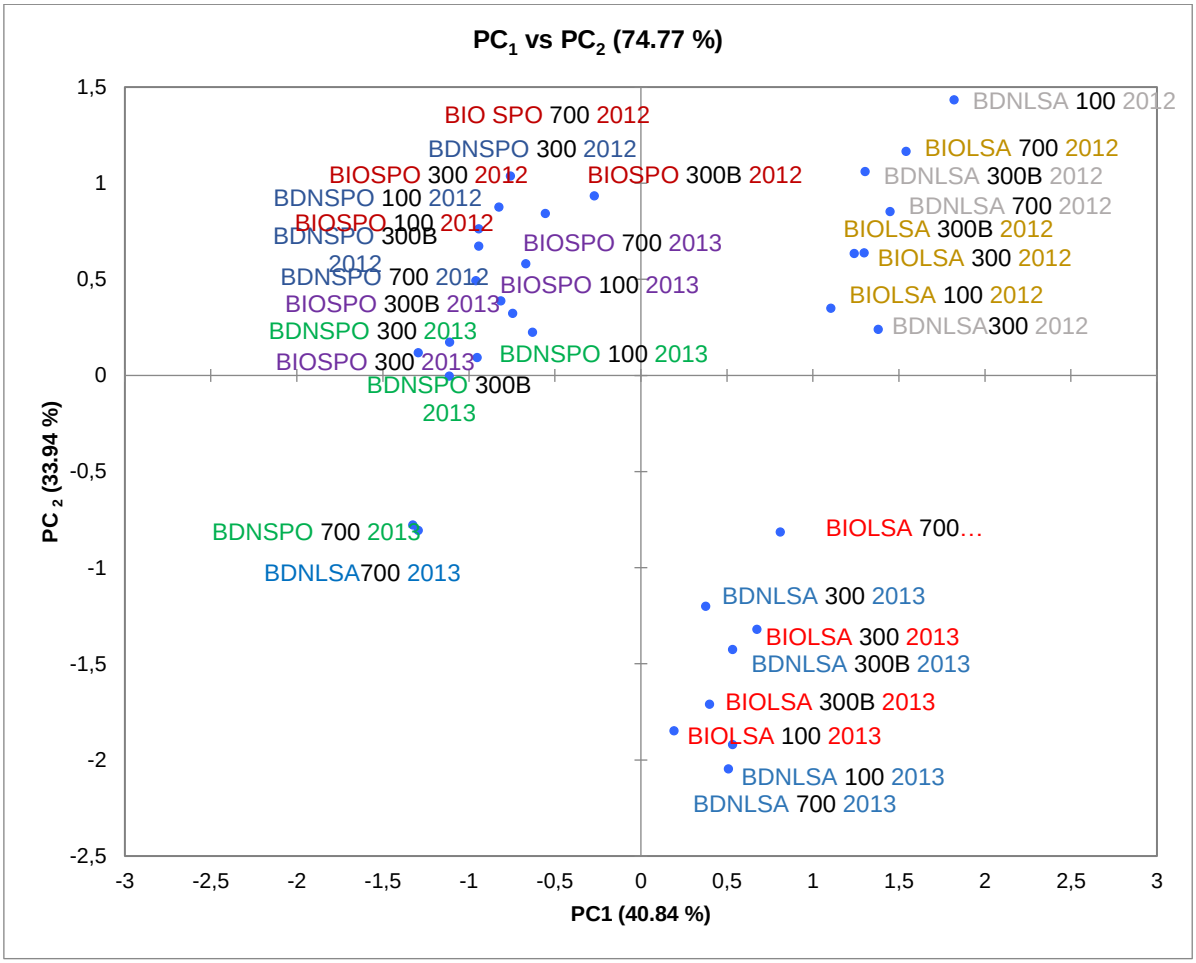

Figure 2.13. *Principal component analysis (PC_1 vs PC_2) of volatile fraction of wines studies after two years of bottle storage.*

Therefore, based on the chemical and sensory analysis performed, it could be seen that the data provided by the synthetic closure manufacturer was not in accordance when oxygen permeation was analyzed.

Moreover, the most permeable closure used in this study (700) was shown to differentiate biodynamic wines from one of the vintages (2013) studied when their volatile fraction was analyzed by flash gas chromatography. Additionally, when analyzing phenolic acids, it showed a higher trend of their content after 650 days of bottling, which could not be indicative of depolymerization of polyphenolic compounds which have higher antioxidant activity, which was corroborated additionally by DPPH antioxidant (data not shown).

2.5. CONCLUSIONS

Oxygen ingress in wines through the closure was shown to have a great impact on wine development during ageing in bottle. The first-order kinetics as well as Peleg's model were observed to explain the change in head space oxygen with time, whereas the change of dissolved oxygen fitted only Peleg's model. The conclusions drawn from the two models disclose the rates for initial level of DO as: 100≤700<300B. On the contrary, towards the end of storage time, the performance of the closures was observed to be as follows: 700<100<300B. A similar trend was also observed in case of headspace oxygen levels. It can be concluded that closure 700 exhibited the best characteristics for Sangiovese red wine evolution. The findings of this chapter can be beneficial for winemaker as well as the closure company to improve the performance of the wine-closure system and its effect on wine evolution, in particular for Sangiovese wines.

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CHAPTER 3

CHAPTER 3

pH CASE STUDY

Abstract

The impact of pH on the physico-chemical and sensory properties of wine was investigated. A *Sangiovese* red wine, vintage of 2013 made from grapes obtained by biodynamic agriculture was divided into five batches with pH adjusted to 3.2, 3.4, 3.6, 3.8 and 4.0. Samples were bottled and subjected to physico-chemical and sensory analysis during a storage period of two years. The parameters tested were anti-oxidant activity with DPPH, color and anthocyanin by Boulton assay, tannins and polymeric compounds by Adams' assay, SO_2 and phenolic acids by HPLC and volatile fractions by electronic nose and Flash-GC periodically. Also, the wines were tested by discriminative sensory analysis by trained panelists at the end of the storage time. After 12 months of storage the volatile acidity was observed to be increasing which was relative to that of pH decrease. The antioxidant activity in contrast was increased during the period, independent of pH. However, the oxidative indicator, sulphur dioxide demonstrated pH dependency in the first year of storage as is observed to be higher in wines with higher acidity. After 24 months, the volatile acidity decreased and antioxidant activity increased independently of the pH. However, the analysis made by Flash-GC was only partially capable of discriminating the volatile fractions of these wines. For further elucidation of the data, mathematical modelling was applied to the data of free and total sulphur dioxide. The results indicate that in general there was no difference in the oxidation of the five wines under study which was also the case in sensory analysis. Therefore, with the parameters evaluated, it can be concluded that the differences in pH – in the range studied and for the time investigated – did not affect the oxidation rate nor the sensory parameters of the studied *Sangiovese* made from grapes of biodynamic agriculture.

3.1. INTRODUCTION

The pH of juice or wine can be defined as the degree of strengthens and concentration of the dissociated acids existing in that medium. It can be calculated using the concentration of hydrogen ions in the formula $pH = -\log_{10}[H^+]$. As it is presented in a logarithm scale, a wine which has a pH 3.0 is ten times more acid than a wine with a pH of 4.0, for example. Wine pH values usually range from 2.8 to 4.2, the lower values typical of whites', whereas the higher values most commonly found in red wines. The pH of wine can be adjusted through the addition of acid or base.

The wine pH is one of the most important parameters to be evaluated regarding wine acidity in grape juice and wines. This parameter affect a number of physico-chemical and microbiological processes, including (i) the malolactic fermentation, which, itself, reduces the total acidity of the wine and increase its pH as a result of decarboxylation of L-malic acid into L-lactic acid (FORNACHON, 1957; LONVAUD-FUNEL, 1995); (ii) the antimicrobial activity of SO_2 that decreases at high pH, as the higher the pH, the easiest it is for microorganisms to survive (DUPUY, 1957). As microbial contamination, undesirable lactic acid bacteria and acetic acid bacteria demonstrate higher vigor at high pH than at pH's on the range of 3.1 – 3.6, which can increase volatile acidity in these wines.

The wine pH also plays an Important role in wine sourness taste, in wine soluble protein stability (MORETTI; BERG, 1965), in potassium bitartrate salts precipitation (BERG; KEEFER, 1958) and in the color stability of red wines (BOULTON, 1980; SOMERS, 1971).

In the past decades, the wine pH has shown an increasing tendency, interfering in wine's taste and preservation. Wine oxidation can be secondarily affected by wine pH, since the it can be stated that higher the pH, many reactions involved in this process have their kinetics changed such as the reduced concentrations of anthocyanins and small polyphenolic compounds, when compared to wines with lower pH (IVANOVA; VOJNOSKI; STEFOVA, 2012; KONTOUDAKIS et al., 2011). Thus, as the pH diminishes, the relative amount of flavylium cation increases, along with the raise of red-colored anthocyanins. Moreover, within the wine pH conditions, a small fraction of sulfur dioxide is found in its free form, and it changes as the wine has its pH modified

(from 6% at pH 3.0 and 0.6% at pH 4.0), and thus the antioxidant capacity of wine changes consequently.

Additionally, wine pH affects the wine not only after its bottling, but the entire process. Within the malolactic fermentation, for example, *Oenococcus oeni* ability to survive in wine environment, like high content of ethanol and low pH may affect its cell redox balance. It has also been observed that the evolution of esters during ageing can be influenced by wine pH (GAROFOLO; PIRACCI, 1994; RAMEY; OUGH, 1980). Ramey and Ough (1980) have shown that the reaction rates for hydrolysis of esters in relation to system pH is presented within a gradual trend that the reaction rates decreased as the pH increases, irrespective to the type of esters. Moreover, according to other studies, the pH , along with alcoholic content of the wine may influence the extraction of volatile compounds from oak, the lower the pH, higher the extraction (MAGA, 1989; PUECH, 1981).

On the other hand, Kontoudakis et al. (2011) conducted a study in which aimed to evaluate the influence of wine pH in the color of red wines when micro-oxygenation was applied. It was found that the pH and the polyphenol concentration affect the color change, as it was seen that there was no evidence of changes when the pH was higher. Another study which conducted a sensory test with 16 judges was able to correlate ethanol level and pH values with wine astringency; while ethanol and pH content increased, the astringency perception was diminished, but pH affected only astringency, whereas ethanol contributed also to the perceived bitterness of tannin oligomers, especially at typical wine ethanol levels (11–15%) (FONTOIN et al., 2008). There are some studies in the literature in which explore the approach the pH and oxygen ingress in Cabernet Sauvignon wines for a storage period and associate them with polyphenolic content, especially tannin structure, but the main studies presented in the literature approach the pH of wine when the most is being prepared of by its relation with potassium salts absorbed by the grapes in steps previous to wine aging and so far there are no studies in which evaluate the wine pH in a short time storage and its relation to possible oxidative markers (BLOUIN; GUIMBERTEAU, 2000; CHAMPAGNOL, 1984; DALLAS; LAUREANO, 1994; GRANT-PREECE et al., 2017; RIZZON; MIELE, 2002; TAMBORRA, 1992; WINKLER et al., 1975).

During storage, the red wines change their composition, depending on the external (e.g. time, temperature, light) and ‘internal’ conditions, i.e. wine composition. In this

view, anthocyanins, proanthocyanidins, flavan-3-ol and other compounds, as flavonols themselves may react among each other affecting their astringency and color (BAKKER; TIMBERLAKE, 1997; DALLAS; RICARDO-DA-SILVA; LAUREANO, 1996; FULCRAND et al., 1998; KOVAC et al., 1992; REVILLA et al., 1999). There are currently many studies in the literature focusing on the levels of these compounds and effects of technological practices during wine making, but little information is available on the evolution of phenolic compounds with time on biodynamic wine at different pH. Thus, as part of this PhD study the effect of pH was investigated in order to elucidate whether and how it affects in terms of oxidative damage biodynamic wines in a medium-term storage.

3.2. MATERIAL AND METHODS

3.2.1 Wines

Sangiovese red wine from biodynamic agriculture and produced by spontaneous fermentation, vintage 2013, harvested, fermented and bottled at TechnoPole of Tebano (Faenza, RA, Emilia Romagna region, Italy) were used in this study as previously described (PARPINELLO et al., 2015). Before bottling the original pH of wine (3.6) was adjusted to 3.2, 3.4, (3.6), 3.8 and 4.0, and then bottled with Nomacorc 300 synthetic closure (24 mm of diameter, length 38 x 44 x 47 mm, foam density of 0.261 g $cm^{3\ -1}$, total density of 0.328 g $cm^{3\ -1}$, and an oxygen ingress of 0.37 mg of O_2 after 3 months, 0.64 mg of O_2 after 6 months, 1.2 mg of O_2 after 12 months, 1.1 mg of O_2 yearly after the first year per bottle). Bottles were stored vertically up to 36 months during which the wine was analyzed by selected physico-chemical and sensory parameters as follows.

3.2.2 Chemicals and reagents

The following chemicals and reagents were from commercial source: methanol, acetonitrile, sodium disulfite and acetaldehyde (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).

Bovine serum albumin (BSA, fraction V, lyophilised powder), sodium dodecyl sulphate (SDS; lauryl sulphate, sodium salt, 95%), triethanolamine (TEA, 98%), $FeCl_3 \bullet 6H_2O$

(98%) used for the Adams Harbertson's assay were purchased from Sigma (Saint Louis, MO).

3.2.3 Antioxidant Activity

Wines' antioxidant activity was determined by its capacity of neutralizing the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and it was performed according to a procedure previously described (BRAND-WILLIAMS; CUVELIER; BERSET, 1995). For the assay, 200 µL of wine was added to 3.0 mL of a methanol solution of the radical DPPH (25 mg L^{-1}), and measured at $\lambda_{517\ nm}$ after 1 h storage in the dark. Antioxidant activity was expressed as percentage of scavenging activity, as 100% was considered the "blank" (200 µL methanol instead of the sample). Measurements were made in a Shimadzu UV-mini 1240 spectrophotometer (Kyoto, Japan) and expressed as percentage of inhibition (DUDONNÉ et al., 2009) as follows:

% DPPH scavenging activity = [(Abs_{DPPH} – Abs_{tannin}) / Abs_{DPPH})] x 100

Where:

Abs_{DPPH} is the absorbance measurement of DPPH solution (2.9 mL) with 0.1 mL of methanol;

Abs_{tannin} is the absorbance measurement of DPPH solution (2.9 mL) with 0.1 mL of tannin solution above mentioned.

3.2.4 Total Polyphenols Index

Total Polyphenolic Index (TPI) was calculated according to previously described (PARDO et al., 1999), where 1 mL of wine was added to 99 mL of distilled water and the absorbance was measured at λ_{280} nm in a 10 mm quartz cuvette (Shimadzu UV-mini 1240 spectrophotometer). The TPI value was calculated by multiplying the absorbance per 100 (dilution factor).

3.2.5 Free and Total SO2; Alcohol content; pH; Total and Volatile acidity

Oenological parameters were measured according to standardized methods of the "Office International de la Vigne et du Vin" (AOAC® Official MethodsSM) (OIV, 2014).

3.2.6 Total color; Copigmentation; SO2 resistant pigments; Total anthocyanins

Before analysis, each wine had the pH adjusted to 3.6. Than total color, copigmentation, SO_2 resistant pigment and total anthocyanins were all measured according to Boulton et al. 1999, where three trials were performed: i) 20 µl of a 10%

(v/v) acetaldehyde solution was added to 2 mL of each of the wines in a plastic cuvette, and then left at room temperature for 45 minutes, when the total color of wine was measured at 520nm; ii) 40 µl of a 20% (w/v) of SO_2 solution was added to 2 mL of each wine in a plastic cuvette, and then the absorbance value of SO_2 resistant pigments was measured at 520 nm; iii) the co-pigmentation was evaluated by diluting each and every wine sample for 1:19 in a wine model solution (12% ethanol, pH 3.60 potassium bitartrate buffer, corrected for the dilution), for further absorbance measurement of 520 nm. The spectrophotometric measurements were done with a Shimadzu 1240 model spectrophotometer (Shimadzu, Milan, Italy).

3.2.7 Small and large polymeric pigments; Tannins

Small and large polymeric pigments (%SPP and %LPP, respectively) relative content and phenolics and tannins content were measured according to Harbertson *et al.* (HARBERTSON; KENNEDY; ADAMS, 2002; HARBERTSON; PICCIOTTO; ADAMS, 2003).

3.2.8 HPLC analysis

A 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 DX600, Milano, Italy) was used for identification and quantification of 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. Knauer C_{18} polar endcapped column (length 150 x 3 mm with precolumn; ~50% hydrophilic endcaping; Eurospher II, Berlin, Germany) was used using the following mobile phases: solvent A (CH_3COOH: H_2O 1:20 v/v) and solvent B (CH_3CN: H_2O, 4:1, v/v), at flow rate of 0.5 mL min^{-1}.

Each chromatographic run had 90 minutes and the proportions of eluent B are as follows: 0 min, 0%; 30 min, 5%; 65 min, 10%; 70 min, 30%; 80 min, 0%; 85 min, 0%.

Protocatecuic acid, p-hydroxybenzoic and vanillic acid were quantified at 256 nm, gallic acid, syringic acid, (+)-catechin and (-)-epicatechin at 280 nm, whereas *p*-coumaric acid and coutaric acid at 308 nm, and caftaric acid and caffeic acid at 324 nm and rutin and quercetin at 365 nm.

3.2.9 Sensory Analysis

In order to evaluate the possible effects of wine pH and their differences among the five wines of this study, a sensory analysis was performed. For that, a discriminant analysis was elaborated, with the application of a sorting test in which astringency, age and preferred wine were ordinated by the assessors. The complete datasheet is available in **APPENDIX B**.

The panelists consisted of 36 volunteers, recruited from students of the BSc program of Oenology and Viticulture (University of Bologna), who regularly consume red wine and are familiarized with astringency standards in wine.

For the analysis, each assessor was asked to use the same five wines to evaluate each parameter in order to complete the test; as they were asked to try the wine at least once for each and every parameter asked.

3.2.10 Eletronic nose

Once wines were open, 10 mL of wine sample were poured versed in a 40 mL vial and left at room there in environmental temperature for equilibration for approx. thirty minutes. Then, the headspace was analyzed with a commercial portable electronic nose PEN2 (Airsense Analytics, Milano, Italy) composed of an array of 10 temperature-moderated metal-oxide sensors (MOS), a sampling system, a data acquisition device and a data processing system. Signal output was measured each second in the intervals of 60 seconds, which is time to most of the sensors to reach the steady state.

3.2.11 Flash Gas Chromatography

For volatile fraction analysis, Flash Gas Chromatography Electronic Nose (Heracles, Alpha MOS®, **Figure 3.1**) was used. Two milliliters of each wine samples were pipetted in vials and immediately closed. They were then placed in the refrigerator (4-6°C) until analysis time.

Before the analysis, samples were incubated for 20 minutes at 40°C in an agitator at 500 rpm.

The injection volume used was 1000 µl by 100 µl per second, with the injection temperature of 200°C for 15 seconds and 10 kPa of pressure. Initial trapping temperature was 40°C, split mode (10 mL/second) for 60 seconds, with 60 kPa of pressure. The programmed temperature and pressure was isotherm mode of 240°C per 93 seconds, 80 kPa of pressure and 10 mL per min of split mode. Valve

temperature was set to 250°C and the initial oven temperature was 50°C (2 seconds), then temperature was set to increase by the rate of 2°C per second until 120°C, then 5°C per second, until 280°C. The flash chromatography was equipped with two columns: MXT®-5 Columns and MXT®-1701 Columns (RESTEK®, Bellefonte, PA, USA). Each column has one Flame Ionization Detectors (FID), both at 280°C.

Figure 3.1 Alpha MOS HERACLES Flash Gas Chromatography Electronic Nose.

3.2.13 Calculation of Kinetic parameter

In chemical kinetics, a chemical reaction is generally expressed in terms of a reaction rate constant or reaction rate coefficient (k), which quantifies the rate of a chemical reaction. In this experiment, in an attempt to modelling the SO_2 consumption rate as a result of wine oxidation, and with the aim to determine the reaction order of some chemical compounds studied, kinetic parameters calculation were performed as previously described (RICCI; PARPINELLO; VERSARI, 2016).

3.2.12 Statistical Analysis

Statistical treatments and data analysis were performed using the XLSTAT Software, Version 2017.1.

3.3. RESULTS AND DISCUSSION

When analyzing the differences in the parameters evaluated within the biodynamic Sangiovese wine in which have its pH adjusted to 3.2, 3.4, 3.6, 3.8 and 4.0 it is important to consider how some chemical factor can affect wine pH (DALLAS; LAUREANO, 1994; SIMS; MORRIS, 1985).

In fact, pH is a measure, which needs to be always considered, since large differences in this parameter can be directly correlated to several other variables, such as color, browning, chemical age, degree of pigment coloration and polymeric pigment color. To simplify the presentation of results, a direct comparison between the two extreme wines, i.e. pH 3.2 and 4.0, was highlighted.

In this study, the chemical parameters related to oxidative change such as sulphur dioxide, phenolic compounds and color did not vary significantly when analyzing the five wines in the same period as it is detailed below.

As expected the LPP increased overt time at a reaction rate constant of 0.02% for wine at pH 3.2 and 0.01% for wine at pH 4.0, whereas SPP decreased as a result of polymerization process at the same rate constant (-0.02% and -0.01%, respectively). However, there is little to any difference between the evolution of polymeric fractions and monomeric fractions (LPP and SPP, respectively) of wines with the lowest (3.2) and higher (4.0) pH (**Figure 3.2**). The large polymeric pigments were slightly higher in relative content in the wine with lowest pH than the one with the higher one.

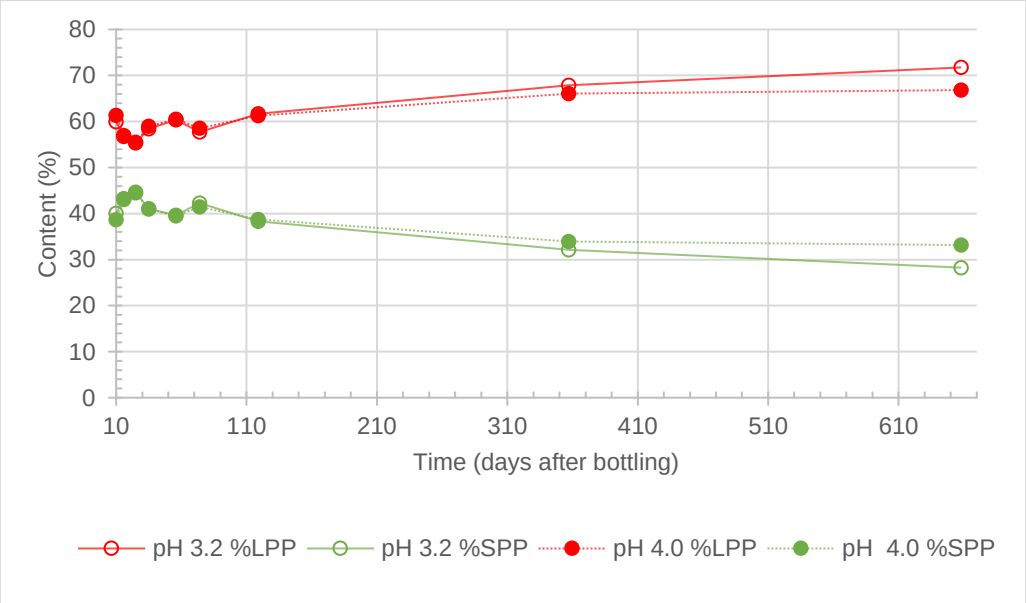


Figure 3.2 *Time course of polymeric pigments of Sangiovese wines with lowest pH (3.2, solid line) and highest pH (4.0, dashed line). Abbreviation: SPP: small polymeric fraction; LPP: large polymeric fraction.*

The total phenolic compounds of these two wines, in the first and last day, were 1665 mg L^{-1}, 1643 mg L^{-1}, 1543 mg L^{-1} and 1689 mg L^{-1}, respectively. The same values were observed for those other three wines with intermediate pH.

As expected, the low pH wine (3.2) showed more total color with time (**Figure 3.3),** (AU 2.89, after 658 days from bottling) compared to those with high pH 4.0 (AU 2.51 after 658 days from bottling).

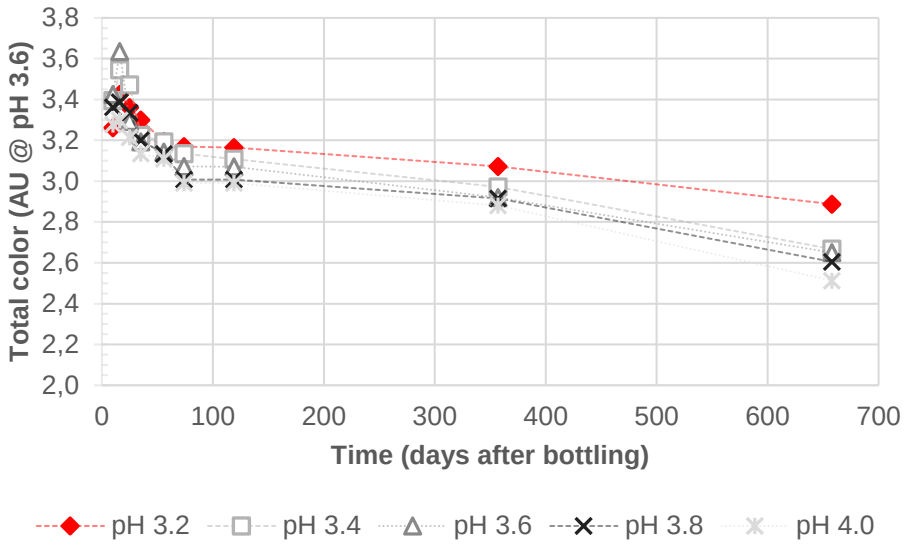


Figure 3.3. *Time course of total color of Sangiovese red wines with different pH.*

Copigmentation (**Figure 3.4**) peaked at approx 100 days, followed by a regular decrease with time most probably consequently to a loss of total color.

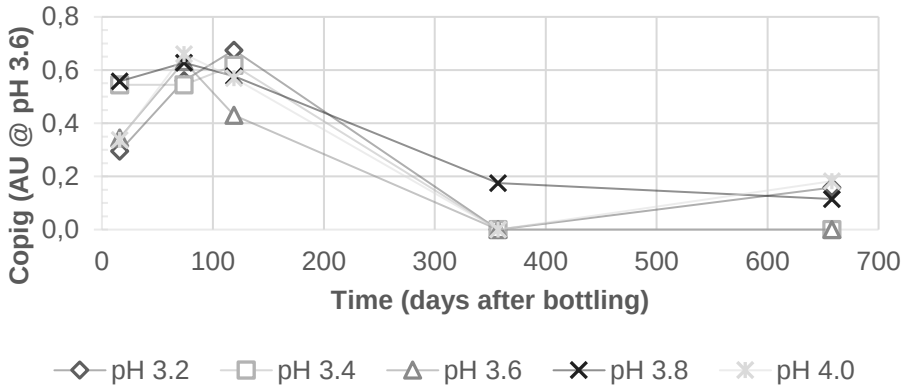


Figure 3.4. *Time course of copigmentation of Sangiovese red wines with different pH*

Instead, total acidity started diminishing around the fourth month after bottling, whereas in around a year after, this decrease was proportional to the pH, when pH 3.2 wine had lost 11% of its total acidity, pH 3.6 wine 14%, and pH 4 wine 15% of its acidity. The volatile acidity, instead, increased along the first year: 15%, 31%, 14%, 21% and 29% for wines with pH 3.2, 3.4, 3.6, 3.8 and 4.0, respectively. From the first year to the second year it drastically diminished to around 38% of its initial content in all wines.

After 658 days, there was an average of 66%, 50%, 71%, 59%, 64% of total SO_2 for wines of pH 3.2, 3.4, 3.6, 3.8, 4.0 respectively, when comparing to the first levels measured after bottling. Moreover, when observing the period from the first year and second after bottling, the wines lost around 20% of the total SO_2 content. The free SO_2 content, instead, presented values of 53%, 100%, 55%, 60% and 37% for wines of pH 3.2, 3.4, 3.6, 3.8, 4.0.

Sulphur dioxide in wine is expected to drop with time as it bounds with other substances presented in wine, such as acetaldehyde, quinone and anthocyanins, or by reacting with reactive oxygen species. SO_2 can also be lost through coupled oxidation with phenolic compounds, and in this case, 1 mole of O_2 results in the loss of 1 mole of SO_2 (BOULTON et al., 1999). SO_2 in wine exist in different chemical forms, such as molecular SO_2 and bisulfite (HSO_3^-), that are present as the chemical equation: $SO_2 + H_2O = HSO_3^- + H^+$ it is known that the more acid the wine, the higher percentage of molecular SO_2 there will be available and by comparing a wine with pH of 3.0 and 4.0, the last one will need 9.45 times the content of SO_2 to be added in order to have the same antioxidant potential. In addition, the molecular form of sulfur dioxide is most importantly responsible for capturing hydrogen peroxide formed due to phenolic oxidation. Molecular SO_2 was presented at concentration of 52 mg L^{-1} for wine at pH 3.2 initially and 38 mg L^{-1} for wine at pH 4.0; whereas these two wines presented the same content of it in the last days of measurement: 13 and 14 mg L^{-1} (**Figure 3.5 a/b**).

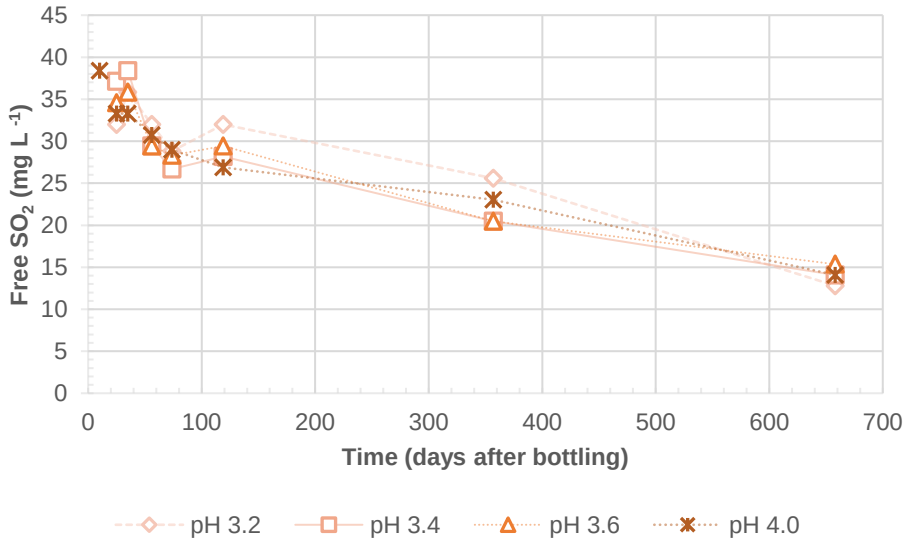


Figure 3.5 (a). *Time course of free SO_2 content of Sangiovese wines at five different pH (3.2, 3.4, 3.6, 3.8, 4.0).*

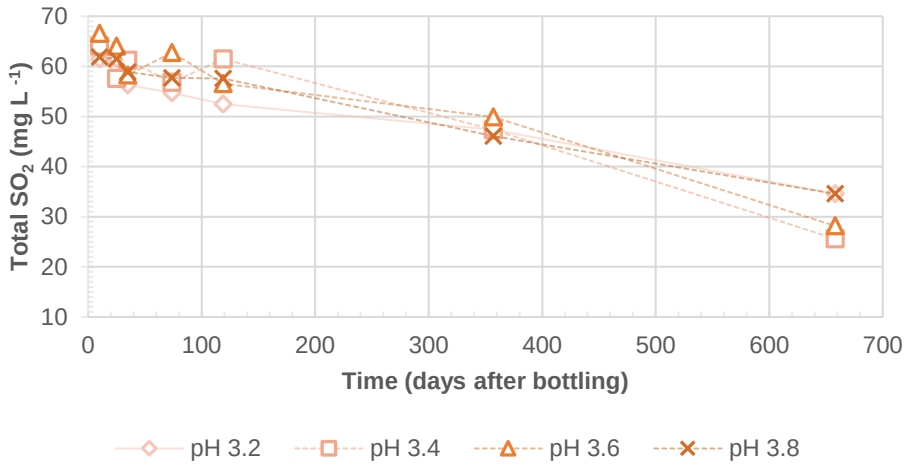


Figure 3.5 (b). *Time course of total SO_2 content of Sangiovese red wines at five different pH (3.2, 3.4, 3.6, 3.8, 4.0).*

In this work, although, the pH was adjusted right before the bottling process, in other words, the wines with five different pH had the same amount of sulfur dioxide.

Therefore, it is expected those wines with lower pH would have less oxidative damage when analyzing the oxidative markers, which did not occur when analyzing it statistically.

Moreover, when considering the calculation of kinetic parameters, it was found that the free and total SO_2 consumption followed a zero-order kinetic rate (**Table 3.1**), which can be understood that the rate-determining step was not influenced by the concentration of products.

Table 3.1. *Kinetic values of free and total sulphur dioxide consumption during two years of bottle storage.*

Wine pH	*k* constant of disappearance Total SO_2 (mg L^{-1})	R^2	*k* constant of disappearance Free SO_2 (mg L^{-1})	R^2
3.2	-0.04	0.94	-0.03	0.92
3.4	-0.05	0.95	-0.03	0.80
3.6	-0.05	0.94	-0.03	0.89
3.8	-0.04	0.99	-0.02	0.86
4.0	-0.04	0.92	-0.03	0.89

The molecular sulphur dioxide is known to be oxidized to sulphate throughout acting as H_2O_2 scavenger, that is provided by the polyphenol oxidation, or by coupled reaction with quinones (BOULTON et al., 2013). Once it reacts with other wine compounds, sulphur dioxide can be presented in different forms of sulphites adducts, which are

product of complexation of these SO_2 molecules and products of polyphenolic oxidation along with metal ions, such as Fe and Cu. Danilewicz (2007) demonstrated that the relation between SO_2 consumption and oxygen ingress through wine is not linear, therefore the formation of these above mentioned adducts are not linear or constant consequently. According to the constant of equilibrium, each adduct formed can affect bounded SO_2 at a different range (DANILEWICZ, 2007; DANILEWICZ; SECCOMBE; WHELAN, 2008; DANILEWICZ; WALLBRIDGE, 2010; RICCI; PARPINELLO; VERSARI, 2016).

Moreover, it is known that within wine pH conditions, a small fraction of sulfur dioxide is found in its free form, and as it changes according to the pH modification, it can be supposed that since a wine with the same amount of total Sulphur dioxide, but with different pH has different range of concentration of molecular SO_2 available: 3.9% for 3.0 wine pH; 2.5% to 3.4 wine pH; 1.6% to 3.6 wine pH; 1% to 3.8 wine pH and 0.7% to 4.0 wine pH), and thus the antioxidant capacity of wine may change consequently. These concepts can explain the slight variation of SO_2 consumption among the five wines used in this experiment, in which had the same composition, but different pH.

Considering the phenolic components, analysis of statistical Pearson's correlation, considering a significance level alpha of α=0.05 can be seen in **Table 3.2**:

Table 3.2. *Pearson correlations (r) among phenolic compounds measured in wines with different pH (3.2, 3.4, 3.6, 3.8, and 4.0) during 658 days of storage.*

Phenolic compound	Protocatecuic acid	Vanillic Acid	Gallic Acid	Syringic Acid	Catechin	Epicatechin	Coutaric acid	caffeic acid	caftaric acid
Protocatecuic acid	**1**								
Vanillic Acid	**-0.876**	**1**							
Gallic Acid	**0.895**	**-0.919**	**1**						
Syringic Acid	**0.873**	**-0.911**	**0.939**	**1**					
Catechin	0.059	-0.384	0.200	0.195	**1**				
Epicatechin	-0.461	0.378	-0.588	-0.586	0.396	**1**			
Coutaric acid	0.721	-0.643	0.552	0.534	0.148	-0.045	**1**		
Caffeic acid	**0.926**	**-0.935**	**0.940**	**0.936**	0.127	-0.586	0.648	**1**	
Caftaric acid	**0.958**	**-0.926**	**0.959**	**0.933**	0.164	-0.505	0.737	**0.960**	**1**

*Values in bold are higher than 0.8

It can be noticed that the highest correlations were among caffeic and caftaric acids with protocatecuic acid (r = 0.926 and 0.958, respectively), gallic with syringic acids (0.939), caffeic with syringic acid (0.936), caffeic with gallic, caftaric and syringic acids (0.940, 0.960, 0.936 respectively). These compounds were correlated positively, ie,

once one increases, the other does likewise proportionally. These compounds have good antioxidant activity, and they all show the same trend in the first weeks of experiment, as it can be seen in **Table 3.3,** which may be due to the formation of polymeric compounds, and after this period, they start increasing their content likewise, showing further reaction, like the release of caffeic acid from the hydrolysis of cinnamoyl-glucoside anthocyanins (MORENO-ARRIBAS; GÓMEZ-CORDOVÉS; MARTÍN-ÁLVAREZ, 2008).

On the other hand, as the vanillic acid diminished, the content of gallic acid increased (Pearson correlation of -0.919), similarly trend was found for vanillic acid with protocatecuic acid (r = -0.876), caffeic acid (r = -0.935) and caftaric acid (r = -0.926). Gallic acid was the most abundant benzoic acid presented in the wines studied, as it was also found in previous studies within wines of other grape varieties (GARCÍA-FALCÓN et al., 2007); Considering the hydroxybenzoic acids in this correlation, among vanillic acid, gallic acid and protocatechuic acid, the first presents less hydroxyl groupments, in which may lead to a less effective antioxidant properties, that will end up decreasing its contents more rapidly, when comparing to those with more potent antioxidant activity. Therefore, while vanilic acid is being oxidized, gallic acid, for instance, is being initially polymerized in larger polyphenolic molecules for further reactions, which will later to the diminishment, it will lead to its content to increase again.

Data of actual concentration of phenolic compounds measures in this study can be seen in **Table 3.3.** Phenolic compounds showed a similar trend with time among the wines, as it can be seen in **Table 3.3.**

Table 3.3. *Phenolic compounds of Sangiovese red wines measured by HPLC-DAD from the first days after bottling until day 658. Values are presented in mg L^{-1}.*

pH	Days	Protocatecuic acid	Vanillic Acid	Gallic Acid	Syringic Acid	Catechin	Epicatechin	Coutaric acid	caffeic acid	caftaric acid	Quercetin
3.2	10	2.9	1.9	20.8	4.8	11.6	14.8	4.2	0.9	34.4	1.7
	16	2.3	2.1	17.2	2.7	16.1	15.2	3.5	0.0	28.5	3.4
	25	2.3	2.1	17.6	2.8	16.2	15.1	3.5	0.1	28.7	3.7
	35	2.3	2.1	18.0	3.0	16.2	14.9	3.5	0.2	29.0	4.0
	56	2.4	2.0	18.8	3.3	16.4	14.6	3.5	0.4	29.6	4.6
	74	2.5	2.0	19.5	3.5	16.5	14.3	3.5	0.5	30.0	5.1
	119	2.7	1.7	19.6	3.1	21.9	14.7	3.3	0.9	29.1	8.9
	357	2.0	1.9	25.2	4.7	19.1	9.1	2.6	1.2	28.2	12.4
	658	5.3	0.0	45.3	13.6	19.5	5.3	4.5	7.3	50.8	22.6
3.4	10	2.4	1.7	20.0	1.9	23.2	20.1	4.2	1.0	32.3	1.4
	16	2.0	1.9	17.3	1.0	21.5	16.3	3.7	0.1	27.5	0.1
	25	2.0	1.9	17.7	1.2	21.5	16.2	3.7	0.2	27.7	0.4
	35	2.1	1.8	18.2	1.4	21.4	16.0	3.7	0.2	28.0	0.8
	56	2.2	1.8	19.2	1.8	21.2	15.7	3.7	0.4	28.6	1.6
	74	2.2	1.8	20.0	2.2	21.1	15.5	3.7	0.6	29.2	2.2
	119	2.7	1.5	22.2	3.2	21.3	12.1	3.6	0.8	29.3	2.1
	357	2.0	1.8	28.9	5.8	14.8	8.2	2.8	1.1	28.9	12.6
	658	5.3	0.0	50.2	15.8	19.1	9.2	4.5	6.8	51.1	24.0
3.6	10	2.5	1.7	19.9	5.1	23.5	14.8	4.3	1.2	34.3	1.6
	16	1.8	2.0	15.2	4.2	22.3	14.6	3.6	0.2	27.8	0.0
	25	1.8	2.0	15.7	4.3	22.3	14.6	3.6	0.3	28.1	0.2
	35	1.9	2.0	16.3	4.4	22.4	14.5	3.6	0.4	28.4	0.6
	56	1.9	1.9	17.4	4.7	22.5	14.4	3.6	0.5	29.1	1.5
	74	2.0	1.9	18.4	4.9	22.6	14.3	3.6	0.7	29.7	2.3
	119	2.0	1.7	20.1	5.7	22.8	15.3	3.6	0.9	28.6	2.5
	357	1.9	1.7	24.4	5.9	21.4	10.4	2.5	0.9	29.0	13.3

	658	5.0	0.0	55.3	13.4	27.3	12.1	4.6	6.0	54.0	28.6
3.8	10	2.0	1.1	19.7	4.0	28.0	24.4	3.9	0.6	30.6	2.0
	16	1.9	1.6	15.3	3.8	23.7	18.9	3.3	0.0	26.7	0.0
	25	1.9	1.6	15.9	3.9	23.8	18.8	3.3	0.0	27.1	0.0
	35	2.0	1.6	16.6	4.1	23.8	18.6	3.3	0.1	27.4	0.4
	56	2.1	1.6	18.0	4.4	23.9	18.3	3.4	0.2	28.2	1.3
	74	2.1	1.5	19.2	4.7	24.0	18.0	3.4	0.4	28.9	2.1
	119	3.1	1.8	21.7	6.8	18.9	11.8	3.0	0.8	30.8	1.4
	357	1.6	1.5	28.6	5.9	25.3	11.6	2.9	0.9	30.3	13.5
	658	5.4	0.0	63.1	15.6	26.9	10.3	4.3	6.0	55.4	28.9
4.0	10	2.5	1.4	19.4	4.4	28.4	23.5	3.9	0.6	30.3	1.9
	16	1.9	1.9	15.4	2.7	25.1	20.3	3.3	0.0	25.8	0.0
	25	1.9	1.8	16.0	2.8	25.1	20.1	3.3	0.1	26.2	0.0
	35	2.0	1.8	16.6	3.0	25.2	20.0	3.3	0.2	26.6	0.4
	56	2.1	1.8	17.9	3.4	25.2	19.6	3.4	0.3	27.5	1.4
	74	2.1	1.7	19.1	3.7	25.3	19.3	3.4	0.4	28.2	2.2
	119	2.9	1.9	21.3	3.7	21.1	16.6	3.2	0.9	30.0	1.4
	357	1.2	1.5	28.3	6.4	26.6	11.0	3.0	0.9	29.9	14.9
	658	6.1	0.0	60.2	15.8	27.5	11.4	4.7	5.2	57.7	29.9

In previous studies it was also found that the total concentration of phenolic acids decreased during storage of the wines in bottles, particularly after the third month (GUTIÉRREZ; LORENZO; ESPINOSA, 2005). In this study, it was found that the phenolic content initially diminished, as a general trend, which may be due to the polymerization of polyphenolic molecules. After a certain period of time, the content of these compounds started increasing again. This phenomenon may be due to breakage of esters, such as tartaric esters of grape hydroxycinnamic acids and caffeoyl-tartaric, which releases caftaric acid molecules, or it also may be due to the breakage of *p*-coumaroyltartaric, which contains a coutaric acid molecule in its structure. It can be observed an increase in the content of caffeic acid along time, when its values increased around six times its original content, when analyzing the wines two years after bottling. This increase may be due to an additional source of caffeic acid from the hydrolysis of cinnamoyl-glucoside anthocyanins (MORENO-ARRIBAS; GÓMEZ-CORDOVÉS; MARTÍN-ÁLVAREZ, 2008) or by the hydrolysis of hydroxycinnamates by cinnamoyl esterases (SOMERS; VÉRETTE; POCOCK, 1987) presented in wine.

In the case of the content of the most common nonflavonoid in grapes, caftaric acid was present initially in concentration around 30 mg L^{-1} in all wines, and around two years after, around 54 mg L^{-1}.

Gallic acid, the unique hydroxybenzoic acid derived from *Vitis vinifera* grapes, is extracted from the seeds during the maceration and fermentation processes (ZOU et al., 2002). It esterifies with ethanol and methanol during fermentation, as what appears to have happened is that there was a cleavage in these molecules, which made gallic acid to appear in its free form, and therefore increasing its concentration in the wines used in this study when comparing it according to time (**Table 3.3)**. Moreover, according to Somers et al. (SOMERS; VÉRETTE; POCOCK, 1987), hydroxycinnamic acids usually appear in the first hours of fermentation and may be due to enzymatic hydrolysis of tartaric esters, and it was also found that these reactions may occur in stainless steel tanks and in oak barrels as well.

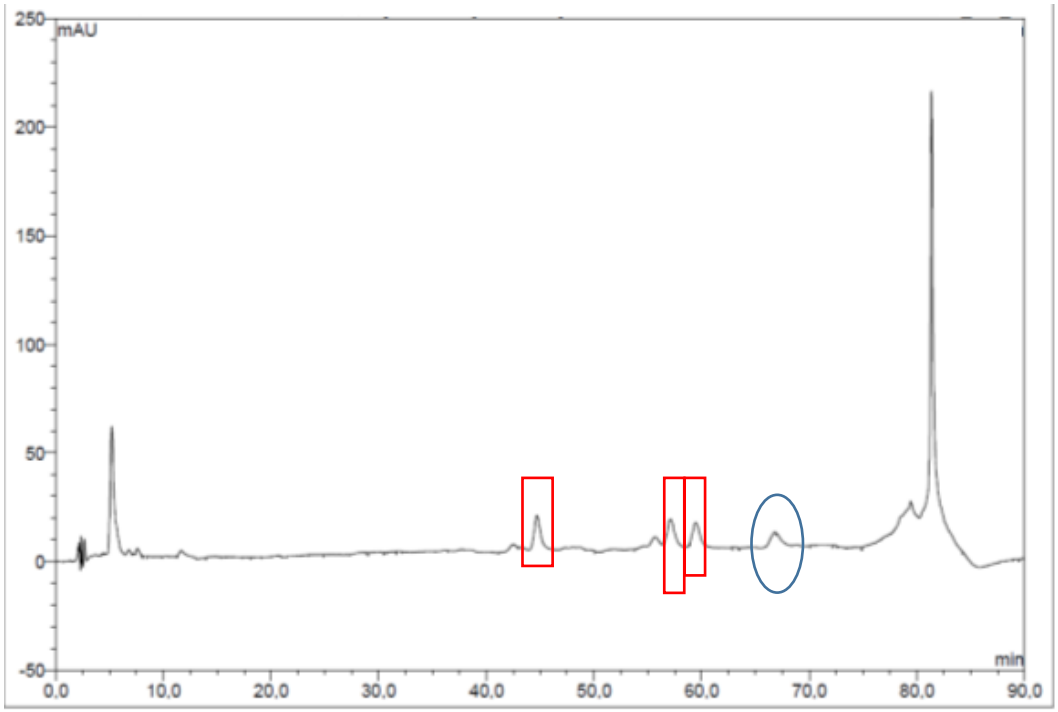
The same trend was observed for the other hydroxybenzoic acids (protocatechuic and syringic acids) studied in these wines. In contradiction to our study, Revilla and González-San José (REVILLA; GONZÁLEZ-SANJOSÉ, 2003) found no significant changes in the total concentration of gallic, protocatechuic and syringic acids in

Tempranillo wines elaborated from vinifications treated with pectolitic enzymes during 24 months of bottle-aging.

For caffeic acid, the increment in concentration was around 6 to 7 times its initial content after two years of bottling, although it was stable during the first year. The evolution of caftaric and caffeic acids may be due to a decline of the concentration of hexose esters of these compounds. However, in the case of coutaric acid, its content was the same during the entire period analyzed.

Catechin had an increase of its content only in the wine with pH 3.2 (11.6 to 1935 mg L^{-1}), whereas the wines with pH 3.4, 3.6, 3.8 and 4.0 showed no difference on its content along the time studied. Catechin concentration can influence the taste and aroma of red wines and it is a precursor of proanthocyanidins. No correlation was found among the parameters studied, but with pH, total acidity and epicatechin content, which have had its values diminished in all wines studied, but having the least final amount on the wine with pH 3.2 (5.3 mg L^{-1}), half of the amount presented in other wines. Instead, vanilic acid content was null after the period considered in the study, whereas after 658 days of bottling three rutin-derivatives were identified based on UV-Vis spectra (**Figure 3.6**).

It indicates that rutin can have been oxidized, since its content diminished in all wines analyzed after 658 of bottling. Further investigations with a mass spectra are necessary to better understand which are those compounds and how the chemical reactions may have occurred.



Retention time (minutes)

Figure 3.6. *Example of chromatogram of a wine after 658 days of bottling, pointing the three rutin derivatives.*

The antioxidant activity of wines studied in this work pointed to a constant increase of the ability to scavenge DPPH˙ radical. In the analysis performed, this ability increases around 10 to 15% in the first month, and it continues increasing. After the first year of bottling, it was necessary to dilute all wines, in order to achieve reliable results in the spectrophotometer. Still, with the dilution, the DPPH radical scavenging arise around 70%.

In the literature, there are some contradictory reports regarding the antioxidant activity of wine after certain periods after bottling. While studies (MANZOCCO; MASTROCOLA; NICOLI, 1998; OKUDA et al., 2002) indicates the decrease of the DPPH radical scavenging activity for older wines, another study (LARRAURI et al., 1999) demonstrates, through an index of antiradical efficiency (parameter in which the combination of the content of total phenolic compounds are necessary to neutralize 50% of the radical), that it increases. It is known though that the antioxidant activity of wines due to phenolic composition may vary according to the concentration of individual and specific phenolic compounds, the storage temperature of the wines, as

the presence of oxygen, closure and sulfur dioxide content (RIBÉREAU-GAYON; GLORIES, 1986).

Moreover, it is also known that the antioxidant activity value determined by this method relies on the selected reaction time, because the kinetics change frequently according to the polyphenolic matrix, in which due to the short time left for reactions, may be leading to underestimated values that do not correspond on the total antioxidant capacity of the wines analyzed (HUANG; OU; PRIOR, 2005; OZGEN et al., 2006). Besides, wine is a complex matrix in which present kinetic pattern constituted by slow and fast oxidative reactions, which presents in DPPH method a rapid absorbance change, followed by a slow one. This dual phase oxidative reactions may be due to a rapid oxidation of phenolic compounds which leads to the formation of semiquinone products that will further dimerize yielding new compounds that contribute to antioxidant activity (HOTTA et al., 2001). Therefore, the results presented in this study may lead to the conclusion that the evolution of polyphenolic content was in direction to those capable of fast neutralizing the DPPH radical, which does not mean that the antioxidant activity increased in absolute value.

The chemical parameters, when using the multivariate analysis of Principal Component analysis, they all differed in time, but all wines (5 pH's groups) were close in the same quadrant, indicating they did not differ among each other.

Moreover, after running principal component analysis, when analyzing the Pearson Correlation Matrix, it could be noticed that the higher the levels of protocatecuic acid, gallic acid, syringic acid and quercetin, the smaller the content of volatile acidity ($r = -0.969, -0.882, -0.854, -0.796$, respectively), considering that values are different from 0 with a significance level of $\alpha=0.05$. These considerations indicate that the concentration of these parameters may be giving wine a limited protection to wine acidity, as these findings indicate that these compounds can be used as oxidative winemakers.

Moreover, as it can be seen in **Figure 3.7**, the eigen values and cumulative variability of factors resulted from the principal component analysis performed considering all chemical parameters measured.

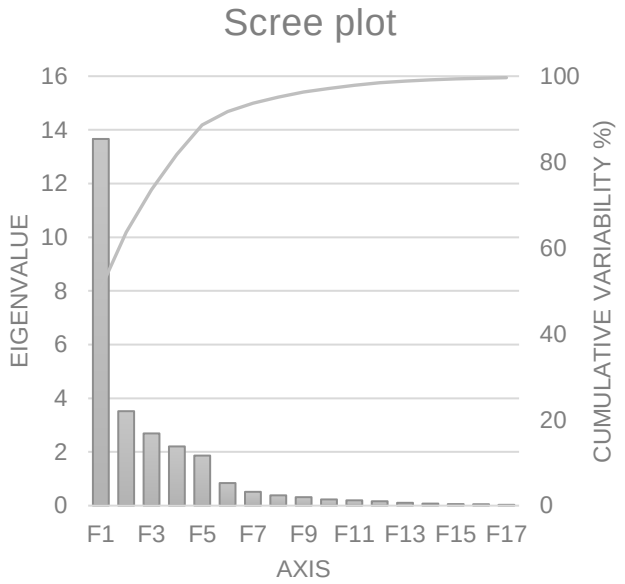


Figure 3.7. *Eigenvalues and cumulative variability of principal components of principal component analysis.*

Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy value was 0.739, proving that the statistical analysis was due to proper analyze de components experimentally investigated in this study.

Figure 3.7 shows the loadings of principal component 1 versus principal component 2, where there can be seen three groups. One of these groups, positive in both PC_1 and PC_2 (**Figure 3.8**), presents the five wines, with all pH tested, after 357 days of bottling. They are all grouped and present no difference among the parameters evaluated.

Another group in which can be seen clearly is the wines with all pH tested in the fourth quadrant, in which are grouped apart from others, the wines after 658 days of bottling, which are different from all others in the period tested. Wines from all pH tested 10, 16 and 25 days after bottling are also part of the same groupment.

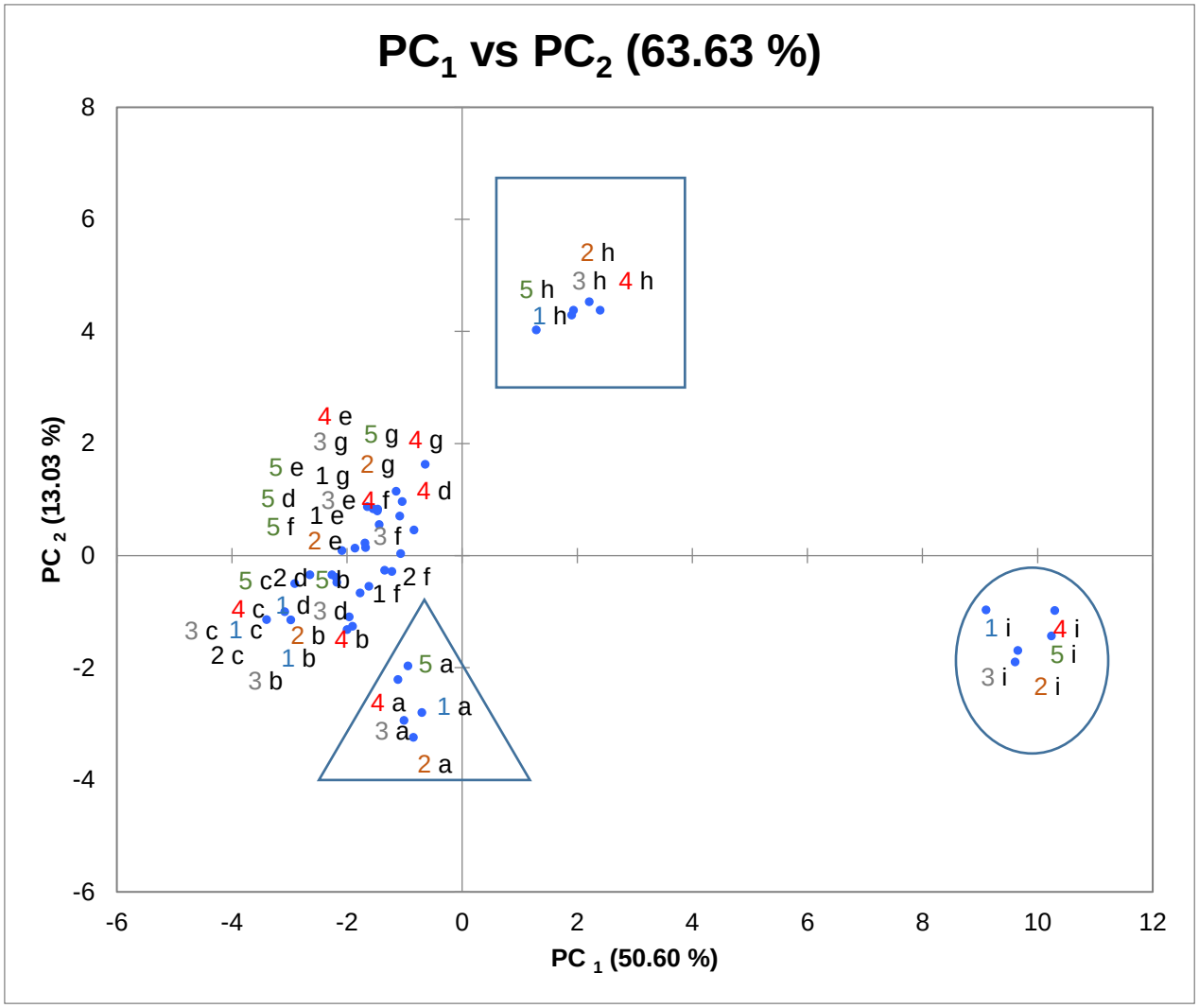


Figure 3.8. *Plot loadings of principal component 1 and 2 (PC_1, PC_2) in which each point represents a sample (wine) according to the subtitle abbreviated. PC_1xPC_2 represent 63.63% of variance of all samples and parameters evaluated. Subtitles: wines with pH 3.2 (1); 3.4 (2); 3.6 (3); 3.8 (4); 4.0 (5). Days after bottling: 10 (a); 16 (b); 25 (c); 35 (d); 56 (e); 74 (f); 119 (g); 357 (h); 658 (i).*

As it can be seen in **Figure 3.9,** the responsible factors (parameters) for the groupment of the wines with different pH 658 days after bottling were: *p*-coumaric acid, gallic acid, syringic acid, total polyphenolic index, polyphenols, caffeic acid, caftaric acid, protocatechuic acid and catechin.

Instead, the parameters responsible for the groupment of those five wines with different pH in the 357 day after bottling were: quercetin, the relative amount of large polymeric phenolic compounds, tannins, pH and DPPH. Phenols are responsible for red wine color, astringency, and bitterness, in addition to contributing to the olfactory profile of the wine.

Figure 3.8 also presents the wines in the periods of 10, 16, 25, 35, 74 and 119 days after bottling are grouped together when considering all the chemical parameters analyzed statistically by principal component analysis.

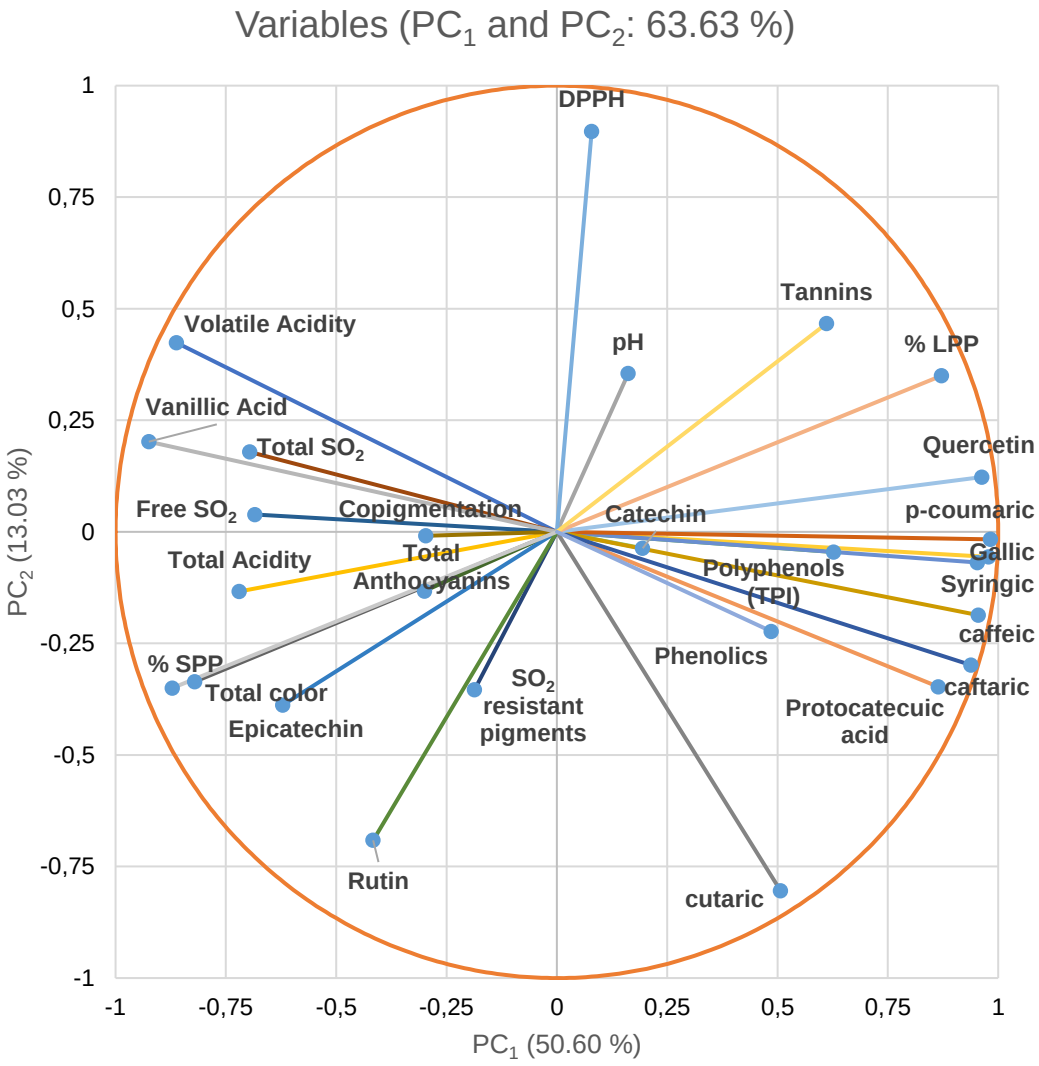


Figure 3.9. *Responsible factors (parameters) for the groupment (PC_1 vs PC_2) of the wines with different pH 658 days after bottling.*

Moreover, it can be seen in **Figure 3.10** the principal component analysis considering the PC_1 vs PC_3 which was responsible for 60.56% of the analysis variability. It corroborates what was previously shown in **Figure 3.8**, and it emphasizes the three distinct groups formed, where all the wines (pH 3.2, 3.4, 3.6, 3.8 and 4.0) were grouped together, but separately from other after 357 and 658 days after bottling. It can lead to

the conclusion that the pH did not alter physico-chemical parameters along the time and wines studied, only regarding to the time after bottling.

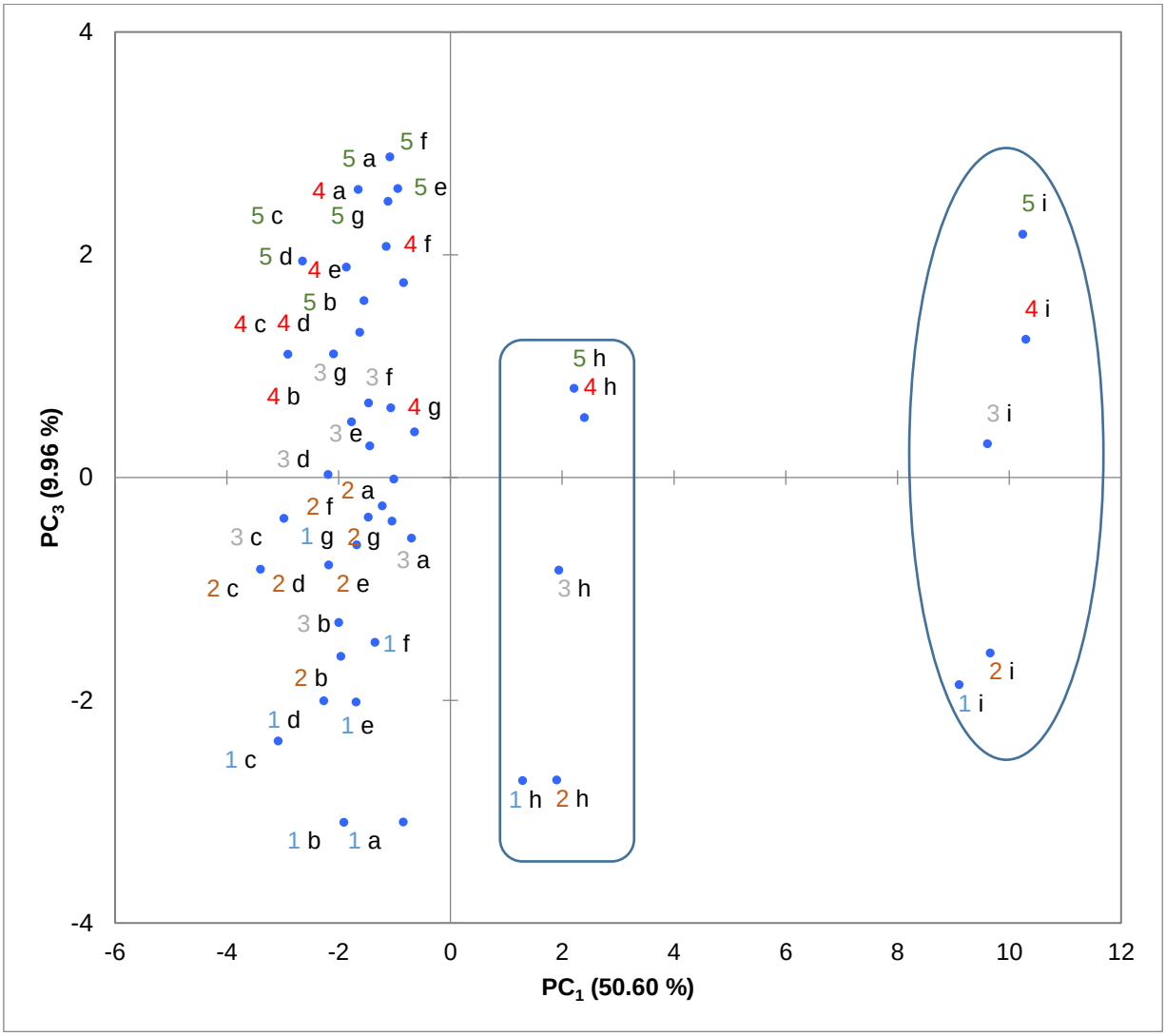


Figure 3.10. *Graphic representation of loadings of principal component 1 (PC_1) and principal component 3 (PC_3), in which each point represents a sample (wine) according to the subtitle abbreviated. PC_1xPC_3 represent 60.56% of variance of all samples and parameters evaluated. Subtitles: wines with pH 3.2 (1); 3.4 (2); 3.6 (3); 3.8 (4); 4.0 (5). Days after bottling: 10 (a); 16 (b); 25 (c); 35 (d); 56 (e); 74 (f); 119 (g); 357 (h); 658 (i).*

The chemical parameters were in accordance with the sensory test performed, where, among the 36 assessors, no significant statistical difference was achieved, indicating that the pH difference was not felt by the senses of human smell and taste.

A number of published works have focused on the essential contributions of phenolic profiles to wine quality and sensory properties. Some studies correlate specially the content of catechin and its influence in color and astringency (KALLITHRAKA; BAKKER; CLIFFORD, 1997; SIMS; MORRIS, 1985). In this study, it was found that

one of the main parameters differing wines among their pH was the catechin content, however there was found no correlation among the wines evaluated, as in the sensory analysis, the testers could not perceive it. It may be due to the fact that this difference was not apparent.

Moreover, electronic nose and fast gas chromatography analysis were performed in order to evaluate possible volatile changes in wine. Results of the analysis performed after two years of bottling using electronic nose (**Figure 3.11**) and Flash gas chromatography (**Figure 3.12**) are presented.

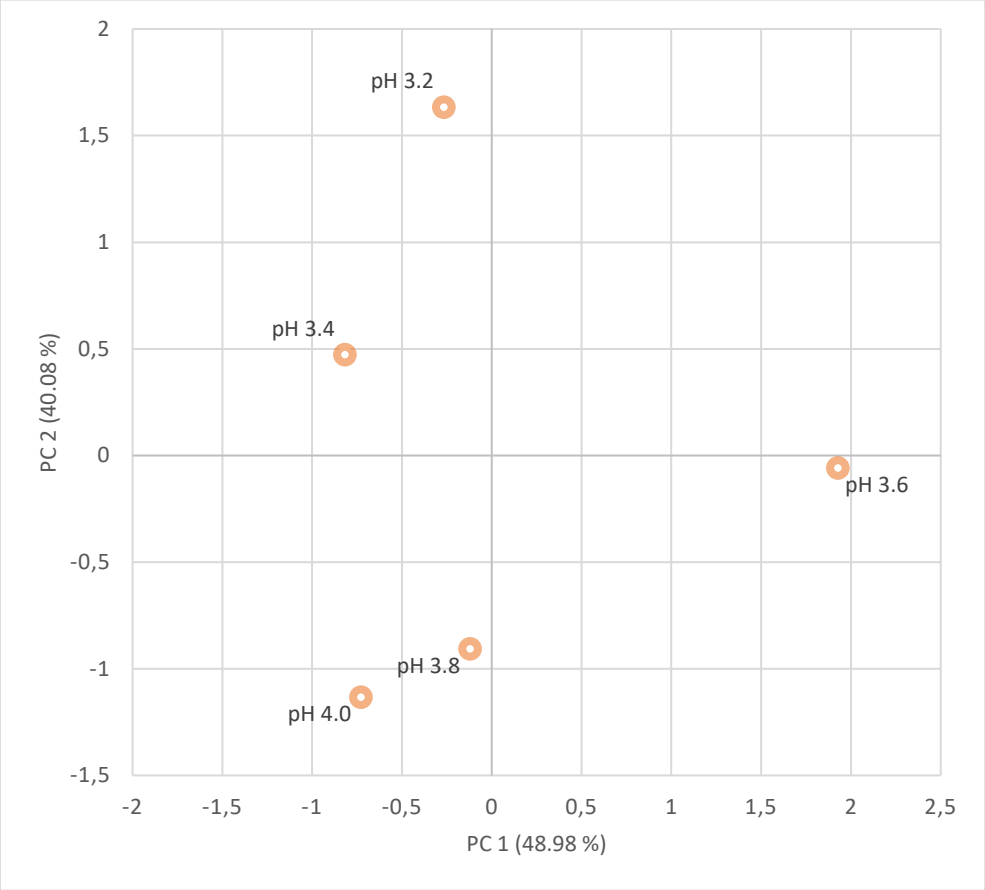


Figure 3.11. *Plot loadings of principal component 1 and 2 (PC_1, PC_2) for electronic nose analysis, in which each point represents a sample (wine).*

For the electronic nose analysis, it was found that the first two principal components were responsible for 89.07% of the total variation resulted in the analysis.PC_2 separated wines according to pH, while PC_1 was able to group all wines in the same left side plot, except the one with pH 3.6. A two-steps mechanism seems to occur, which involve a common pathway for all wines along PC_1, whereas only the wine at pH

3,6 seems to be involved at some extent in the second step of reaction as highlighted by PC_2.

Regretfully, the Flash Gas Chromatography was able to distinguish the wines at pH 3.8 and 4.0, only (**Figure 3.12**).

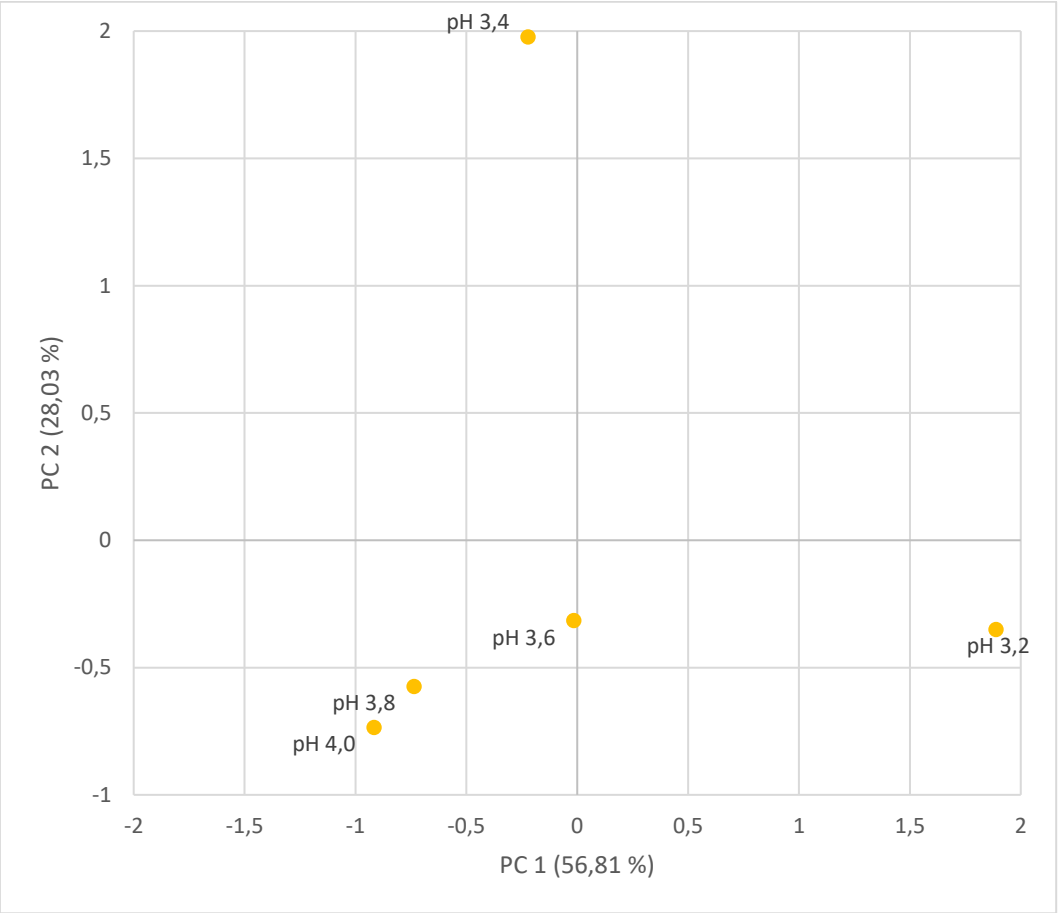


Figure 12. *Plot loadings of principal component 1 and 2 (PC_1, PC_2) for Flash Gas Chromatography analysis, in which each point represents a sample (wine).*

The different results may be due to the sensitivity/selectivity of each analytical method. The electronic nose does not separate the volatile fraction in terms of polarity, for example, but only by the temperature in which they are detected by one of the twelve sensors used in the measurements. In the contrary, the Flash Gas Chromatography is equipped with two columns and a FID detector, where selected explanatory variables (area of chromatographic peaks) characterized by the biggest variability of the response were used for discriminant analysis. A study used the two methods to classify samples of agricultural distillates into quality classes using a linear discriminant function, and it was found that the prototype of electronic nose used provided correct classification of 70 % of the samples when compared to the Flash Gas Chromatograph,

which was the same used in the present study (DYMERSKI; GĘBICKI; NAMIEŚNIK, 2014). Therefore, flash gas chromatography was able to detect volatile fraction similarities among the five wines studies that the e-nose device was not able to, due to sensitivity limitation.

3.4. CONCLUSIONS

The changes in pH did not interfere in the wine oxidation parameters along the studied period, although some small changes have been seen, they were not significant to the overall quality of wines studied. The hypothesis of marked complexity where contra-effects tended to balance out pH effect, was formulated.

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CHAPTER 4

CHAPTER 4
DISCLOSURE OENOLOGICAL TANNINS

ABSTRACT

Oenological tannins are polyphenolic compounds extracted from oak, chestnut, or birch woods and other suitable plant sources, including grape seeds and skins. Chemically they can be classified in two groups: condensed and hydrolysable tannins. Of great importance in winemaking process, they have several uses in the wine industry, as new studies and applications to them arise from scientific works nowadays. Their use in the avoidance of oxidative damages in wine has been reported, however as the manufacturers give little scientific information on their regard, studies have been performed in this PhD in order to better elucidate the complex chemistry in which are involved, as to elaborate screening methods for the industry on the better evaluation of this 'natural bioactive adjuvant'. In order to achieve it, thirty samples of commercial oenological tannins with several origins and suppliers were analyzed either for its antioxidant activity by DPPH method, and by FTIR. With the use of chemometrics through Partial Least Square regression with full cross-validation, a fast screening method for the classification of oenological tannins was developed.

4.1. Tannins - General Introduction

Originally the term "tannin" means *oak* in Celtic language and it has been extensively used commercially due to its chemical properties which makes them capable of producing stable combinations with proteins and other plant polymers (HASLAM, 1998; RIBÉREAU-GAYON et al., 2006a).

Tannins are secondary plant metabolites that belong to the polyphenolic compounds class that have the ability to precipitate proteins and complex carbohydrates. This property is only found in polyphenols above a certain molecular weight (500-3000) (PUECH; FEUILLAT; MOSEDALE, 1999; RIBÉREAU-GAYON et al., 2006b). Henceforth tannins are natural occurring compounds that exist inside grape skins, seeds and stems and they can be chemically divided into two groups namely *hydrolysable* (or proanthocyanidins, originated from oak) and *condensed* (originated from grapes), as it is detailed in **Figure 4.1.**

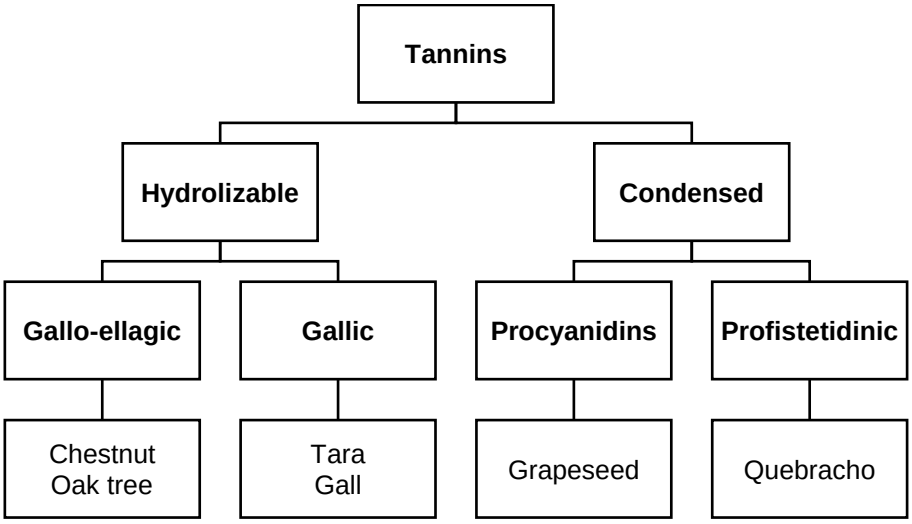

Figure 4.1 *Chemical division of tannins.*

They are one of the main constituents in red wine, as they contribute to it structure, reinforce the color and play an active role both promoting or protecting from wine oxidation, as they also act on the sensorial quality, e.g. astringency (VERSARI; DU TOIT; PARPINELLO, 2013). The use of oenological tannins (food grade) is accepted by the International Œnological CODEX of Organization Internationale de la Vigne et du Vin (OIV, 2012) as part of 'fining' enological practice, however tannins can be used for further purposes redox buffer; sun-damaged fruit; unripe grape tannins; structural/textural, mouth feel modification; increased substrate for micro-oxidation; limit the activity of laccase; assist to precipitate proteins; help to modify aromas, including vegetative aromas; help increase aging potential and help stabilize red wine color (LURTON, 2002; PEÑA-NEIRA, 2000, ZOECKLEIN, 2005).

Commercial tannins can be added to wine for the above-mentioned reasons depending on the different country's legislation. However, it should be noted that all these claimed properties of commercial tannins have not been fully scientifically confirmed.

Moreover, due to several factors, some wines are olfactive poor and with an inadequate phenolic content, which makes it necessary the use of oenological tannins, in order to improve their final quality and to obtain wines with greater distinctiveness, complexity and palate balance.

Part of this PhD study has been dedicated to evaluate the use of oenological tannins, particularly regarding their analysis in order to investigate their characterization.

This chapter is inferred from a scientific manuscript aimed to publication, which is currently accepted by the *Italian Journal of Food Science,* entitled “Rapid screening method to assess tannin antioxidant activity in food-grade botanical extract” authored by Palma, Ricci, Parpinello, Versari.

Moreover, a second published work is presented in **APPENDIX C** in which was a collaborative work entitled “Analytical profiling of food-grade extracts from grape (*Vitis vinifera* sp) seeds and skins, green tea (*Camellia Sinensis*) leaves and Limousin oak (*Quercus robur*) heartwood using MALDI-TOF-MS, ICP-MS and spectrophotometric methods" published in *Journal of Food Composition and Analysis* and authored by Ricci, Parpinello, Palma, Teslić, Brilli, Pizzi, Versari.

Tannins are, in general terms, large phenol molecules produced by the polymerization of elementary molecules with phenolic functions. Condensed and hydrolysable tannins vary in their monomer constituents which comprise their oligomeric structures. Independently on their classification, they are all polyphenols with considerable amount of hydroxyl moieties. It is due to the hydroxyl groups that provide these molecules their physical and chemical properties which are responsible for their industrial usage (SANTOS-BUELGA; SCALBERT, 2000).

4.2.1 Condensed Tannins

Condensed tannins, also known as proanthocyanidins, are formed in grapes by the polymerization of catechins (flavan-3-ols). They can be classified according to their nature of their flavonoid unit, bonding, esterification to other compounds, or functional properties (JACKSON, 2014). Generally, they are formed by flavanol units (**Figure 4.2**) polymerized, which are composed by either catechin, epicatechin, epigallocatechin, gallocatechin or epicatechin gallate (PRIEUR et al., 1994; SOUQUET et al., 1996).

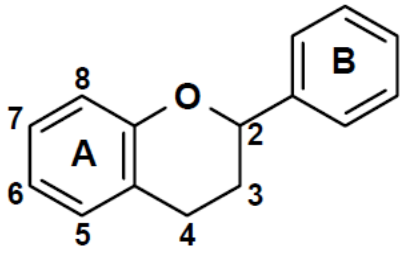

Figure 4.2 *Basic flavonoid skeleton structure*

One of procyanidins classification can be according to the nature of the molecular flavonoid monomer nature, which can be either proanthocyanidins or prodelphinidins,

which is based on their cleavage under low pH, which can release cyanidin or delphinidin, respectively (JACKSON, 2014).

Procyanidins containing only a single covalent carbon bond between adjacent flavonoid sub-units are the most common as typical catechin subunits in grapes are (+)-catechin, (-)-epicatechin, (-)-epicatechin-gallate, and less commonly (-)-epigallocatechin.

(+)-Catechin and (-)-epicatechin (**Figure 4.3**) are differentiated basically by their stereochemistry; while (+)-catechin have its C3 hydroxyl group in a plane opposite to B-ring, (-)-epicatechin has it both C3 hydroxyl groups in the plane of B-ring. Instead, (-)-Epigallocatechin and epicatechin are differentiated by the first having a third hydroxyl group in its B-ring, while (-)-epicatechin gallate has a gallic acid esterified to C3 (JACKSON, 2014).

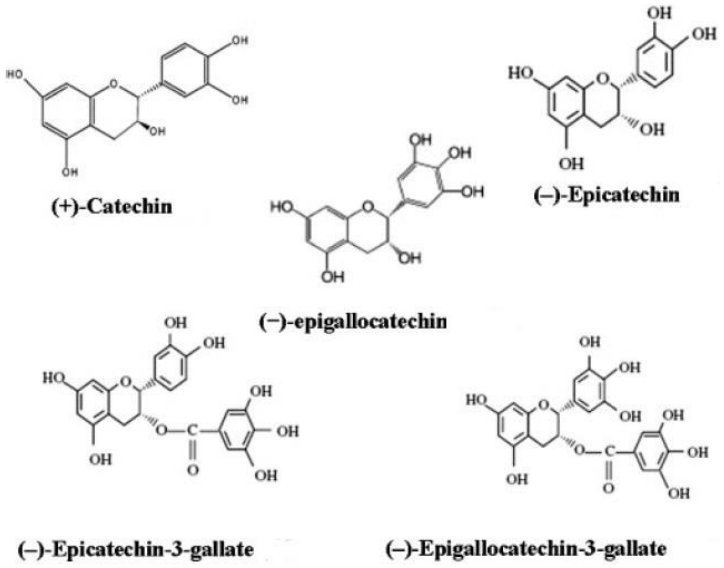

Figure 4.3 *Chemical Structures of (+)-catechin, (-)-epicatechin, (-)epigallocatechin, (-)-epicatechin-3-gallate, (-)epigallocatechin-3-gallate. Source: Modified from (CHATTERJEE et al., 2012)*

Usually in grapes skin catechin is the most typical terminal unit, whereas catechin, epicatechin and epicatechin-gallate occur as terminal units in seeds tannins. Most of procyanidins in wine are originally from grapes, while few are extracted from oak. Red wines have around 20 times more procyanidins than the white ones. There are structural differences among skin, stem and seed procyanidins as they vary in type

and concentration according to the cultivar (JACKSON, 2014; KOVAC et al., 1990). Tannins present in seeds are less polymerized than those in the skin, which contain around 74 flavanol moieties against 28 flavonol moieties in the first mentioned (HAYASAKA et al., 2003; LABARBE et al., 1999).

When the wine is young, most of the procyanidins extracted are dimmers and trimers, and while it ages, these procyanidins bind with monomeric units of flavonoids (around 8 to 14 units), generating polymers, which can be than called tannins. It is important noticing that this procyanidins are not only polymerizing among themselves, but also with anthocyanins and polysaccharides. It is due to the presence of unconjugated hydroxy-phenolic groups in flavonoid polymers that grant tannins the ability of binding proteins and the bigger the polymer, the more insoluble it gets, and the less is their ability to react with proteins (JACKSON, 2014).

Condensed tannins (CT) are hence distinguished from hydrolysable or complex and mixed tannins by the type of elementary molecules and from their chemical characteristic of acquiring red color when diluted in acid. This phenomenon happens when the interflavanyl bond is hydrolysed and colored anthocyanidins are formed (**Figure 4.4**). The low pH weakens the bonding between the hydrogen and oxygen atoms of the associated moieties. On the other hand, hydrolysable tannins are more stable under acidic conditions, which sticks together by covalent bonds. Therefore, hydrolysable tannins do not form colored compounds under the same conditions (ROUX, 1992; SANTOS-BUELGA; SCALBERT, 2000; SCHOFIELD; MBUGUA; PELL, 2001; SERRANO et al., 2009).

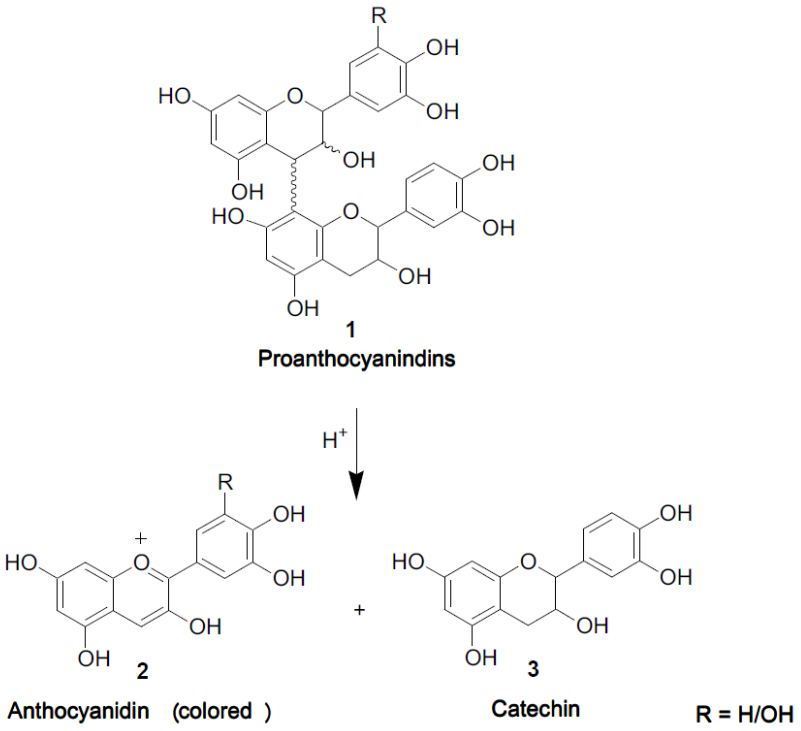

Figure 4.4. *Hydrolysis of the interflavanyl bond to form anthocyanidins (JORDAAN, 2013)*

In this context, among the beneficial usage of exogenous tannins in wines, Saucier et al. (SAUCIER et al., 2004) has described that the color of wine enhances with aging, as the polyphenols molecular weight, which is due to the polymerization between anthocyanins and proanthocyanidins. Another study (LIU et al., 2013) evaluated the addition of 5 exogenous tannins from different botanical sources into Cabernet Sauvignon wines. They found that condensed tannins contributed to the redness of wine, most probably due to the ability of tannins to protect wine against oxidation, as contribute to the color copigmentation and the formation polymeric pigments.

4.2.2. Hydrolyzable Tannins

Hydrolyzable tannins are not naturally present in grapes (except gallic acid), although they are the main commercial tannins legally authorized as wine additives. They are mainly derived from oak and in those wines which do not age in it, they derivate of hydroxycinnamic and hydroxy-benzoic acids, which are primarily in cell vacuoles of skin and pulp, and therefore extracted during crushing process of winemaking.

These tannins (**Figure 4.5)** are nonflavonoids (C6-C3 skeleton) with a simple structure, and they can be classified as either gallotannins or ellagitannins, according to the type

of acid formed (PUECH; FEUILLAT; MOSEDALE, 1999). A polyhydric alcohol is the basic structural unit, where hydroxyl groups are esterified by gallic acid or hexahydroxydiphenic acid (HAGERMAN, 2002; HAGERMAN; BUTLER, 1991). These tannins are hydrolyzed straightforwardly either enzymatically, in acid or basic media, forming free gallic acid or hexahydroxydiphenic acid. Hexahydroxydiphenic acid hydrolyzes to ellagic acid (PUECH; FEUILLAT; MOSEDALE, 1999).

Common examples are caftaric, coutaric and fertaric acids and their tartaric esters. In minor amounts the caftaric and coutaric acid derivatives which undergo oxidation can give a gold coloration to white wines.

Hydrolyzable tannins signify around 10% of the dry weight of oak heartwood. Therefore, the maturation in oak gives wine high levels of hydroxybenzoic acid derivatives, remarkably ellagic acid, which are formed by the association of two molecules of gallic acid. Moreover, esters of ellagic acid increase the red wine color by copigmentation with anthocyanins. Their ability to oxidize quickly also makes them important in the redox status of wine, where they consume the oxygen in wine matured in oak cooperage.

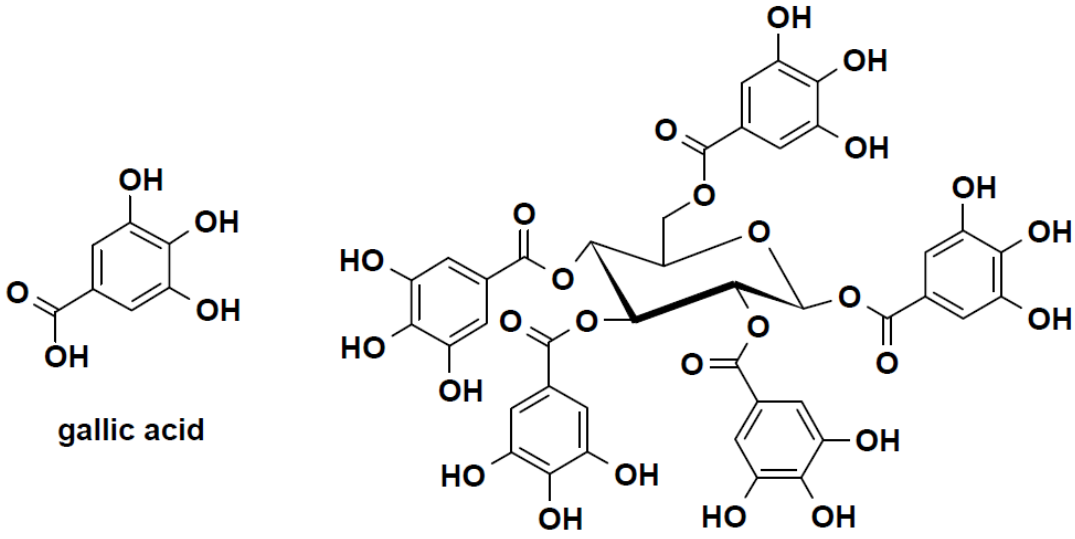

Figure 4.5 *General structure of the simplest hydrolysable tannin, a gallotannin (HAGERMAN, 2002).*

Hydroxyl groups present in hydrolysable tannins are responsible to most of their properties, which can vary according to the number location of these groups. These hydroxyl groups confer tannins antioxidant properties, which can act in two ways: it can be due to the fact that hydrolysable tannins may be easily oxidized, thus

diminishing the oxygen availability for other reactions; or by chelating metal cations, which are catalysts for oxidation reactions. By acting as free radical scavengers, hydrolysable tannins can also possibly combine with quinones and inhibit free radical formation (PUECH; FEUILLAT; MOSEDALE, 1999; VIVAS; GLORIES, 1996; WATERHOUSE; LAURIE, 2006).

Concerning a comparison between catechin and ellagitanins, Vivas and Glories (1996) demonstrated that ellagitanins oxidation is diminished when sulphur dioxide is added, since it competes for binding with oxygen, and that ellagitannins are more effective than catechins, regarding oxygen consumption rate, when used as a supplement in red wines. It may be due to the comparative number of hydroxyl functions in both molecules. In the same study, they also showed that as these two molecules compete by the oxygen, and the ellagitannis (which consumes more the oxygen) protects catechins from oxidation when they are together in the same media, because they compete for the oxygen.

4.3. Commercial Tannins

Commercially, hydrolysable tannins are still limited due to the small quantities available in the commerce. As example, some of these tannins, such as oak and chestnuts extracts, can be used to produce high-value specialty leathers.

On the contrary, proanthocyanidins are more available in the market, including the heartwood of *Schinopsis balansae* and *Schinopsis lorentzii* that can be extracted in order to obtain quebracho extract.

4.3.1 Oenological Tannins

In oenology, commercial tannins have been extensively studied and used (BAUTISTA-ORTÍN et al., 2005; OBRADOVIC; SCHULZ; OATEY, 2005; VERSARI; DU TOIT; PARPINELLO, 2013) for several reasons, as it follows:

a) Precipitation of proteins;
b) Improvement of mouthfeel sensation and wine aroma / flavour;
c) Inhibiting laccase activity;
d) Stabilization of red wine colour;
e) Decrease reductive off-flavours.

Table 4.1 summarizes some of the most used tannins in agriculture and its agricultural source.

Table 4.1. Origin of oenological tannins

Origin	Tannins Nature
Galls of *Rhus semialata*	Gallotannins
Fruits of *Terminalia chebula*	Gallo / ellagitannins
Wood of *Castanea sativa*	Ellagitannins
Wood of *Quercus robur*	Ellagitannins
Wood of *Quercus petraea*	Ellagitannins
Galls of *Quercus infectoria*	Gallotannins
Galls of *Robinia speudoaccacia*	Gallotannins
Fruits and pods of *Caesalpina spinose*	Gallotannins
Fruits and seeds of *Vitis vinifera*	Procyanidines

Sources: (TANG; HANCOCK; COVINGTON, 1992; VIVAS, 1997)

As it can be seen, tannins used in the winery industry are derived from several sources, including those that have their origin from the grape, or as lignified plant parts, from fruits and protuberances produced by pathogenic agents (galls). The most used tannins source currently comes from different vegetal species, such as different oak species (Quercus sp.), chestnut (*Castanea sativa* Mill.), quebracho (Schinopsis sp.), mirabolano (*Terminalia chebula*), etc (CRESPY, 2006; MOUTOUNET et al., 2004; VIVAS, 2001).

In addition, besides the botanical source, according to Versari et al. (2013), tannins available for commercial use can also be classified according to their methods of extraction, way of processing and purification, as by their degree of polymerization. Moreover, they are mostly obtained by water or steam extraction, following by drying and milling process (ZOECKLEIN, 2007).

Because of those above-mentioned uses and the extensive use of tannins in the winemaking process, several studies have been developed regarding the positive characteristics acquired by the wine after the addition of these oenological tannins. Moreover, there are new insights on the property of tannins as antioxidants, as there was a study proving that the addition of ellagitannins and gallotannins to wine have limited wine oxidation in time (BOSSO et al., 2001).

Additionally, another study demonstrated that the addition of oenological tannins can also be positive to wine aroma, correspondingly by their ability to inhibit the oxidation process of musts and wines when low sulfur dioxide content is used. They act as antioxidants and avoid the oxidation of some ethyl-esters, preserving the aroma quality of wines (BELLACHIOMA et al., 2008; SONNI et al., 2009).

Based on the wide range of commercial tannins, the necessity of deep investigations becomes necessary, in order to stablish its potential as enological input to be applied as adjuvants for fine and complex wine with high quality.

Therefore, the aim of this PhD chapter was to set-up a rapid method for monitoring the antioxidant activity of tannins, in order to give wine industry a practical tool for daily use.

4.4. Materials and Methods

4.4.1 Samples and Chemicals

4.4.1.1 Samples

Thirty samples of commercial oenological tannins as lyophilized powders (**Table 4.2**) with several origins and suppliers were analyzed. Before use the tannins were dissolved in model wine solution as follows.

4.4.1.2 Model Wine Solution

Model wine solution was made with 12% of ethanol content in distilled water, L-tartaric acid (0.033 M) and pH adjustments with NaOH and HCL 0.1N until pH was 3.6 (L-tartaric acid (≃100%) and pure ethanol (<99%) from Merck Darmstadt, DE).

Solutions of tannins were made in a concentration of 1 g L^{-1} in the model wine solution, which was in accordance with previous works (RICCI et al., 2016), corresponding to 3.45 mM of (+)-catechin.

Table 4.2. Chemical classification and origin of tannins studied

No	Chemical classification	Origin
1	Condensed	Grape skin and seeds
2	Condensed	not specified
3	Condensed	red grapes
4	Condensed	Grape seeds
5	Condensed	Malbec's grape seeds
6	Condensed	Quebracho

7	Condensed	Unfermented grape skins
8	Hydrolysable	Chestnut wood
9	Hydrolysable	American oak
10	Hydrolysable	Allier French Oak
11	Hydrolysable	Limousin French Oak
12	Hydrolysable	wood Quercus
13	Hydrolysable	Selected woods
14	Hydrolysable	Allier French Oak
15	Hydrolysable	Chestnut
16	Hydrolysable	Galls
17	Hydrolysable	Not specified
18	Hydrolysable	Oak heartwood
19	Hydrolysable	Not specified
20	Hydrolysable	Wood (not specified)
21	Hydrolysable	Oak Wood
22	Hydrolysable / condensed	Not specified
23	Hydrolysable / condensed	Grapes
24	Hydrolysable / condensed	Not specified
25	Hydrolysable / condensed	Not specified
26	Hydrolysable / condensed	French Oak Wood
27	Hydrolysable / condensed	Toasted oak and grapes
28	Hydrolysable stabilized with natural polysaccharides	Red berries
29	Not specified	Grapes
30	Not specified	Not specified

Tannin's origin given by the producer.

4.4.2 Antioxidant Activity

The antioxidant activity of oenological tannins was evaluated according to the DPPH• radical scavenging activity. The analysis was performed according to literature (BRAND-WILLIAMS; CUVELIER; BERSET, 1995) (VILLANO et al., 2007).

In the assay 100µl of tannins solution (0.2 mM of catechin equivalent per liter of solution) were added to 2.9 mL of 200µM of DPPH solution in methanol. These solutions were than incubated in dark at room temperature for one hour, when they had their absorbance measured at 517 nm wavelength in 10 mm plastic cuvettes and the blank solution was pure methanol. Measurements were performed in a Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan) and expressed as percentage of inhibition(DUDONNÉ et al., 2009) as it follows:

$$\% \text{ DPPH scavenging activity} = [(Abs_{DPPH} - Abs_{tannin}) / Abs_{DPPH})] \times 100$$

Where:

Abs_{DPPH} is the absorbance measurement of DPPH solution (2.9 mL) with 0.1 mL of methanol;

Abs_{tannin} is the absorbance measurement of DPPH solution (2.9 mL) with 0.1 mL of tannin solution above mentioned.

4.4.3 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier-Transform Mid-Infrared (FT-MIR) spectral analysis was performed using a Tensor 27 spectrometer (Bruker Optics) (**Figure 4.6**) equipped with a horizontal attenuated total reflectance (ATR) zinc selenide (ZnSe) crystal (HATR, PIKE Technologies, Madison, US) (**Figure 4.7**).

One milliliter of sample was used for each and every tannin analysis. They were kept at 40 ±1°C for the whole duration of measurements.

The spectra obtained had a spectral resolution of 4 cm^{-1} with 64 scans averaged for each spectrum were recorded in duplicates from 4000 to 700 cm^{-1} for all samples. Accordingly, the same number of scans was used for background subtraction.

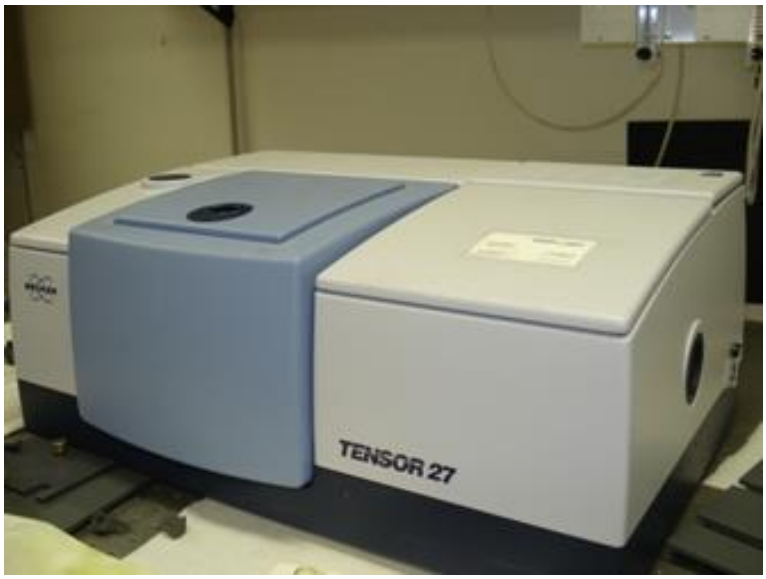

Figure 4.6. Tensor 27 spectrometer (Bruker Optics) used in the study.

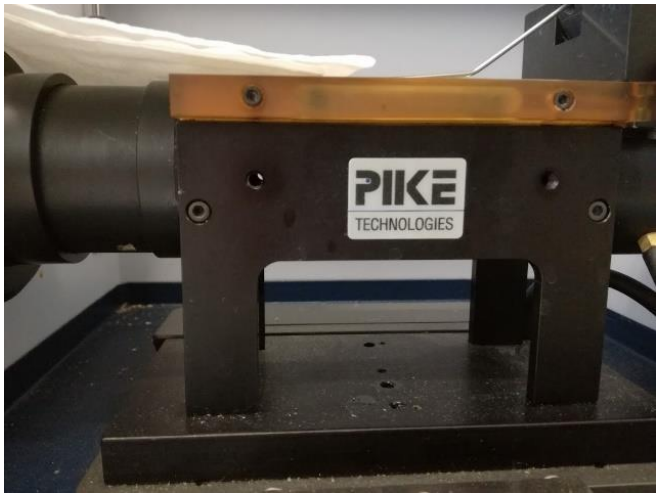

Figure 4.7. Horizontal attenuated total reflectance (ATR) zinc selenide (ZnSe) crystal (HATR, PIKE Technologies, Madison, US).

Before data analysis, each spectrum was corrected for the variation in effective path-length using the ATR correction option available in the Spectrum One 5.3.1 software (Perkin–Elmer, Waltham, MA). Spectra were than exported in ASCII format to the statistical software for statistical analysis.

4.4.4 Statistical Analysis

Chemometrics and data analysis were performed with Unscrambler software (Unscrambler 9.7, Camo, Oslo, Norway). Partial Least Square regression (PLS) was performed to model the relationships between the antioxidant activity of oenological tannins and wavelengths of FTIR spectra. Multivariate analyses were made using full cross-validation.

4.4.5 MALDI-TOF analysis

According to Singhal et al. (2015), the analysis by MALDI consists in mixing the analyte with a solution which is energy-absorbent, which will result in a matrix. Than it is dried until its crystallization. Then the sample, which is within the matrix, is ionized by a laser beam. This ionization in addition to a desorption will generate singly protonated ions from the sample, which will be accelerated at a certain potential that allows them to separate from each other, based on their mass-charge ratio (m/z). Once the analytes are charged, they can be detected and measured by time of flight (TOF) analyzers.

Basically, the couple MALDI-TOF allows the m/z ratio of an ion to be measured by the determination of time in which is necessary for it to travel the length of the flight tube. MALDI-TOF instrumentation were used for a collaborative work within commercial tannins analysis, in which is further summarized in this Chapter.

For the analysis, MALDI-TOF spectra were recorded using a Kratos compact MALDI Axima Performance TOF 2 instrument (Shimadzu Biotech, Manchester, UK), furnished with a nitrogen laser (337 nm), an ion gate for the selection of precursor ions, and a collision cell, in accordance to previous cited in the literature (LAGEL et al. 2014).

4.5. RESULTS AND DISCUSSION

The infra-red (IR) data obtained, which the signal was ranging from 4000 to 700 cm^{-1} (example found in **Figure 4.8**) was analyzed and a specific region was chosen to be further chemo metrically analyzed.

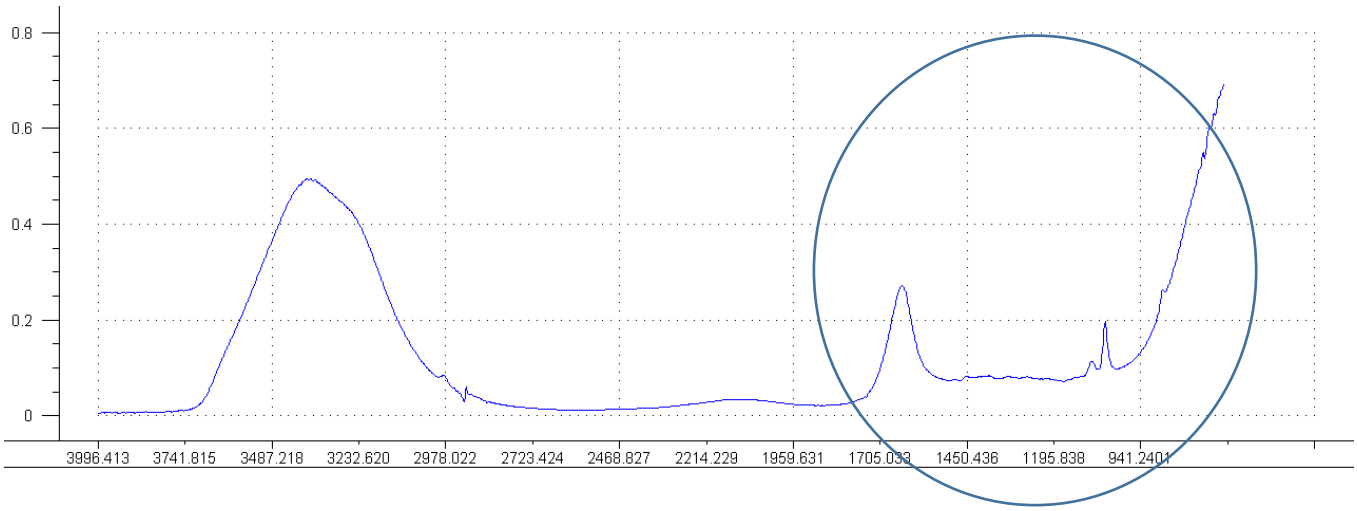

Figure 4.8. *Example of IR spectra obtained from the oenological tannins analysis.*

By analyzing the IR spectra obtained, a specific region of them was chosen (1750-900 cm^{-1}) for further chemometric analysis. This region is known as 'fingerprint' (are highlighted within the circle, ***Figure 4.8****) for polyphenolic compounds and it was previously used in other studies (JENSEN; EGEBO; MEYER, 2008; RICCI et al., 2015).*

The DPPH values obtained in the analysis ranged from 18.7% to 49.6%, indicating a wide difference among oenological tannins antioxidant activity which were considered in this study.

Partial Least Squares Regression (PLS) was then used with the attempt to model FTIR spectra according to the antioxidant activity of oenological tannins analyzed in the study. In this chemometric analysis, the use of six latent variables which explain 77% of total X-variance and 61% of total Y-variance.

Spectroscopic results obtained from the MIR screening demonstrated an acceptable correlation between the actual values of commercial tannins' scavenging capacity of DPPH radical towards predict values. Data are shown in **Table 4.3** and **Figure 4.9.**

Table 4.3. PLS Validation Statistics for fast prediction of antioxidant activity of commercial tannins

PARAMETER	VALUE
R	0.817
Slope	0.82
RMSECV	6.6

Abbreviation: RMSECV (root mean square error of full cross-validation); r (correlation)

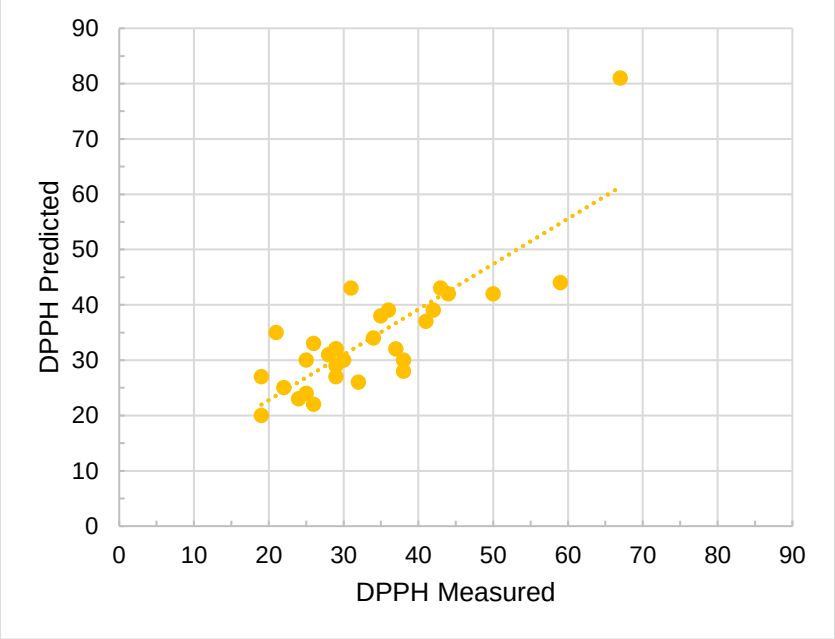

Figure 4.9. *Regression Plot for the prediction of antioxidant activity measured through DPPH radical scavenging of commercial tannins using FTIR and Full Cross PLS Validation.*

Results found in this work are consistent with previously reported in the literature (VERSARI et al., 2010) and it can be stablished that the use of FTIR along with PLS

regression as a chemometric approach is a fast method which can support the food and beverage industry in their choice of commercial tannins to be used as an additive. Several researches have been made in order to develop an analytical method for determination of tannins, either commercial or those existing in wine. These methods include precipitating tannins in the sample with proteins (HARBERTSON; PICCIOTTO; ADAMS, 2003), precipitation using polymers (SARNECKIS et al., 2006), other spectrophotometric assays for tannins detection (PORTER; HRSTICH; CHAN, 1985; STEVANATO; FABRIS; MOMO, 2004; SUN; RICARDO-DA-SILVA; SPRANGER, 1998), and precipitation of tannins with different reagents (ANTOINE; SIMON; PIZZI, 2004; SILANIKOVE et al., 1996; SINGLETON, 1974).

The results obtained with these methods can vary, especially when condensed tannins are being analyzed (ALEIXANDRE-TUDO et al., 2015). Therefore, it is difficult to use data obtained in the literature or resulted from different methods. Besides, the tannin content in such a complex matrix may not only vary according to the purity of the product obtained commercially, but also regarding its properties, such as antioxidant activity.

Therefore, the use of our fast screening method may be useful to indicate one of the main properties in which tannins are applied in food industry: their antioxidant activity.

4.6. ADVANCED STUDY

As previously mentioned, a collaborative work was performed with the aim of explore food grade tannins regarding the analysis of its composition in order to properly identify them concerning their declared botanical origin stated commercially. For this purpose, a combination of Matrix-assisted laser desorption/ionization allied to a time of flight Mass Spectrometry detector (MALDI-TOF) and spectrophotometric analysis were employed.

Among the four commercial tannins originated from different botanical sources (grape seed and skin proanthocyanidins, green tea procyanidins, and Limousin oak ellagitannin), the highest content of polyphenols was seen in the skin proanthocyanidins extract (93%), whereas the grape seed presented the smaller content. The antioxidant capacity of the extracts evaluated by DPPH radical scavenging demonstrated to be in accordance to the polyphenol measurements.

Moreover, when considering the chemical composition of the polyphenolic fraction, it was found that the main discriminant fingerprints between the plant extracts evaluated

as authentication tools were found: the composition in flavonoid and their degree of galloylation for all samples analyzed, but for Limousin Oak ellagitannins, which have had specific glycosylation patterns.

In **Figure 4.10** it is demonstrated as an attempt to exemplify the MALDI-TOF profile analysis of a grape seed sample, where galloylation patterns are highlighted due to their importance as marker for tannins extraction from grapes (SOUQUET, 1996, RICCI et al., 2017).

In this figure monomers from grape seed tannins are exemplified, where monomers of flavonoid compounds derived from fragmentation patters (231 Da) can be seen, the 271 Peak was referred to a catechin with a hydroxyl groupment missing, whereas the 291 Da peak, to catechin in its protoned form.

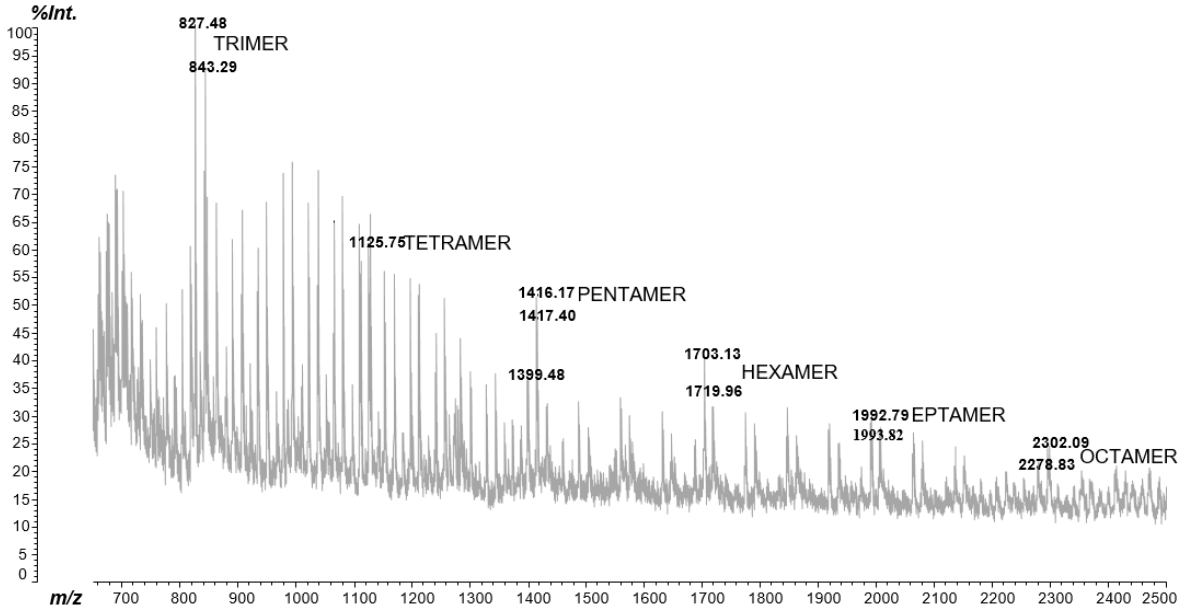

Figure 4.10 *The MALDI-TOF MS spectrum of sample SEP (linear positive mode, ion gate: 400 Da) recorded in the range 650–2500 Da: procyanidins series (repeat unit: catechins) for the grape seed extract (Source: RICCI et al., 2017).*

Therefore, it can be inferred that the aim of developing indicators for the certification of commercial tannins according to the declared botanical origin was achieved throughout the combination of MALDI-TOF MS and UV–vis spectrophotometric methods, whereas the chemical composition of the polyphenolic fraction the main discriminant fingerprints between the plant extracts evaluated as authentication tools were found: the composition in flavonoid and their degree of galloylation for grapes skin and seed tannins (RICCI et al., 2017).

4.7. CONCLUSIONS

The findings of the works performed in this thesis suggested that (i) FTIR spectroscopy is appropriate for a rapid screening tool to provide information on antioxidant activity of commercial tannins and (ii) using a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) to profile tannins succeeded in their characterization of their occurrence and influence regarding technological process.

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CHAPTER 5

CHAPTER 5

Wine Copigmentation study

Abstract

Anthocyanins are phenolic molecules glycosides of anthocyanidins, polyhydroxy derivates of flavylium ion. They can bind with other phenolic compounds, which leads to a phenomenon called copigmentation. These phenolic composites may be non-colored organic compounds from wine, as some pigments form molecular association and complexes, in which result in the enhancement of color of wine. It may shift the wavelength, once the absorbance values are taken. This chapter aims to give an overview regarding how this phenomenon occurs and the factors in which are involved in it as how the resulting wine color is changed.

5.1 INTRODUCTION

In grapes, anthocyanins are mainly located in their skins. They are natural pigments well known in due to its characteristics in plants and vegetables. It is known that some factors may influence the chemical structure of these pigments, such as pH, temperature, and the presence of acids, sugars, metallic ions and copigments in the environment containing them.

Anthocyanins are phenolic substances, glycosides from anthocyanidins polyhydroxy derivate from flavylium ion (**Figure 5.1**).

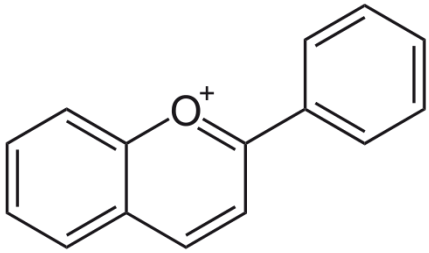
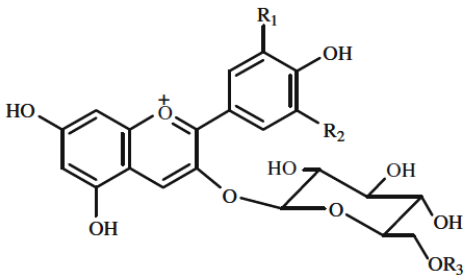


Figure 5.2 *Chemical structure of Flavylium ion*

The differences among anthocyanins are centered in the number of hydroxyl groups (-OH), in the methylation degree of these groups, in the nature and number of sugars linked in these molecules and in the position of them. They can also vary in the nature and number of aliphatic / aromatic acids linked to the sugar of anthocyanin molecule.
Moreover, anthocyanins are structurally characterized by a C_6–C_3–C_6 skeleton, therefore they can be associated with non-anthocyanin flavonoids. Glucose, arabinose, galactose and rhamnose are the most common sugars bonded to anthocyanins, and they also may combine to form di and trisaccharides, and then bind anthocyanins (TIMBERLAKE; BRIDLE, 1975).
Anthocyanins can be glycosylated by different sugars in positions 3, 5 and 7, but they are always glycosylated in C-3 position. In some cases, the sugar moiety is also acylated p-coumaric, caffeic and ferulic acid (MAZZA; BROUILLARD, 1987).
Hydroxyl and methoxy groupments, the presence of acids and sugars may influence in the color and stability of anthocyanins. Therefore, one anthocyanin may have different color, depending on the pH, concentration in the solution and the presence of copigments among other factors.
The anthocyanins recognized in grape skins and wines from *Vitis vinifera* are the 3-O-monoglucosides and the 3-O-acylated monoglucosides of five key anthocyanidins: delphinidin, cyanidin, petunidin, peonidin and malvidin, which are shown in **Figure 5.2.**



Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

R_3= $-CO-CH_3$ (-acetyl) $-CO-CH=CH-C_6H_4-OH$ (-*p*-coumaroyl) $-CO-CH=CH-C_6H_3(OH)_2$ (-caffeoyl)

Figure 5.3 *Chemical structures of anthocyanins present in wine (MONAGAS; BARTOLOMÉ, 2009).*

Within the wine pH (3.0 – 4.0), anthocyanins present a red coloration, which has its intensity decreased once the pH increases. In acid aqueous solution, there may exist four types of anthocyanin structures in equilibrium among themselves: quinoidal base (A), flavylium cation (AH^+), carbinol preudo-base B and chalcone (C) (**Figure 5.3**).

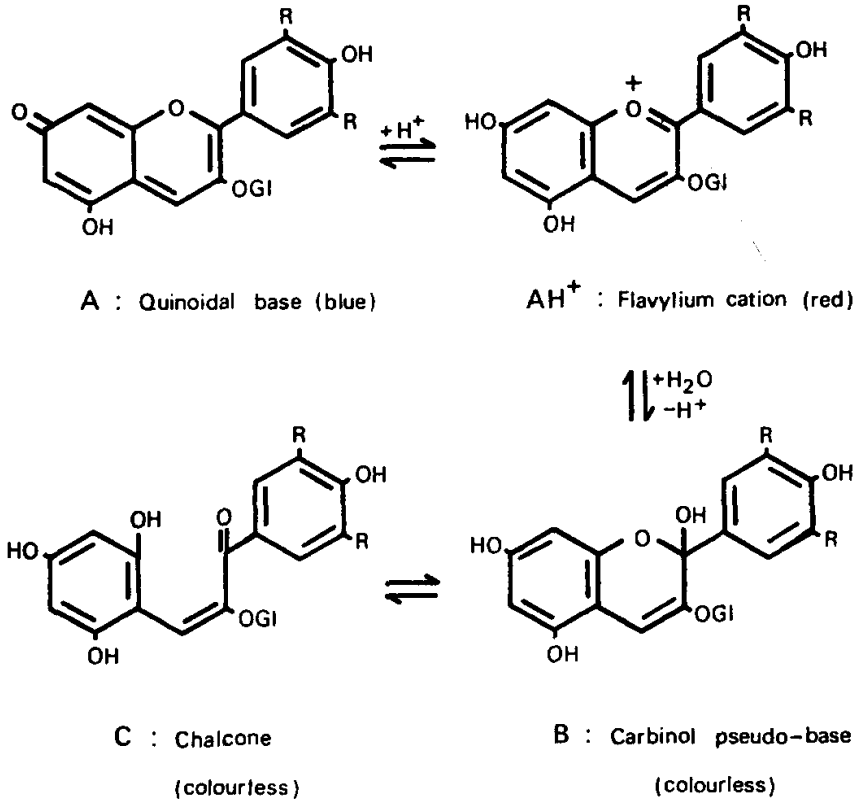


Figure 5.4 *Anthocyanin (Malvidin 3-glucoside) structural transformations with pH (TIMBERLAKE, 1980).*

In acid conditions, there is an equilibrium between the anthocyanins in AH^+ form and B, with the existence of a transitory specie: A, which is a structure obtained by le deprotonation of flavylium cation (HRAZDINA; IREDALE; MATTICK, 1977).

Therefore, depending on pH, anthocyanins can behave as electrophiles as a flavylium form trough their positions C-2 and C-4 in C ring, or as nucleophiles in the hemiketal form through their C-6 and C-8 positions (A-ring) (MONAGAS; BARTOLOMÉ, 2009).

Anthocyanin solutions are strongly bleached in the presence of sulfur dioxide. Within wine range pH, 3.2, 96% of the sulfur dioxide (SO_2) consists of HSO_3 (bisulfite), which are anions that are able to react with the flavylium cation, most probably on carbon 2 by analogy with the hydration reaction. The product formed is colorless:

$$A^+ + HSO_3^- \overset{K_s}{\rightleftharpoons} ASHO_3$$

.

Therefore, if water of bisulphite molecules bonds the flavylium cation in positions C-2 or C-4, respectively, by a nucleophile addition, anthocyanins are bleached. This color loss does not happen (BERKÉ et al., 1998; CHEMINAT; BROUILLARD, 1986), however, if the anthocyanins are stabilized by copigmentation.

Copigmentation can account for between 30 and 50% of the color in young red wines (BOULTON, 2001) and it consists by the hydrophobic interaction of the polarizable planar nuclei of the colored form of anthocyanins (flavylium cation and quinoidal base) with another molecule or copigment (intermolecular copigmentation) or with an aromatic residue linked to the pigment (intramolecular copigmentation). Over this interaction, the nucleophilic attack of water at C-2 is partly diminished (HE et al., 2012). Thus, the red wine color is positively influenced by the copigmentation phenomenon, which occurs by molecular associations between pigments and other organic molecules within wine solution. In general, the factors affecting copigmentation phenomena are pH, ethanol, temperature, molecular structure of the copigments and concentration ratio between anthocyanins and copigments (BOULTON, 2001; LEVENGOOD; BOULTON, 2004).

Additionally, it is proposed that this phenomenon may have great importance in the evolution of wine color towards its aging, by influencing the rate of polymerization reactions, as by avoiding the degradation of anthocyanin pigments (BOULTON, 2001). According to Boulton (2001), copigmentation phenomenon is a planar polarizable nuclei of colored forms of anthocyanins along with other organic components, in which result in the formation of vertical stacking complexes held by low energy bonds (Van

der Waals, hydrophobic interactions) (**Figure 5.4**) which are stabilized by the removal of sugar molecules on the outside, in which establish hydrogen bonds among themselves. Therefore, molecules of water do not have access into the interior of these complexes, resulting in the prevention of anthocyanins' hydration, which shifts the equilibrium toward colored anthocyanin forms.

These phenomenon usually result in increased absorbance intensity (hyperchromism) and a positive shift in wavelength (bathocromism) (ASEN; STEWART; NORRIS, 1972).

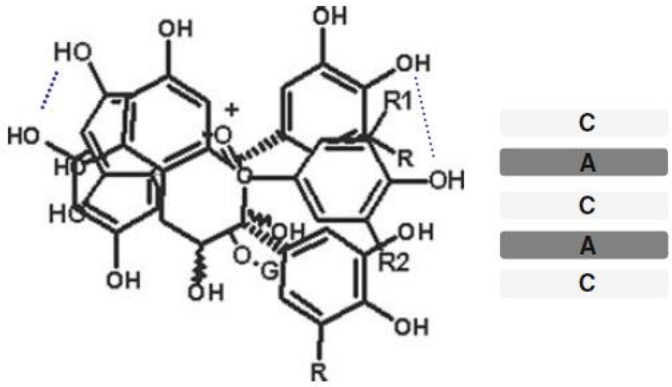


Figure 5.4. *Vertical π–π stacking between an anthocyanin (A) and a copigment (C) by (SANTOS-BUELGA; DE FREITAS, 2009)*

Studies have pointed to several phenolic compounds which can act as anthocyanin copigments (ASEN; STEWART; NORRIS, 1972; MAZZA; BROUILLARD, 1990). Additionally, it is stated that flavonoids and hydroxycinnamic acids appears to be the most capable compounds to perform as anthocyanins copigments. Gonzalez et al. (GONZÁLEZ-MANZANO et al., 2009) have shown that flavan-3-ols demonstrate small strength in copigmentation phenomenon, when compared to other compounds, due to their non-planar structure. Nevertheless, due to their high concentrations in wine, their copigmentation with anthocyanins contribute significantly in wine color.

According to Terrier et al. (TERRIER; PONCET-LEGRAND; CHEYNIER, 2009), copigmentation phenomenon happens due to hydrophobic vertical π- π stacking (**Figure 5.4**) between the anthocyanin and the copigment molecule, which forms a waterless complex. The flavylium cation and quinonoidal base are planar hydrophobic structures that can be involved in such complexes, while the hemiketal form0 cannot. The complex formation leads to the displacement of the anthocyanin hydration

equilibrium from the colorless hemiketal to the red flavylium form, which can be easily visualized by spectrophotometry.

Copigmentation can be either intramolecular, intermolecular or self-association process. Intermolecular copigmentation occurs when anthocyanins bind other molecules; self-association, instead, contains anthocyanins themselves; intramolecular copigmentation happens when the anthocyanin chromophore interacts with other residues of its own molecule (SANTOS-BUELGA; DE FREITAS, 2009).

Intramolecular copigmentation is limited to anthocyanins which are acylated by phenolic acids linked to the anthocyanidin through a appropriate inset, allowing, therefore, the molecule to fold in a certain matter in which the aromatic acyl group(s) will be capable to interact with the flavylium nucleus and keep it from hydration (DANGLES; SAITO; BROUILLARD, 1993).

According to Boulton (2001), anthocyanins present in *Vitis vinifera* possess only one sugar moiety and mostly one hydroxycinnamoyl, therefore the contents of acylated anthocyanins are diminished in most of grape varieties, having even less representatives in red wines. Under the mentioned conditions, it can be stated that intramolecular copigmentation may not constitute an important mechanism for the improvement of red wine color, though acylated anthocyanins may be more involved in intermolecular copigmentation than non-acylated ones (BOULTON, 2001).

In the case of self-association, there is a positive deviation from Beer's law which occurs on increasing the concentration of anthocyanins in the medium. However, Boulton (2001), based on studies of circular dichroism, settled that self-association was not much pertinent to the improvement of color in young red wines, nonetheless intermolecular copigmentation between anthocyanins and different phenolic compounds would be mainly responsible for the nonlinear color deviations observed.

Furthermore, bathochromic effect can also be produced due to the proton transfer equilibrium between the flavylium cation and the quinoidal base and/or preferred association between quinoidal forms and copigments. As the type of anthocyanins and copigments vary, as their concentrations, there may be variations in color hue and intensity. Therefore, color stabilization and variation can be obtained (BROUILLARD; CHASSAING; FOUGEROUSSE, 2003).

Moreover, the copigmentation phenomenon, besides affecting color definition of red wines, also influence its stability. Some chemical reactions occurring in wine, like

oxidation and polymerization, are, according to Boulton (2001), probable to be connected with the free concentrations of phenolic substrates. These substrates are partially involved in the copigmentation reaction, which present low rates, therefore evolution might be expected in wines with greater copigmentation degree.

Within this above described complex anthocyanin reactions in wine environment, an experimental procedure is described and discussed forward in the present chapter, aiming to helpfully provide a guide for the copigmentation phenomenon to be better understood in classes.

5.2 Experimental

One can make use of experimental procedures in order to better elucidate chemistry within teaching mechanisms. Experiments can facilitate interpreting the comprehensive nature of science.

In this context, the experimental approach and laboratory procedure are stated herein, with an aim to act as an agent to facilitate the learning process in chemistry, in particular, with regard to food science and Enology, independently for both university and technical courses.

Laboratory practice is an important tool in the chemistry. The preliminary step is observation as it is essential for the further steps of the process and is the key for hypothesis proposal, outcome prediction, prediction testing and upgradation of initial hypothesis.

As already mentioned in previous existing laboratory procedures, there are studies regarding anthocyanins in the introduction of chemical equilibrium (DI MEO et al., 2012), through acid−base chemistry (CURTRIGHT; RYNEARSON; MARKWELL, 1994; DEWPRASHAD; HADIR, 2009; FORSTER, 1978; LECH; DOUNIN, 2011; MARKWELL; CURTRIGHT; RYNEARSON, 1996; STODDARD; MCINDOE, 2013; SUZUKI, 1991), chromatography (CURTRIGHT; EMRY; MARKWELL, 1999; MARKWELL; CURTRIGHT; RYNEARSON, 1996), plant pigment identification (GARBER; ODENDAAL; CARLSON, 2013), investigative approaches for antioxidant activity (GALLOWAY; BRETZ; NOVAK, 2014), extraction and quantification from natural products (ROSSI III et al., 2012).

Therefore, it is of importance to understand and develop a suitable procedure for an approach which could combine the concepts of anthocyanins complexation and wine color, in the complex chemical matrix of wine which is constantly reacting.

With the scientific background of standing procedures, a method for students to well understand the chemistry involved on wine color, especially concerning the anthocyanins and polyphenols interaction was developed and is proposed herewith.
This experiment also provides students an opportunity for testing their abilities of being skeptical and upgrading their hypothesis simultaneously throughout experimentation with the use of spectrophotometry, which is a simple method that has been used in chemical analysis over a huge number of fields.

5.2.1 EXPERIMENTAL PROCEDURE

Once the subject is developed in theoretical classes, before the laboratory session, it is good for the students to have cleared in mind:
- the concept of red wine color and the importance of this parameter as one variable of sensory analysis that most characterizes and describes the wine. One of the main factor to be remembered is that young red wine shows a red-purple hue, due to monomeric fractions of anthocyanins and their copigmentation, which turns red-brown as wine ages, due to the presence of polymeric pigments (CASTANEDA-OVANDO et al., 2009);
- the chemical properties of anthocyanins, which belongs to the chemical group of flavonoids, with two aromatic rings linked through C-C bonds and a third heterocyclic ring containing oxygen;
- the mechanisms of intra- and intermolecular copigmentation as well as to have clear concept of the linearity of Lambert-Beer's law.
- hypothesize that, anthocyanins are reactive due to their chemical composition; thus, susceptible to degradation and complexation, the latter mechanism forming anthocyanin-derived pigments. Particularly, during wine ageing the anthocyanins can react with other constituents of wine, such as tannins, and their complexation is favored by the presence of acetaldehyde (VERSARI; DU TOIT; PARPINELLO, 2013).
After the theoretical review, students were instructed to proceed to the laboratory session. In this session, a method is proposed to be used to initiate students to formulate hypothesis on wine co-pigmentation.
The observation phase is suggested to be the first step of the procedure, where caffeic acid is added to red wine as this propels the complexation reactions. One to ten drops of 0.1% solution of caffeic acid are added into a red wine. During this stage, students should be observing it and taking their notes regarding the change in wine color, as to

theorize the possible chemical reactions that lead to color change based on their observations and make note of their hypotheses.

Chemical students, as viticulture and enology technicians and students must be able to seek experimental answers, as carry out chemical experiments, analyze the data, and transfer the significant findings.

Following the hypothesis part, students can be asked to perform the proposed spectrophotometric assay.

Firstly, they need to prepare a model wine by dissolving 2.5 g of potassium bitartrate ($KC_4H_5O_6$) and 120 mL of ethanol (analytical reagent quality) and making up with distilled water for 1L of reagent.

Furtherly, students can be asked to sample their wines twice: i) undiluted and ii) diluted 20-fold with model wine. The experimental procedure is to read these wines in UV-vis spectrophotometer using 520nm wavelength. The undiluted wine is asked to be filled in a 0.5 cm path cuvette whereas the 20-fold diluted wine, in 1.0 cm path cuvette. Different cuvettes are used to adjust for the dilution and have accurate readings of anthocyanins.

Finally, the reading of two prepared samples at the specified wavelength can be performed. Data needs to be corrected to unit pathway (1 cm) and results can be thereby analyzed by comparison of data obtained with the two sampling methods using a XY plot of the two absorbance readings, both at 520 nm.

5.3 DISCUSSION

It can be seen in **Figure 5.5** that the relationship between the Abs reading (520 nm) of the (i) undiluted wine in a 0.5 cm path cuvette, and (ii) the 20-fold diluted wine in a 1 cm path cuvette, with values corrected to unit pathway (1 cm). Although the Abs at 520 nm are linearly correlated ($R^2 = 0.9855$), the XY plot with regression line enabled visual evaluation of results and disagreement of fitted regression line and identity line ($R^2 = 1$) revealing small constant (i.e. intercept) but large proportional systematic error (i.e. regression line's slope) that determines the increased response due to copigmentation.

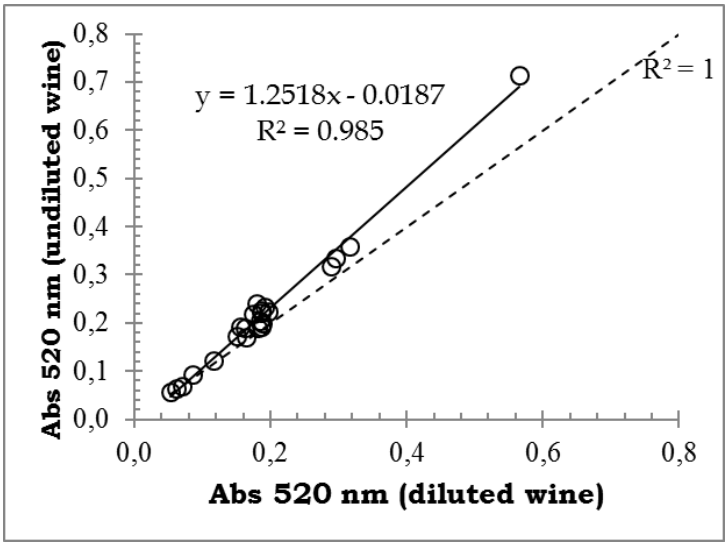


Figure 5.5. *Relationship between the Abs reading (520 nm) of the undiluted wine in a 0.5 cm path cuvette (Y-axis), and the 20-fold diluted wine in a 1 cm path cuvette (X-axis), with values corrected to unit pathway (1 cm). Dotted line (---): theoretical regression with slope =1 and intercept = 0. Full line (—): experimental finding.*

As described in the literature (MAZZA; BROUILLARD, 1987; VERSARI; DU TOIT; PARPINELLO, 2013), intermolecular copigmentation among anthocyanins and with other compounds can produce an increase in the color intensity (hyperchromic effect) and a dislocation in the maximum absorbance wavelength (bathochromic shift). It can be seen properly when compared to a compound which does not undergo copigmentation, such as a calibration curve of a standard using regularly in the lab (such as caffeic acid, for example).

Additionally, in accordance to Lambert-Beer's law, by diluting the wine 20-fold the absorbance should decrease linearly, however the presence of proportional error was proven (**Figure 5.5**) and must be considered in the application of the Lambert-Beer's law. For example, according to the Lambert-Beer law a red wine with 10 A.U @520 nm as it is should give a final absorbance value of 0.5 A.U @520 nm with 20-fold sample dilution (20 x 0.5 = 10); however, with ca. 40% copigmentation the absorbance @520 nm of wine with 20-fold dilution drops to 0.1967 A.U. (**Figure 5.6**). The decrease of red wine absorbance at 520 nm by dilution is due to the breakdown of copigmented anthocyanins that are in a dissociable equilibrium with free copigments as follows:

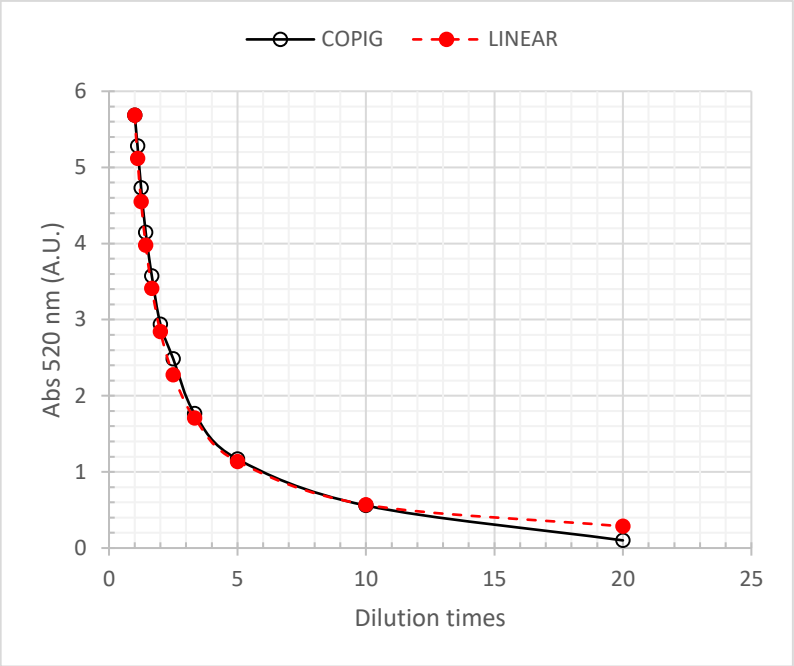


Figure 5.6. *Relationship between absorbance and dilution factor of red wine. Legend: (-●-) lack of copigmentation (i.e. linear); (-○-) copigmentation effect.*

In this view, it is important to students to evaluate to what extent copigmentation happens, if any, and associate it with the wine composition. Therefore, after students set the data they had from the analysis and plots as described in Figure 1 and 2, were asked to discuss results within the classroom.

5.4 CONCLUSIONS

Anthocyanin copigmentation is an important phenomenon for the wine quality, whereas it may be difficult to be learnt. In this thesis' section, a suggestion of practical method was made, as an attempt to simplify the process of learning in this particular topic. This simple analytical experiment proposed will therefore make it easier for students to understand copigmentation in red wine by visual observation followed by its quantification by spectrophotometry.

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APPENDIX

APPENDIX A

DOC and DOCG wines made with Sangiovese grape varietal.

Alcamo	Alghero Sangiovese	Arborea Sangiovese rosato
Arborea Sangiovese rosso	Assisi novello	Assisi rosato
Assisi rosso	Barco Reale di Carmignano	Bolgheri rosato
Bolgheri rosso	Bolgheri Vin Santo Occhio di Pernice	Bolgheri Vin Santo Occhio di Pernice riserva
Botticino (vino)	Botticino riserva	Brunello di Montalcino
Capalbio rosato	Capalbio rosso	Capalbio rosso riserva
Capalbio Sangiovese	Capriano del Colle novello rosso	Capriano del Colle rosso
Capriano del Colle rosso riserva	Carmignano (vino)	Carmignano riserva
Carmignano rosso	Castel San Lorenzo rosato	Castel San Lorenzo rosso
Castelli Romani rosato	Castelli Romani rosso	Cerveteri rosato
Cerveteri rosato frizzante	Cerveteri rosso amabile	Cerveteri rosso novello
Cerveteri rosso secco	Chianti (vino)	Chianti Classico (vino)
Chianti Colli Aretini	Chianti Colli Fiorentini	Chianti Colli Senesi
Chianti Colline Pisane	Chianti Montalbano	Chianti Montespertoli
Chianti Rufina	Chianti Superiore	Cilento rosato
Circeo Sangiovese	Circeo Sangiovese rosato	Colli Altotiberini rosato
Colli Altotiberini rosso	Colli d'Imola Sangiovese	Colli d'Imola Sangiovese riserva
Colli del Trasimeno rosato	Colli del Trasimeno rosso	Colli del Trasimeno rosso frizzante
Colli del Trasimeno rosso novello	Colli del Trasimeno rosso riserva	Colli del Trasimeno rosso scelto

Colli dell'Etruria Centrale	Colli della Sabina rosato	Colli della Sabina rosato frizzante
Colli della Sabina rosso	Colli della Sabina rosso frizzante	Colli della Sabina rosso novello
Colli della Sabina rosso spumante	Colli di Faenza rosso	Colli di Faenza rosso riserva
Colli di Faenza Sangiovese	Colli di Faenza Sangiovese riserva	Colli di Luni rosso
Colli di Luni rosso riserva	Colli di Rimini rosso	Colli di Scandiano e di Canossa Cabernet Sauvignon
Colli di Scandiano e di Canossa Cabernet Sauvignon riserva	Colli Etruschi Viterbesi novello	Colli Etruschi Viterbesi Procanico
Colli Etruschi Viterbesi rosato	Colli Etruschi Viterbesi rosso	Colli Etruschi Viterbesi Sangiovese rosato
Colli Martani Sangiovese	Colli Martani Sangiovese riserva	Colli Perugini rosato
Colli Perugini rosso	Colli Pesaresi Focara rosso	Colli Pesaresi rosso
Colline di Levanto novello	Colline di Levanto rosso	Colline Lucchesi rosso
Colline Lucchesi rosso riserva	Colline Lucchesi Sangiovese	Colline Lucchesi Sangiovese riserva
Conero (vino)	Contea di Sclafani Sangiovese	Contea di Sclafani Sangiovese riserva
Cortona (vino)	Delia Nivolelli novello rosso	Delia Nivolelli rosso
Delia Nivolelli Sangiovese	Elba rosato	Elba rosso
Elba rosso riserva	Elba Vin Santo Occhio di Pernice	Esino novello
Esino rosso	Faro (vino)	Garda Bresciano Chiaretto

Garda Bresciano rosso	Garda Bresciano rosso novello	Garda Bresciano spumante rosé
Garda Bresciano superiore	Garda classico Chiaretto	Garda classico Rosso
Garda classico Rosso superiore	Genazzano rosso	Guardiolo rosato
Guardiolo rosso	Guardiolo rosso riserva	Lago di Corbara (vino)
Leverano novello	Leverano rosato	Leverano rosso
Leverano rosso riserva	Menfi Bonera	Menfi Bonera riserva
Menfi rosso	Menfi Sangiovese	Merlot di Aprilia
Molise Sangiovese	Monreale rosato	Monreale Sangiovese
Montecarlo rosso	Montecarlo rosso riserva	Montecarlo Vin Santo
Montecucco rosso	Montecucco rosso riserva	Montecucco Sangiovese
Montecucco Sangiovese riserva	Montefalco rosso	Montefalco rosso riserva
Montepulciano d'Abruzzo Colline Teramane	Montepulciano d'Abruzzo Colline Teramane DOCG	Montepulciano d'Abruzzo Colline Teramane riserva
Monteregio di Massa Marittima novello	Monteregio di Massa Marittima rosato	Monteregio di Massa Marittima rosso
Monteregio di Massa Marittima rosso riserva	Monteregio di Massa Marittima Vin Santo Occhio di Pernice	Montescudaio rosso
Morellino di Scansano	Morellino di Scansano riserva	Ortanova rosato
Ortanova rosso	Parrina rosato	Parrina rosso
Parrina rosso riserva	Pentro di Isernia rosato	Pentro di Isernia rosso
Pomino rosso	Pomino rosso riserva	Pomino Vin Santo rosso
Rosso Conero	Rosso dei Colli Amerini	Rosso di Montalcino

Rosso di Montalcino Vigna	Rosso di Montepulciano	Rosso di Torgiano
Rosso Orvietano	Rosso Orvietano Sangiovese	Rosso Piceno
Rosso Piceno novello	Rosso Piceno superiore	Rosso superiore dei Colli Amerini
Sambuca di Sicilia Sangiovese	San Gimignano novello	San Gimignano rosato
San Gimignano rosso	San Gimignano rosso riserva	San Gimignano Vin Santo Occhio di Pernice
San Severo rosso o rosato	Sangiovese di Aprilia	Sangiovese di Romagna
Sangiovese di Romagna novello	Sangiovese di Romagna riserva	Sangiovese di Romagna superiore
Sannio rosato	Sannio rosato frizzante	Sannio rosso
Sannio rosso frizzante	Sannio rosso novello	Sant'Antimo Vin Santo Occhio di Pernice
Sant'Antimo Vin Santo riserva	Santa Margherita di Belice rosso	Santa Margherita di Belice Sangiovese
Sciacca rosato	Sciacca rosso	Sciacca rosso riserva
Sciacca Sangiovese	Solopaca rosato	Solopaca rosso
Solopaca rosso superiore	Sovana rosato	Sovana rosso riserva
Sovana rosso riserva Sangiovese	Sovana rosso superiore	Sovana rosso superiore Sangiovese
Taburno rosso	Tarquinia rosato	Tarquinia rosso amabile
Tarquinia rosso novello	Tarquinia rosso secco	Torgiano rosso riserva
Trebbiano di Aprilia	Val di Cornia Campiglia Marittima riserva	Val di Cornia Campiglia Marittima rosato

Val di Cornia Campiglia Marittima rosso	Val di Cornia Piombino riserva	Val di Cornia Piombino rosato
Val di Cornia Piombino rosso	Val di Cornia rosato	Val di Cornia rosso
Val di Cornia rosso riserva	Val di Cornia San Vincenzo riserva	Val di Cornia San Vincenzo rosato
Val di Cornia San Vincenzo rosso	Val di Cornia Suvereto riserva	Val di Cornia Suvereto rosato
Val di Cornia Suvereto rosso	Valdichiana rosato	Valdichiana rosso
Valdichiana Sangiovese	Velletri rosso	Velletri rosso riserva
Vignanello rosato	Vignanello rosso	Vignanello rosso novello
Vignanello rosso riserva	Vin Santo del Chianti Classico Occhio di Pernice	Vin Santo di Carmignano Occhio di Pernice
Vin Santo di Carmignano Occhio di Pernice riserva	Vin Santo Montepulciano Occhio di Pernice	Vino Nobile di Montepulciano
Vino Nobile di Montepulciano riserva		

APPENDIX B

	More Adstringent			Less Adstringent		Older			Less old		Prefered			Less Prefered	
Assessor 1	1	3	2	5	4	1	2	4	5	3	3	4	5	2	1
Assessor 2	5	2	3	4	1	5	4	3	1	2	2	1	3	4	5
Assessor 3	1	2	3	4	5	3	5	4	2	1	4	5	3	2	1
Assessor 4	4	1	3	5	2	3	4	2	1	5	1	3	5	2	4
Assessor 5	1	3	4	2	5	3	1	5	2	4	1	5	2	4	3
Assessor 6	3	1	5	2	4	2	4	1	5	3	3	1	2	4	5
Assessor 7	4	5	2	1	5	1	2	4	3	5	4	2	5	1	3
Assessor 8	1	4	2	3	5	3	1	2	4	5	1	4	3	2	5
Assessor 9	1	3	2	4	5	2	4	1	3	5	3	2	1	4	5
Assessor 10	5	2	3	4	1	3	5	4	2	1	5	2	4	1	3
Assessor 11	2	4	5	1	3	4	2	1	5	3	3	2	4	1	5
Assessor 12	5	2	4	1	3	4	3	5	2	1	4	2	5	1	3
Assessor 13	1	2	3	4	5	3	5	4	2	1	2	4	1	3	5
Assessor 14	5	4	2	1	3	3	4	1	5	2	4	1	3	2	5
Assessor 15	2	3	1	5	4	1	3	2	4	5	1	3	5	2	4
Assessor 16	2	4	1	3	5	1	2	4	5	3	4	2	5	1	3
Assessor 17	5	3	2	1	4	3	5	4	2	1	3	5	4	2	1
Assessor 18	1	2	4	5	3	3	4	5	2	1	3	5	4	2	1
Assessor 19	1	3	2	4	5	1	3	2	5	4	3	1	2	4	5
Assessor 20	2	1	3	4	5	4	5	3	2	1	2	4	3	5	1
Assessor 21	4	2	3	1	5	5	3	4	2	1	5	4	2	1	3
Assessor 22	4	3	1	5	2	3	5	4	2	1	1	5	4	3	2
Assessor 23	2	3	4	1	5	3	4	5	2	1	5	1	4	2	3
Assessor 24	5	2	3	4	1	3	5	4	2	1	5	4	3	1	2
Assessor 25	1	2	5	4	3	3	5	4	1	2	1	2	5	3	4
Assessor 26	2	5	3	1	4	2	3	4	5	1	3	5	1	2	4
Assessor 27	2	3	1	5	4	3	1	2	4	5	5	2	4	1	3
Assessor 28	5	3	2	4	1	3	5	4	2	1	2	1	3	4	5
Assessor 29	5	3	2	1	4	3	5	2	1	4	5	3	4	1	2
Assessor 30	2	5	1	4	3	1	4	2	5	3	4	3	2	5	1
Assessor 31	2	4	5	1	3	5	4	2	1	3	5	1	4	2	3
Assessor 32	2	5	3	4	1	5	1	4	3	2	4	3	1	2	5
Assessor 33	2	3	1	5	4	5	4	1	2	3	2	3	4	1	5
Assessor 34	1	3	2	5	4	4	5	2	3	1	5	1	3	4	2
Assessor 35	2	3	1	4	5	3	1	4	2	5	5	4	1	3	2
Assessor 36	1	5	4	2	3	2	1	4	5	3	1	2	3	4	5

Wine: BDN SPO 100 2013 bottled on april of 2014

1: pH3,2
2: pH 3,4
3: pH 3,6
4: pH 3,8
5: pH 4.0

APPENDIX C

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Original Research Article

Analytical profiling of food-grade extracts from grape (*Vitis vinifera* sp.) seeds and skins, green tea (*Camellia sinensis*) leaves and Limousin oak (*Quercus robur*) heartwood using MALDI-TOF-MS, ICP-MS and spectrophotometric methods

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ABSTRACT

Tannins are food additives widely used due to their antioxidant and antimicrobial activities, and flavouring properties. Nevertheless, the information provided by the manufacturers are often generic, and data on the presence of specific phenolic compounds and potentially toxic elements are needed. In this work a selection of food-grade plant extracts – also called 'commercial tannins' – from different botanical sources: *Vitis vinifera* sp., *Camellia Sinensis*, *Quercus robur*, were profiled using UV–vis spectrophotometry, matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS), and inductively coupled plasma-mass spectrometry (ICP-MS). The combined analytical approach was suitable for quality control of the polyphenolic fraction to highlight authenticity markers (e.g. galloylated flavonoids, glycosides, ellagitannins), and to ascertain the content of toxic elements (Cu, Sr, As, Co, Cr, Fe, Zn, Li, Ba and Pb) respect to their legal and/or recommended limits.

1. Introduction

There is an increasing interest in the exploitation of phyto-chemicals from agri-food by-products and wastes. In this perspective, tannins – secondary phenolic metabolites produced by plants – have gained increasing interest for their exploitation in the food industry, due to their application in human and animal nutrition as natural antioxidants (Arogba, 2000; Chung et al., 1998) and feed flavourings (EFSA FEEDAP Panel, 2014).

Commercial tannins are natural polyphenolic compounds occurring in plant woods, fruits, seeds, and extracted from different plant tissues. Few selected species are employed in the food technologies, due to their high content in bioactive compounds which reflects their technological performances. Procyanidins are condensed molecular structures composed of flavan-3-ol monomeric units; the main natural sources are constituted by grape seed, skin and leaves, mimosa bark, Quebracho wood, and tea leaves. Hydrolysable tannins consists of glycosylated units of gallic and ellagic acid monomers and polymers; the oak, chestnut and gallnut woods provide primary sources of hydrolysable tannins for the food industry (Versari et al., 2013).

Tannins consist of a complex matrix including polymerized fraction and additional components such as phenolic monomers, oligomeric fractions, phenolic aldehydes, stilbenes, sugars and glycosylated compounds, among others (Versari et al., 2013). The properties of tannins are strongly dependent on the composition of specific classes of bioactive compounds occurring as a consequence of the extraction process, and on the lack of contaminants (Vivas et al., 2004a). In particular, the phenolic compounds can

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remove free radicals and chelate transition metals and proteins, improving quality and stability of food and beverages (Haslam, 1998). However, tannins could induce astringent, antinutritional and toxic effects (Avallone et al., 1997; Makkar, 2003). Therefore their analytical characterization is a suitable tool for the valorisation of plant extracts (e.g. tea leaves) and for their exploitation as sources of food supplements for human nutrition and animal feed. In particular, the bioaccessibility and fermentability of tannins by colonic microflora is highly affected by their degree of polymerisation (Serrano et al., 2009).

The use of tannins in the food industry is regulated by Communitarian and International legislations (FDA- Code of Federal Regulations Title 21; Directive 2012/12/EU of the European Parliament and of the Council, amending Council Directive 2001/112/EC; Beer Judge Certification Program (BJCP) Style Guidelines 2015; Resolution OIV-OENO 554-2015, among others) but limited recommendations are provided for the authentication and typification of raw materials. The composition of the monomeric polyphenolic fraction and building blocks of oligomers are specific for each botanical class, and provide information on the effective origin of the extracts. The main markers of quality and authenticity of commercial tannins are related to the content of condensed and hydrolysate structures, also including (i) the degree of galloylation of polygalloylglucose chains and flavanol monomers, (ii) the glycosylation patterns derived from degradation of plant tissues, and (iii) their molecular arrangement following extraction (Amakura et al., 2009; Nonier et al., 2005)

One of the most beneficial groups of polyphenolic compounds obtained by natural extracts is constituted by proanthocyanidins, with procyanidins as the most prominent subclass; mixtures of procyanidolic oligomers naturally occur in fruits, with red fruits and grapefruit as the main sources, and tea leaves. The related extracts have technological and nutritional impact, being exploited both in the beverages industry and as food supplement. The grape extracts are commonly composed of flavonoid monomers and procyanidin oligomers and polymers extracted from *Vitis vinifera* sp. grape seeds and skin; among them, a large amount of flavan-3-ol monomers esterified with gallic acid units has been observed in the grape seed procyanidins (Krueger et al., 2000), resulting in an enhanced bioactivity and a stronger sensory impact when compared to the skin extract.

Due to the steric hindrance produced by esterification of building monomers, the degree of polymerisation of procyanidins could reach a maximum of 16 units; the occurrence of higher polymers may derive from oxidative condensation of oligomers following extraction (Prieur et al., 1994). The grape skin extract is characterised by a higher degree of polymerisation, related to the low degree of esterification of flavan-3-ol monomeric units. Both skin and seed extracts from grape are effective antioxidants and can be added to foods and beverages to retard deterioration.

Green tea (*Camellia sinensis*) leaves are recognized as a major source of galloflavonoid and flavonoid gallates, with a prevalence of (−)-epigallocatechin gallates (Graham, 1992; Yoshizawa et al., 1987); the peculiar composition makes the extract a suitable candidate as a protection from oxidative stress (Frazier et al., 2010; Perumalla and Hettiarachchy, 2011). Nevertheless, the ability of galloylated structure to interact with proteins is also responsible for the astringent perception induced by gallic acid-based compounds (Harbertson et al., 2012; Obreque-Slier et al., 2010); the use of both grape seed extract and green tea tannins would require a detailed investigation on the chemical composition of the phenolic fraction and on the sensory impact for their addition.

The water-soluble hydrolysable tannins contained in European oak (*Quercus* species) heartwood have been historically exploited for cooperage, and they are recognized as providing beneficial stabilising and flavouring effects. Oak wood is mainly used for the conservation of fine beverages: aging wines, spirits, and balsamic vinegars are traditionally stored in barrels, where the continuous contact with wood naturally extracts and releases ellagitannins in solution; ellagitannins contained in the oak extracts increase the antioxidant activity, due to the high content in gallic and ellagic acids-based compounds (Landete, 2011). Several scientific surveys have explored the differences between European and American *Quercus* species used for cooperage, to determine the most suitable formulation for food industry needs. Among them, the work of Chatonnet and Dubourdieu (1998) has highlighted the supremacy of oak obtained from Limousin forests in relation to its content in water-soluble precursors (Chatonnet and Dubourdieu, 1998). Limousin oak forests are located in the southwest area of France, with a prevalence of *Q. robur* trees; this species has a high content in ellagitannins, vanillin and phenolic aldehydes when compared to *Q. petraea* and *Q. alba* oak species (Chatonnet and Dubourdieu, 1998).

Since there is no regulation constraining manufacturers to provide a detailed composition of the extracts or the methods of extraction used, information on adulteration and possible contamination is not available to the consumer, and the purity and the healthiness of commercial tannins can be questionable. Monitoring the composition of food additives is a key step to guarantee consumer safety, through the identification of markers (both qualitative and quantitative) which inform on their effective content in bioactive compounds, their authenticity and non-toxicity (Li and Vederas, 2009; Pennington, 2002). Moreover, a correct balance in macroelements (Ca, K, Mg, Zn, among others) could enhance the nutritional value of this food additive (Sulaiman et al., 2011; Vin et al., 2014).

Due to the large variety of commercial botanical extracts used in the food industry, there is a need to explore both targeted and non-targeted approaches to assess the quality/authenticity of commercial tannin formulations. In this work, the compositional parameters of four tannins employed for human consumption and selected to represent commercially available extracts from: grape seed, grape skin, green tea leaves, and Limousin oak heartwood, were investigated, and quality and authenticity markers were identified. MALDI-TOF MS, ICP-MS and spectrophotometric assays were used for qualitative and quantitative analysis; in particular, the elemental and molecular composition, along with the polyphenolic content and antiradical activity were assessed for these commercial food additives, and briefly discussed.

2. Experimental

2.1. Chemicals

Solvents, L-tartaric acid (≈100%) pure ethanol (>99%) to prepare tannin solutions, and HNO_3 and H_2O_2 reagent grade used for ICP-MS analysis were supplied by Merck (Darmstadt, Germany). Bovine serum albumin (BSA), anhydrous $FeCl_3$ and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) for spectrophotometric determinations were purchased by Sigma Aldrich (St. Louis, MO). Phosphorus red reference standard (99,999%) used for MALDI-TOF analyses was purchased from Acros Organics (Fair Lawn, NJ).

2.2. Samples preparation

Four food-grade commercial tannins from different botanical sources: grape seed (SEP) and skin (SKP) proanthocyanidins, green tea procyanidins (GTP), and Limousin oak ellagitannin (LOE), were purchased from the Enologica Vason company (Enologica Vason S. p.A., Verona, Italy) as lyophilized powders and stored at room temperature; any purification treatment was carried out on commercial samples before analyses. According to Harbertson

et al. (2002) 1 g/L of tannin powders were dissolved in hydro-alcoholic solution (12% (*v/v*) ethanol in distilled water, with addition of L-0.033 M tartaric acid and 1 M NaOH to reach pH 3.6) to obtain a 'stock solutions' of each sample; then, stock solutions were properly diluted in distilled water for spectrophotometric assays.

For MALDI-TOF experiments, powder samples were dissolved in acetone/water 50/50% (*v/v*) at a concentration of 5 mg/mL, and then the solutions were mixed (1:1, *v/v*) with a standard solution of 2,5-dihydroxybenzoic acid at 10 mg/mL dissolved in the same solvent. Then, 1.5 μL of the mixed solution were spotted on a 384-well MALDI-TOF plate, followed by evaporation of the solvent at ambient temperature before analysis.

For ICP-MS analysis the stock solutions (5 mL) were added with 3 mL of HNO_3 and 2 mL of H_2O_2 reagent grade, then heated to 130 °C (digester DigiBlock ED365; LabTech, Hopkinton, MA) for 3 h; subsequently the mixture was diluted with 25 mL of ultrapure H_2O before analysis.

2.3. Determination of total polyphenols, tannin fraction and in vitro antioxidant capacity

Total (iron reactive) polyphenols and tannins were quantified using the method of Harbertson et al. (Harbertson et al., 2002) which is based (i) on the ability of protein (i.e. bovine serum albumin, BSA) to precipitate tannins, and (ii) on the reactivity of ferric chloride with phenolic compounds that possess *ortho*-dihydroxyl groups, as previously described (Versari et al., 2007). This method is particularly suitable for commercial tannins analysis as it estimates the degree of purity and the amount of iron-reactive polyphenolics, which play a critical role in redox systems.

The *in vitro* antioxidant capacity of tannin samples was determined using the DPPH$^{\bullet}$ radical scavenging method (Brand-Williams et al., 1995) that evaluate of the decay of radical absorbance at 517 nm; results were expressed as Trolox equivalents (mmol TE/L).

All spectrophotometric determinations were performed using a Shimadzu UV Mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. MALDI–TOF mass spectrometry

MALDI-TOF spectra were recorded using a Kratos compact MALDI Axima Performance TOF 2 instrument (Shimadzu Biotech, Manchester, UK), equipped with a nitrogen laser (337 nm), an ion gate for the selection of precursor ions, and a collision cell, according to Lagel et al. (2014). The windows for separation of precursor ions were approximately 4 Da. Argon has been used as the collision gas. All data were obtained in positive ion linear mode applying the accumulation of 441 scans per spectrum. Linear negative mode was used for the investigation of hydrolysable and glycosylated molecular patterns which characterise the Limousin oak extract. The calibration of the linear modes was done using phosphorus red pigment as a reference over a mass range up to 2500 Da. NaCl was added in the sampling wells as the salt to enhance ion formation previous deposition of samples[S1] . The MALDI-TOF target was then analysed to give the resulting spectra, using a raster analysis over the target; Maldi-MS software was used for data treatment (Shimadzu Biotech, Manchester, UK).

2.5. ICP-MS analyses

Metals were analyzed using an Agilent ICP-MS equipped with a 7700 Q Apex + Spiro TMD (Agilent Technology, Santa Clara, CA); the standard nebulizer for sample introduction was replaced with a desolvation system. The APEX Q atomizes the sample into a cyclonic chamber heated to 140 °C and subsequently cooled, thus inducing the removal of most of the aqueous component of the sample. After condensation; this process enables concentration of the sample by about 10 times. The Spiro TMD (Teflon membrane desolvator) is composed of a spiral interfaced with a Teflon membrane heated at 100 °C that further removes the water vapor from the sample aerosol stream, enabling only the dry component to reach the plasma. The acid digestion step and the combined use of APEX Q and Spiro TMD technologies enable the simultaneous determination of macro, micro and trace elements, and it is routinely used as an internal laboratory protocol for elemental profiling; nevertheless, in this work only the first two categories showed significant concentrations and are accordingly discussed in the Results and Discussion section. The instrument is also equipped with a collision chamber sparged with He for the removal of interferences, such as oxides (below 0.1%); analyses were performed with and without He stream, to avoid overestimation due to occasional adducts formed.

Internal standards Ge and Tl were added through a tee into the Apex, before the introduction of the sample and used for quantitation.

2.6. Statistical analysis

Microsoft Excel was used for data entry, and statistical analysis was performed with Unscrambler X.1 (Camo ASA, Oslo, Norway). All analyses were performed in triplicate and the results expressed as mean ± standard deviation (SD). The statistically significant level was considered at $\alpha = 0.05$.

3. Results and discussion

3.1. Total (iron reactive) polyphenols, tannin fraction and in vitro antioxidant capacity

The total polyphenol content, tannin fraction and the *in vitro* antioxidant capacity of the four commercial extracts (Table 1) was

Table 1
Phytochemical composition and antioxidant capacity of selected commercial tannins. Results from replicates ($n = 3$) are reported as mean values ± standard deviation. Legend: grape seed (SEP) and skin (SKP) proanthocyanidins, green tea procyanidins (GTP), Limousin oak ellagitannin (LOE).

	Tannin samples			
	SEP	SKP	GTP	LOE
Total polyphenols (mM CE[a])	1.62 ± 0.12	2.83 ± 0.01	2.11 ± 0.01	2.34 ± 0.03
Tannins (mM CE)	1.49 ± 0.02	1.23 ± 0.01	1.04 ± 0.02	1.42 ± 0.0
*Tannins/total polyphenols (%)	93.8	42.9	47.6	60.9
DPPH (mM TE[b])	0.26 ± 0.0	0.24 ± 0.01	0.42 ± 0.01	0.39 ± 0.01
#Non-phenolic compounds* (%)	46.7	6.7	30.0	23.3

[a]Expressed as (+)-catechin equivalent. [b]Expressed as Trolox equivalent. *Calculated as% weight: tannins/total polyphenols. #Calculated as% weight: 1 g/L dry powder – estimated total polyphenols.

carried out to set-up a rapid screening approach to provide both qualitative and quantitative information useful for quality control of commercial products. Samples were ranked in terms of total polyphenols content as follows: SKP > LOE > GTP > SEP.

The SKP extract peaked in the total polyphenol content (2.83 mM CE), of which 42.9% is the polymeric tannin fraction (1.23 mM CE). The seed procyanidin (SEP) was lowest in total polyphenols (1.62 mM CE), most of which (93%) comprised the polymerised fraction (flavonoid oligomers and polymers). The SKP/SEP ratio of proanthocyanidins estimated in this work (value = 1.3) is almost doubled when compared to the value of 0.55 obtained by Vivas et al. (2004a, b) when studying similar commercial formulations. This result emphasised the high variability of bioactive compounds in commercial extracts, which is affected by several factors, including the extraction process (e.g., time, temperature, solvent, etc.) and the raw material (e.g., grape variety and maturity). Although the tannin fraction (% of the total polyphenol content) defines the effectiveness of the extraction process and influences the technological potential of the extracts for industrial applications (Kallithraka et al., 1995), the monomeric fraction contributes to the antioxidant properties, and constitutes an important parameter to be monitored. The green tea extract showed a high content in polyphenolic compounds (2.11 mM CE), part of which was tannins (47.6%), and the highest antioxidant capacity as radical scavenging (0.42 mM TE). The green tea leaves are rich in flavonoid-based monomers with a high degree of galloylation (Perumalla and Hettiarachchy, 2011), and the effectiveness of the galloylation patterns in the radical scavenging activity was also observed in the SEP sample (0.26 mM TE) compared to SKP (0.24 mM TE). The enhanced antioxidant capacity of Limousin oak extract (0.39 mM TE) compared to the grape extracts can be explained by the high content in total polyphenols (2.34 mM CE), with a great content in hydrolysable tannins (60.9%) that provides an effective protection against oxidation.

In general, samples showed a variable content in non-phenolic compounds (SEP > GTP > LOE > SKP), which was attributable to the presence of degradation by-products following extraction or addition of stabilising additives during processing, i.e. arabic gum powder, proteinaceous material, cellulose (Romani et al., 2006).

3.2. Targeted analysis by MALDI-TOF mass spectrometry

3.2.1. Seed proanthocyanidin (SEP)

The MALDI-TOF profile of SEP sample highlighted the galloylation patterns that are a valuable marker of extracts from grape seeds and skin (Souquet et al., 1996), the latter with high polymerization index and less astringent sensation (Vidal et al., 2003). The SEP showed monomers from fragmentation patterns of flavonoid compounds (231 Da), whereas the 271 Da peak was attributable to fisetinidin (or catechin with loss in —OH), while the catechin was present in protonated form (291 Da). The occurrence of (−)-gallocatechin/(−)-epigallocatechin compounds was represented by the peak at 303 Da, together with typical fragments 258–220 Da [MS^2 (m/z)]. The 152 Da fragment was related to the presence of galloyl units released during fragmentation. The major flavonoid monomeric units were found to be (+)-catechin/(−)-epicatechin (mass increment: 289 Da). However the building block of the first series of polymeric structures was represented by a fisitinedin-fisetinidin dimer (540 Da), increasing its degree of polymerisation with catechins as repeating unit (Fig. 1): 827 Da (trimer); 1125 Da (tetramer); 1416 Da (pentamer); 1703 Da (hexamer); 1992 Da (heptamer); 2278 Da (octamer). Although the MALDI-TOF analysis was not able to discriminate between stereoisomers, due to the steric hindrance provided by flavanol

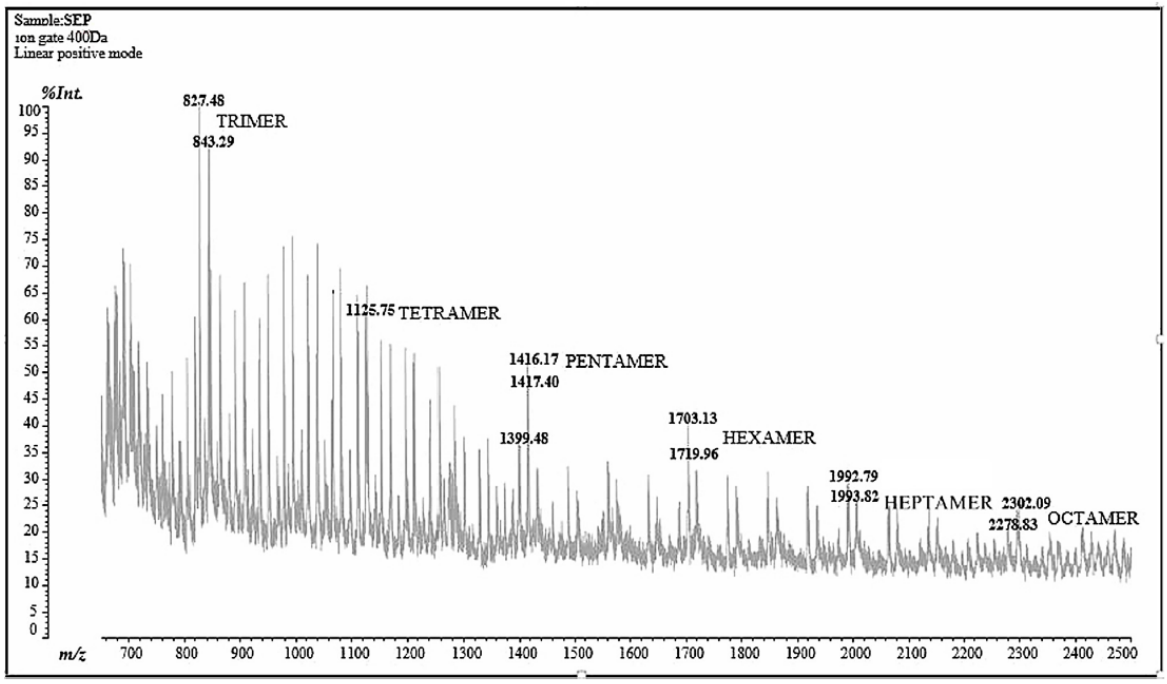

Fig. 1. The MALDI-TOF MS spectrum of sample SEP (linear positive mode, ion gate: 400 Da) recorded in the range 650–2500 Da: procyanidins series (repeat unit: catechins) for the grape seed extract.

Table 2
Calculated and experimental MALDI-TOF peaks related to the galloylated procyanidins series in the SEP sample.

DP*	no. of galloyl units (+152 Da)	Calculated (M+Na$^+$)	Observed(M+Na$^+$) *Linear positive mode*
Trimer	0	890	891
	1	1041	–
	2	1194	1194
	3	1344	1344
Tetramer	0	1178	1178
	1	1330	1328
	2	1482	1482
	3	1634	1633
Pentamer	0	1466	–
	1	1618	–
	2	1770	–
	3	1922	1922
	4	2074	2080
Hexamer	0	1754	–
	1	1906	–
	2	2058	–
	3	2210	–
	4	2363	–
Heptamer	0	2042	2037
	1	2194	2197
	2	2347	2348
	3	2495	2497
	4	2939	–
Octamer	0	2331	–
	1	2483	2480
	2	2635	–
	3	2787	–
	4	2939	–

*DP: degree of polymerisation. (–) Not present.

units, we hypothesized a (–)-epicatechin-like structure as a building block for grape seed procyanidins, with (+)-catechin as a possible terminal unit (Vivas et al., 2004b). The second series of polymeric structures was characterised by the presence of galloylated compounds with a variable number of galloyl units, as summarized in Table 2. Flavonoid compounds were also found in the grape seed extract, as sodium adducts (309 Da, kaempferol; 324 Da, quercetin); on the basis of their molecular weight, the 463 peak was attributed to the quercetin-3-glycoside monomer, and the 609 Da peak was related to a quercetin-3-rutinoside (rutin) – like structure.

3.2.2. Skin proanthocyanidin (SKP)

The MALDI-TOF spectra of the grape skin proanthocyanidin (SKP) showed (+)-catechin/(–)-epicatechin (peak 283 Da) and gallocatechin/epigallocatechin (peak 303 Da with additional fragments 250 and 226 Da) as the main units, in agreement with the literature (Souquet et al., 1996). Noteworthy, the peaks at 166/169 Da (gallic acid), 436 Da ((–)-epicatechin-3-gallate) and 462–464 Da ((–)-epicatechin-3-gallate sodium adduct) were markers for the presence of galloylated units. Catechin gallates are generally recognized as the main constituents of grape seed extracts (Vivas et al., 2004b; Yang and Chien, 2000), with a variable degree of galloylation in grape skins, mainly featured as (–)-epicatechin-3-*O*-gallate structures (Souquet, Cheynier, Brossaud, & Moutounet, 1996). The 463 Da peak could be alternatively assigned to the occurrence of quercetin-3-glycoside (isoquercetin), although not associated with complimentary fragmentation patterns. The only confirmation for this attribution derived from the presence of the peak at 617 Da, as a possible isoquercetin gallate dimer. The 605 Da peak was referred to as quercetin-3-rutinoside (rutin) with loss in protons.

The composition of procyanidins for the SKP sample is listed in Table 3. It is mainly composed of three building blocks: catechin dimers (procyanidins B), catechin-gallocatechin dimers, fisetinidin dimers; among these three combinations, the main repeat unit consists of catechins (+288 Da, Fig. 2).

3.2.3. Green tea procyanidins (GTP)

The green tea leaves were characterized by prodelphinidin compounds and flavonol-glycosides (Table 4). According to the procyanidins composition, two series were detected in the MALDI-TOF spectra: the first series was characterised by procyanidin dimers, trimers and tetramers (mostly prodelphinidin), with various combinations of monomeric units. The 467 Da peak was attributed to catechin gallate or robinetinidin gallate, whereas the 628 Da was tentatively attributed to a catechin digallate protonated adduct that might represent a marker for authenticity of the

Table 3
Calculated and experimental MALDI-TOF peaks for procyanidins detected in the SKP sample.

Calculated (M, Da)	Observed (M, Da) *Linear positive mode*	Attribution
542	544	Fisetinidin dimer
572	572	Catechin-gallocatechin with loss of water
578	581	A-/B- type procyanidins
591	592	Catechin-gallocatechin dimer
604	600	B-type procyanidin, (+Na$^+$)
633	634	Gallocatechin-gallocatechin dimer (+Na$^+$)
715	713	Catechin gallate + catechin dimer, with loss in –OH
823	828	Gallocatechin + catechin + flavanol fragment, trimer
847	847	Gallocatechin + catechin + flavanol fragment,trimer (+Na$^+$)
1111	1114	Gallocatechin + 2*catechin + flavanol fragment, tetramer
1129	1131	2*gallocatechin + catechin + flavanol fragment, tetramer
Oligomers with increase in catechin unit (+288 Da)		
1419	1415	Pentamer
1707	1702	Hexamer
1995	1990	Heptamer
2300	2298	Octamer (+gallocatechin*)

*Octamer, gallocatechin as a possible terminal unit.

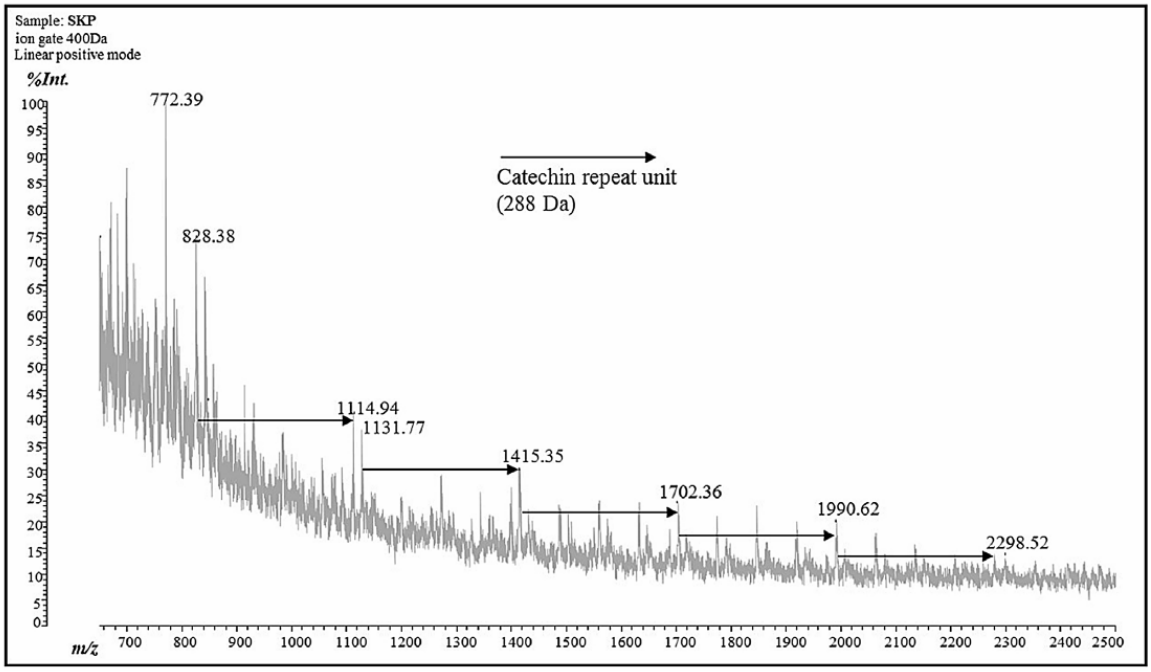

Fig. 2. The MALDI-TOF MS spectrum of sample SKP (linear positive mode, ion gate: 400 Da) recorded in the range 650–2500 Da: procyanidins series (repeat unit: catechins) for the grape skin extract.

extract. Despite the presence of the 574 Da peak – attributed to a procyanidins B-like structure – the oligomers series was mainly characterised by gallate compounds: 735 Da (catechin gallate-fisetinidin, dimer); 889 Da (catechin gallate-fisetinidin gallate, dimer); 903 Da (catechin gallate-catechin gallate, dimer); 1775 Da (3 catechin gallates units-fisetinidin gallate, tetramer). The 1978 Da and 2419 Da were assigned to the same series and were composed of catechin gallate unit linked to higher oligomers.

Remarkably, the mass region of ion peaks of the second series (951-1112-1274-1436-1602-1765-1927-2088-2247-2406), with occasional loss in protonated adducts, was observed with a peak-to-peak mass difference of 162 Da, consistent with the repeating unit of a hexose structure, possibly glucose (Fig. 3). This finding suggested the presence of glycosylated chains, either derived from degradation products of the foliar tissue or related to rearrangement of glucose release during extraction, since flavonoids in green tea are predominantly present as glycosides rather than non-glycosylated forms (Wang and Helliwell, 2001). As an alternative hypothesis, the use of a sugar-based chain as a stabilising agent was formulated (although not labelled by suppliers).

Table 4
Calculated and experimental MALDI-TOF peaks for flavonol and flavonol glycosides detected in the GTP sample.

Calculated (M, Da)	Observed (M, Da) *Linear positive mode*	Attribution
309	309	Kaempferol (+Na^+)
318	318	Myricetin
325	325	Quercetin (+Na^+)
448	444	Kaempferol-3-glycoside (astragalin)
464	464	Quercetin-3-glycoside (isoquercetin)
510	510	Myricetin-3-glycoside (+Na^+)
610	609	Quercetin-3-rutinoside (rutin)
617	617	Kaempferol-3-rutinoside (+Na^+)
847	847	Quercetin-3-rutinoside (+Na^+)

3.2.4. Limousin oak ellagitannin (LOE)

The Limousin oak heartwood extract was rich in distinctive compounds, such as valoneic acid, polygalloylglucose structures, aldehyde derivatives and ellagitannins. The occurrence of a hydrolysable tannin is unambiguously confirmed by the presence of fragmentation patterns related to the polygalloylglucose structures: the 127 Da peak is related to cleavage mechanisms involving the glucose ring; the 152 Da peak is attributed to galloyl moieties (coupled to the 166 Da peak, due to the presence of gallic acid molecules). Moreover, ellagic acid (305 Da) and hexahydroxydiphenic acid (HHDP, 343 Da) monomers were detected in the extract, which are typically found in ellagitannin-based extracts. Several patterns were attributed to the fragmentation of polygalloyl compounds and sugar chains, which are likely to derive from oak wood following extraction; the basic unit for this series was a glucose dimer (glucose + glucose fragment, 219 Da; sodium adduct: 248 Da). Table 5 provides a list of the fragmentation patterns produced by the Limousin oak tannin, including polygalloyl glucoses: the gallic acid multiprotonated residues linked as esters, through carboxylic acid moiety, to sugar or gallic acid molecules (Fig. 4). Vescalagin/castalagin (934 Da) were detected as the main constituents of the extract, as confirmed by the 593, 623 and 609 Da peaks attributed to castalin/vescalin fragments (most likely castalin with loss in —OH moieties, and castalagin molecule with loss in an ellagic acid function and a water molecule, respectively). The occurrence of these compounds in the *Q. robur* extract is consistent with the literature (Nonier et al., 2005). The

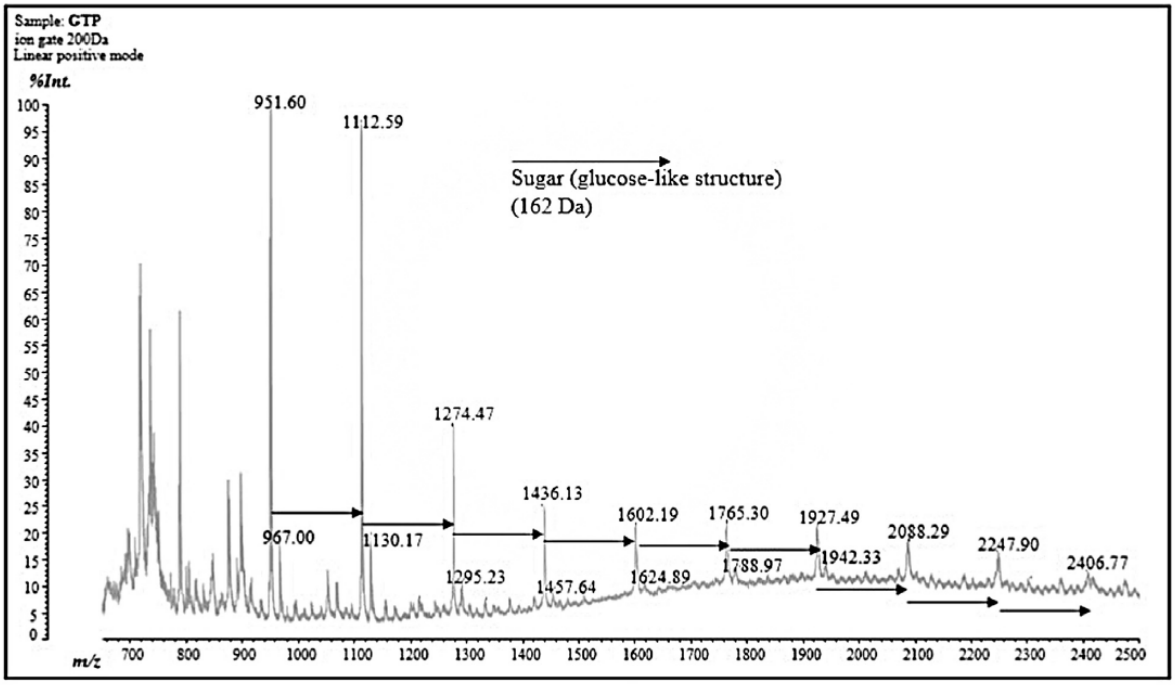

Fig. 3. The MALDI-TOF MS spectrum of sample GTP (linear positive mode, ion gate: 200 Da) recorded in the range 650–2500 Da: glycosylated chain series.

odium adduct of roburin A/roburin D dimers (1873 Da peak) can ıe considered a marker for the *Quercus* wood extract.

.3. Elemental profiling

Besides four macroelements (^{24}Mg, ^{39}K, ^{44}Ca and ^{55}Mn), twelve race isotopes (^{7}Li, ^{27}Al, ^{52}Cr, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{75}As, ^{38}Ba and ^{207}Pb) were investigated as possible contaminants lerived from the botanical source or processing. Overall, the oncentrations of macroelements ranged from 0.08 to 0.5 ppm for Ag, and from 0.2 to 0.4 ppm for Ca. The microelements ranged ıetween 0.001–0.01 ppm for Mn, up to 0.99 ppm for Zn, and round 0.0001 ppm for Co (Table 6). Iron and copper were enerally present in samples at very low levels (GTP sample: .06 ppm ^{56}Fe and 0.04 ppm ^{63}Cu, respectively) which is suitable or beverage application. Cu, Fe and Mn are important catalysts of xidation of organic substrates (Danilewicz, 2007) and they form table complexes with polyphenols, affecting the aroma, taste and olor of many beverages, including wine (Waterhouse and Laurie, 006) and beer (Bamforth, 2011).

As, Ni, Cu, and Ba, with respective security limits of 0.01, 0.07, 2.00, and 0.70 ppm recommended by WHO (2004), were far below the legal limits for all samples assayed. Although Cr, Li, Al and Sr are not included in the WHO guidelines, their concentrations lay within the ppb level in the four samples. In particular aluminum, which is likely to induce clouding and decreasing wine stability at increasing pH values (Mrak et al., 1937), was below the limit of quantitation (in the order of ppt) for all samples investigated. The content of Cr, which is crucial in foods due to its toxicity, ranged between 8.2–10.7 ppb, which is far below the ppm levels previously published for tannin extracts (Zmozinski et al., 2015; Mehra et al., 2013).

The Pb levels exceeded the limit set up by WHO for drinking waters (Table 6); the occurrence of the same Pb level in all samples suggested that a possible source of contamination occurred during processing rather than from the botanical sources themselves. Besides the WHO recommendations, several Community regulations have focused on exposure of humans to lead through food, and the SCF suggested a PTWI (provisional tolerable weekly intake) of 25 μg/kg bw (Reports of the Scientific Committee for Food, 32nd

able 5
ALDI-TOF fragmentation patterns for ellagitannins and polygalloylglucoside compounds detected in the LOE sample.

Calculated (M, Da)	Observed (M, Da) *Linear negative mode*	Attribution
364	362	Gallic acid dimer + sugar fragment
390	394	Galloyl moiety + sugar + sugar fragment
424	424	Gallic acid dimer + sugar fragment
463	463	Ellagic acid hexoside
493	494	Valoneic acid dilactone (+Na^+)
507; 506	506	Digalloyl glucose (+Na^+); Valoneic acid
567	566	Gallic acid trimer + sugar fragment (+Na^+)_loss in two −OH
634	633–634	Galloyl HHDO glucose
1035	1038	Esamer[S2] : 2 gallic acids and 4 hexosyl polysaccharide + glucose residue
1463	1463	Castalagin or pentagalloylglucose linked to a nonahydroxythriphenoic acid (+Na^+)

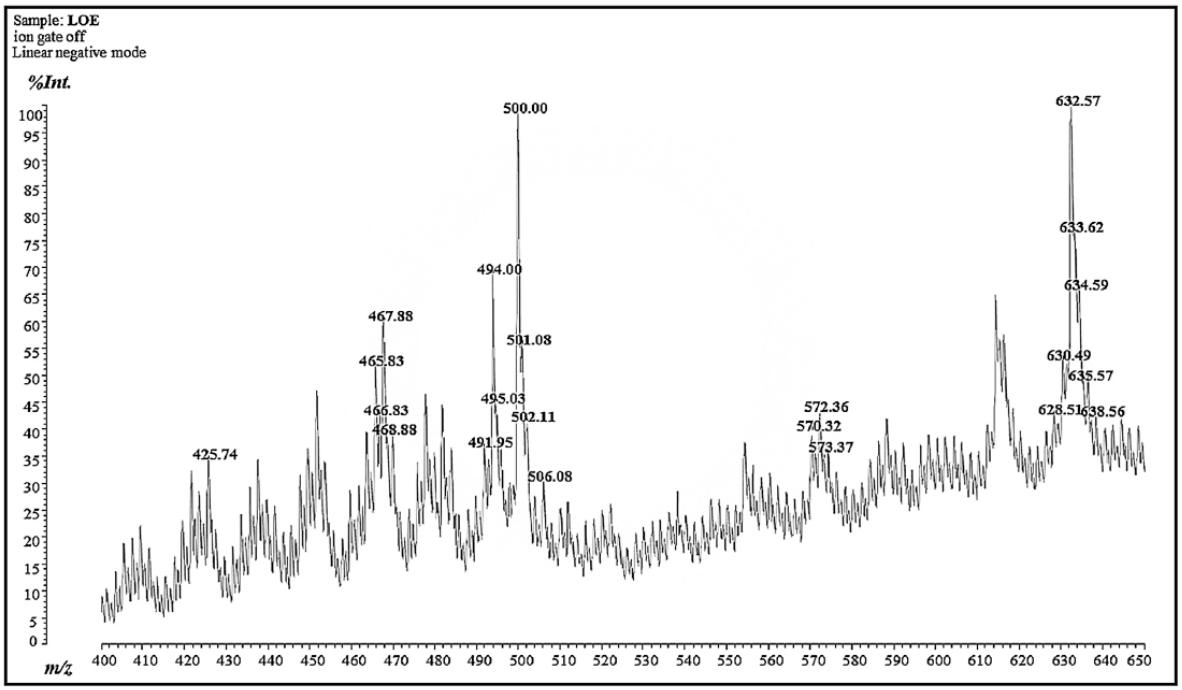

Fig. 4. The MALDI-TOF MS spectrum of sample LOE (linear negative mode, ion gate: off) recorded in the range 400–650 Da showed basic units of ellagitannins and glycosylation patterns (see Table 5).

Table 6
Elemental composition of selected commercial tannins used as food additives; results are provided as mean values (n = 3).

	Tannin samples (ppm)				(ppm)	(%)	Guideline values (WHO, 2004)
Element	SEP	SKP	GTP	LOE	LOD	RSD*	
^{7}Li	0.001	0.001	0.002	0.002	8.53E–06	2.44	na
^{24}Mg [He]	0.080	0.090	0.500	0.200	1.34E–04	1.64	na
^{27}Al	nd	nd	nd	nd	1.01E–03	2.48	0.200
^{39}K [He]	1.400	1.200	0.800	0.600	2.31E–04	1.39	na
^{44}Ca [He]	0.200	0.220	0.400	0.400	7.81E–04	2.28	na
^{52}Cr [He]	0.009	0.011	0.008	0.008	9.09E–06	1.89	0.050
^{55}Mn [He]	0.001	0.002	0.010	0.004	5.81E–06	1.96	0.400
^{56}Fe	0.050	0.050	0.060	0.030	9.77E–05	2.28	1.000–3.000
^{59}Co	0.0001	0.0001	0.0001	0.0001	3.30E–07	2.03	na
^{60}Ni	nd	nd	nd	nd	7.95E–05	1.94	0.070
^{63}Cu	0.020	0.020	0.040	0.020	1.35E–05	2.06	2.000
^{66}Zn	nd	nd	0.990	0.250	5.51E–04	1.95	3.000
^{75}As	0.002	0.002	0.002	0.002	1.30E–05	2.52	0.010
^{88}Sr	0.003	0.004	0.020	0.020	2.22E–05	1.96	na
^{137}Ba	0.010	0.010	0.020	0.020	7.02E–04	2.17	0.700
^{207}Pb	0.031	0.033	0.033	0.033	5.74E–05	1.61	0.010

RSD*: Average values for each element. nd: not detected or below the quantitation limit; na: not available. [He]: analysis was performed under He stream conditions.

series, 19 June 1992); in more recent times, the Scientific Committee has stressed the hazard related to metal contaminant intake with a directive that encourage the minimisation of lead levels in food (Reports on tasks for scientific co-operation, task 3.2.11: Assessment of dietary exposure to arsenic, cadmium, lead and mercury of the population of the EU Member States, Directive 93/5/CEE, 2004).

It has to be noticed that the maximum concentration of Pb allowed in wine is 0.3 mg/L according to the International Organization of Vine and Wine (OIV); accordingly, the lead level in the extracts lies far below the safety threshold defined for enological applications (Ivanova-Petropulos et al., 2013). Regardless of the ambiguity in the definition of a common risk threshold for the Pb content, the hypothesis of occasional contaminations occurring along the supply chain would require a detailed investigation in a representative number of samples to be confirmed, and the implementation of purification processes.

4. Conclusions

In this work, the composition of commercially available food-grade tannins was explored to achieve two objectives: (i) identify

markers for the authentication of commercial products according to the declared botanical origin, and (ii) monitor the presence of potentially toxic contaminants. Objective (i) was achieved through the combined use of MALDI-TOF MS and UV–vis spectrophotometric methods which showed variable degree of purity and variable percentage of tannins; in particular, the highest content in polyphenols was reached by the SKP extract (93.3%), followed by LOE > GTP > SEP. The quantitative analysis of polyphenols accounted for the antiradical activity of the extracts, which reflects the nutritional properties of additives. According to the chemical composition of the polyphenolic fraction the main discriminant fingerprints between the plant extracts evaluated as authentication tools were found: the composition in flavonoid and their degree of galloylation for SEP, SKP and GTP samples, and the specific glycosylation patterns and castalagin/vescalagin derivatives occurring in the LOE sample. The GTP sample showed a high variety of molecular fragments attributable to flavonols, which have a high antioxidant power and may legitimize the use of this extract as a food supplement. Recognition patterns were detected for the GTP sample (628 Da protonated fragment) and for the LOE extract (sodium adduct of roburin dimer at 1873 Da); the two molecular fingerprints were suggested as authenticity markers for the tea leaves and *Quercus* wood extracts. In objective (ii), the ICP-MS elemental profile confirmed the levels of potentially toxic contaminants; Pb levels were found to exceed the level suggested by the WHO regulations for drinking water, despite higher concentrations are allowed in other food applications. The present work has highlighted the need for correct production practices along the supply chain, to minimize the occurrence of external contamination sources. To our knowledge, the elemental composition of a Limousin oak food-grade tannin was reported for the first time.

The present work constitute a preliminary study, and will be implemented increasing the number and variety of samples to build a database of commercial products currently available in the market, thus contributing to their proper exploitation in the food industry.

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