Factors influencing the colour and phenolic composition of Shiraz wine during winemaking

by

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DECLARATION

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SUMMARY

Phenolic compounds are considered important quality contributors of red wines. However, lot of unknowns exist in terms of their role in wine due to their complexity and continuous evolution which starts in fresh grapes, during alcoholic fermentation and wine ageing. In this study, we have evaluated different parameters which could affect the colour and phenolic composition of Shiraz wines. All the work has been carried out within the same vineyard during three harvest seasons. This study has interconnected topics in order to reach a broader understanding on how certain factors influencing the phenolic composition in fresh grapes can affect phenolic extractability and subsequent wine ageing.

In the first part of the study, the colour and phenolic composition of wines was changed by harvesting the grapes at different ripening levels. Differences observed in the phenolic composition in young red wines were also followed during prolonged wine ageing. The initial phenolic composition of young wines influenced the wines' colour and phenolic evolution during bottle storage. The sensory profiles of the wines were also influenced by the grape ripening and ageing.

This work also evaluated the impact of vintage, grape ripeness and alcoholic fermentation on the transformation of the grape berry cell wall. All these factors have been shown to affect the final colour and phenolic composition of young red wines. Grape ripeness was relevant for the study, but the vintage effect was shown to have the major impact on the phenolic content and the cell wall composition in fresh grapes. Nonetheless, compositional changes in the grape berry cell wall, partly influenced by the de-pectination during grape ripening and fermentation, also affected the release of grape phenolics into the wines. In addition, the extraction of grape proanthocyanidins with a longer polymer length was also partly influenced by the cell wall breakdown during fermentation, enhanced in riper berries.

The last part of the study has shown the influence of different anthocyanin/tannin ratios and oxidation on the evolution of colour, phenolics and the precipitate formed over time in a wine-like system (WL). The use of advanced chromatographic and untargeted techniques has allowed us to measure the impact of different seed additions on the phenolic composition and development of the WL. A larger amount of tannins, extracted from higher additions of grape seeds, influenced the initial phenolic concentration and also the polymerisation reactions over time, which were enhanced in the presence of oxygen. Differences in the precipitate formed over time were also influenced by the initial amount of seeds used. This work highlights the importance of a better understanding of the grape cell wall composition and its evolution. This can lead to better control of the extraction of grape phenolic compounds and their ratios during the winemaking process, which can also influence the ageing of the wines.

OPSOMMING

Fenoliese verbindings word beskou as belangrike bydraers tot die gehalte van rooiwyne. Daar is egter nog baie onduidelikheid oor hul rol in wyn as gevolg van hul kompleksiteit en voortdurende evolusie wat begin in vars druiwe, tydens alkoholiese fermentasie en tydens veroudering. In hierdie studie het ons verskillende faktore geëvalueer wat die kleur en fenoliese samestelling van Shiraz-wyne kan beïnvloed. Al die werk is gedoen op dieselfde wingerd oor 'n periode van drie oesseisoene. Hierdie studie omvat onderlinge verwante onderwerpe om 'n breër begrip te verkry van hoe sekere faktore wat die fenoliese samestelling in vars druiwe beïnvloed, fenoliese ekstraksie kan beïnvloed en die daaropvolgende wynveroudering.

In die eerste deel van die studie is die kleur en fenoliese samestelling van wyne verander deur die druiwe op verskillende rypheidsvlakke te oes. Verskille wat waargeneem is in die fenoliese samestelling van jong rooiwyne is ook waargeneem tydens langdurige wynveroudering. Die aanvanklike fenoliese samestelling van jong wyne het die kleur- en fenoliese evolusie van die wyne tydens bottel veroudering beïnvloed. Die sensoriese profiele van die wyne is ook deur druif rypwording en veroudering beïnvloed.

Hierdie werk het ook die impak van oesjaar, druifrypheid en alkoholiese fermentasie op die transformasie van die druifkorrel-selwandsamestelling geëvalueer. Al hierdie faktore het die finale kleur en fenoliese samestelling van jong rooiwyne beïnvloed. Druif rypheid was relevant vir die studie, maar daar is aangetoon dat die seisoenale effek die grootste impak op die fenoliese inhoud en die selwandsamestelling van vars druiwe het. Tog het komposisionele veranderinge in die druifkorrel selwand, deels beïnvloed deur depektienase tydens druif rypwording en fermentasie, ook die vrystelling van druif fenole in die wyne beïnvloed. Daarbenewens was die ekstraksie van druif proantosianidiene met 'n langer polimeerlengte ook deels beïnvloed deur die afbraak van die selwand tydens fermentasie, hierdie verskynsel is verhoog in ryper druiwe.

Die laaste deel van die studie het die invloed van verskillende antosianien/tannienverhoudings en oksidasie op die evolusie van die kleur, fenoliese samestelling en die presipitaat wat oor tyd in 'n wynagtige medium gevorm het, getoon. Die gebruik van gevorderde chromatografie en ongeteikende tegnieke het ons toegelaat om die impak van verskillende druiwepit toevoegings op die fenoliese samestelling en ontwikkeling van die wynagtige medium te meet. Die groter hoeveelheid tanniene, verkry uit die hoër toevoegings van druiwepitte, het die aanvanklike fenoliese konsentrasie sowel as die polimerisasie-reaksies oor tyd beïnvloed. Hierdie reaksies was ook verhoog in die teenwoordigheid van suurstof. Die verskille in die presipitaat wat oor tyd gevorm het, is beïnvloed deur die aanvanklike hoeveelhede sade wat gebruik is. Hierdie werk beklemtoon die belangrikheid van 'n beter begrip van die selwand se samestelling en evolusie, met die doel om die

ekstraksie van druif fenoliese verbindings en hul verhoudings beter te beheer tydens die wynmaak proses, wat ook wynveroudering ook kan beïnvloed.

This dissertation is dedicated to my family

Biographical sketch

Gonzalo Garrido Bañuelos was born on 3rd April 1988 in Logroño, La Rioja, Spain. In 2006, he enrolled for his Licenciatura en Biología at Universidad de Salamanca (Salamanca, Spain), completing the specialisation in fundamental biology and biotechnology in 2011. That same year, he moved to Bordeaux, France, obtaining his MSc. degree in Oenology and Wine environment at Université de Bordeaux in 2013. He completed his MSc. project at the Institut des Sciences de la Vigne et du Vin (ISVV). He enrolled for his PhD in Oenology in 2014.

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Preface

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the *South African Journal of Enology and Viticulture*. Each chapter should be regarded as an individual entity and therefore some repetition between chapters may occur.

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Chapter 1

Introduction and project aims

1.1 INTRODUCTION

"A bottle of wine contains more philosophy than all the books in the world" Louis Pasteur mentioned. All these secrets make wine the ideal metaphor of life. Any cultivar, white or red, in any wine world region, shows a distinctive and special character as the diversity of humanity. A wine's life starts in the vineyard, growing new grapes every season, under different environmental and climatic circumstances just as parents raises their children based on their different life styles. Once mature, children tend to leave their homes to explore the world or pursue their education comparable to the moment when the harvested grapes get into the cellars. And from then onwards, a large number of chemical reactions and interactions take place during wine maceration and ageing, leading to the evolution of the product profile, as with social interactions and apprenticeships enriching a person's life, before getting, for both cases, into the final market.

Currently, South Africa is the 8th largest wine producer and as the rest of the global wine industry, is seeking to increase its offering of high-quality wines. After Cabernet Sauvignon, Shiraz is the second most planted red cultivar in South Africa. In the recent times, its importance in the South African wine industry has increased, being the most planted cultivar from 2000 to 2010 and now accounts for 10.4% of the total vineyard surface area in the country. In a worldwide context, South Africa is the 4th largest Shiraz grower (ShirazSA; Wines of South Africa).

Grape and wine phenolic compounds are very important contributors to wine quality, especially in Shiraz wines. It is for this reason that a deeper understanding of the phenolic content and evolution during the entire winemaking process is essential. These molecules are a group of secondary metabolites, with a large diversity of structures commonly found in plants, plants derivatives and beverages (Monagas, *et al.*, 2005; Cheynier, *et al.*, 2006). Wine phenolics contribute not only to the colour, but also to the main sensorial and organoleptic properties of the wine throughout the winemaking process (Somers & Evans, 1974; Singleton, 1987; Brossaud, *et al.*, 2000; Cheynier, *et al.*, 2006; Ribéreau-Gayon, *et al.*, 2006). The high reactivity of these phenolic compounds leads to a continuous evolution, with the formation of new compounds over time (Singleton & Trousdale, 1992; He, *et al.*, 2012; Arapitsas, *et al.*, 2014).

The phenolic profile can differ between the fresh grapes and their corresponding wines, but certain positive correlations between grapes and wines have been found (Du Toit & Visagie, 2012). A red wine's chemical composition cannot be understood or predicted without a good knowledge of the grape's phenolic composition, potential and factors affecting their extraction during alcoholic fermentation. Several studies have reported different phenolic profiles in grape cultivars (Ryan & Revilla, 2003; Pérez-Magariño & González-SanJosé, 2004; Jensen, *et al.*, 2008; Río Segade, *et al.*, 2009) and how changes in their concentrations are affected by berry development, grape ripening (Adams, 2006; Fournand, *et al.*, 2006; Cagnasso, *et al.*, 2011;

Bautista-Ortín, *et al.*, 2012), different harvesting seasons (Boido, *et al.*, 2006; Lorrain, *et al.*, 2011) and different vineyard management practices (Zoecklein, *et al.*, 2008; Gil-Muñoz, *et al.*, 2009; Mota, *et al.*, 2011). Additionally, different winemaking decisions and techniques, such as the time of harvest, cold soaking (Álvarez, *et al.*, 2006; González-Neves, *et al.*, 2015) or the use of specific enzymes (Ortega-Heras *et al.*, 2012; Río-Segade *et al.*, 2015; Gao, *et al.*, 2016), can also play an important role in the extractability of these compounds into the wines (Sacchi, *et al.*, 2005; Smith, *et al.*, 2015). The final concentration and nature of these phenolic compounds extracted into the wines will influence their ageing potential (Pascual, *et al.*, 2016; Picariello, *et al.*, 2017).

Complementary to this, understanding of the grape cell wall architecture is crucial in order to control the phenolic extractability during alcoholic fermentation. The biosynthesis and accumulation of phenolic compounds occurring in the cells of different grape berry tissue layers (Amrani Joutei, *et al.*, 1994; Fournand, *et al.*, 2006) is accompanied by a series of conformational changes in the grape's cell walls. Thus, the release of phenolic compounds during alcoholic fermentation is related to the structural breakdown occurring in the grape cell walls (Gao *et al.*, 2016). However, there is a lack of information concerning the relationship between the grape phenolics and cell wall proteins and polysaccharides. To date, most research groups have approached studies on the grape berry cell walls from its monosaccharide composition as a fractionation product of the cell wall polysaccharides. However, the use of new techniques (Moller, *et al.*, 2007) allows for the investigation of the cell wall composition and its evolution during grape ripeness and fermentation at a polymer level. It is a relevant topic for the wine industry as interactions between cell wall polymers and phenolics can take place not only in fresh grapes, but also in wines, thereby affecting the phenolic stability (Riou, *et al.*, 2002; Bindon, *et al.*, 2016) and mouth-feel properties of the wines (Vidal, *et al.*, 2004). The structural diversity and complexity of phenolic compounds and their continuous evolution in wine make research on this topic a difficult undertaking. For this reason, most work on wine phenolics has been carried out in young or shortly aged red wines. Little is known on how different phenolic proportions/compositions in young red wines evolve during wine ageing, and due to the complexity of the wine matrix, most of these studies were done in wine-like solutions (Timberlake & Bridle, 1977; Es-Safi, *et al.*, 1999; He, *et al.*, 2008). The role of oxygen during the ageing of red wine has also not been elucidated completely. A better understanding of factors affecting the phenolic extractability during alcoholic fermentation is therefore essential, not only for the young wines composition, but also to interpret their ageing potential, as the proportion of phenolic compounds can affect a wine's colour and phenolic stability over time (Picariello, *et al.*, 2017).

1.2 PROJECT AIMS

The broad aims of this work were to obtain a better understanding of factors affecting the extraction and evolution of phenolics in Shiraz wine. The link between changes in the berry cell wall and the final phenolic composition in red wines will be explored. The present work also aims to investigate the colour and phenolic evolution during ageing as a consequence of naturally altered phenolic ratios in model wine and real Shiraz wines. The entire study was done using grapes from an experimental, well characterised, *Vitis vinifera* cv. L. Shiraz vineyard situated on the Welgevallen experimental farm of Stellenbosch University. All wines produced, during three harvesting seasons (2014, 2015 and 2016) were made from this vineyard.

The specific aims of this project were:

I. Evaluate the changes occurring over time in the phenolic and sensorial properties of Shiraz wines with different initial phenolic profiles made from grapes from different vineyard treatments.

II. Explore the association between the structural and compositional changes in grape skin cell walls and the phenolic content during ripening.

III. Explore the association between the structural and compositional changes in fermented grape pomace and the phenolic extractability occurring during alcoholic fermentation.

IV. Determine the impact of oxygen and of three altered anthocyanin/tannin ratios on the colour and phenolic evolution and precipitate in a model wine system.

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Chapter 2

Literature review

Factors influencing the colour and phenolic composition in red wines.

Chapter 2: Factors influencing the colour and phenolic composition in red wines.

2. 1 INTRODUCTION

Wine phenolics are considered important quality indicators, especially in red wine. A greater number of variables in the red winemaking process influences the wine's matrix in red wines compared to that of white wines. Part of this complexity is due to a larger polyphenol content found in red wine. These non-odorant compounds play an essential role in the colour stabilization, the sensory properties and the ageing potential of red wines (Sáenz-Navajas *et al.*, 2012; Casassa, 2017).

Grape phenolics, with sugars, acids and aroma compounds will determine the berry quality and are determining factors in the final chemical profile of red wines. These groups are further subdivided according to their structure. This literature review aims to give an overview of the importance of the phenolic compounds from the grapes to the final wine product. It also shows the relevance of the grape phenolic potential to the wine phenolic composition and its ageing potential.

2.2 PHENOLICS IN GRAPE AND WINE

As illustrated in Figure 2.1, the berry structure consists mainly of the skin, pulp, and seeds. In a grape berry, phenolic compounds are found these three areas. The colour pigments, anthocyanins, are in most red cultivars exclusively found in the grape skin. This layer also contains other phenolic compounds, such as flavonols and tannins. The main phenolic compounds found in the pulp are phenolic acids. Lastly, the seeds are characterized by the presence of tannins (Prieur, *et al.*, 1994; Harbertson, *et al.*, 2002; Ribéreau-Gayon, *et al.*, 2006). Phenolic compounds can be divided in two main groups: non-flavonoids and flavonoids. The former group are generally found in lower concentrations in grape and wines compared to flavonoids (Ribéreau-Gayon, *et al.*, 2006). Non-flavonoids are mainly represented by benzoic acids derivatives, cinnamic acids derivatives, and stilbenes. Some of these compounds, such as hydroxycinnamic acids, can contribute to the wine colour as copigments (Aleixandre-Tudó, *et al.*, 2013; Bimpilas, *et al.*, 2016).

Flavonoids are the major constituents of a red wine's phenolic composition, contributing to a greater extend to the wine's colour and flavour than non-flavonoids. This group of molecules is characterised by a C6-C3-C6 skeleton, two benzene rings (A and B) bound by a heterocyclic pyran ring (Cheynier, *et al.*, 2006). Flavonoids can be subdivided in different subgroups according to the unsaturation of their pyran ring. The main classes are flavonols (Figure 2.1A), anthocyanins (Figure 2.1B), flavan-3-ols (Figure 2.1C) and condensed tannins (also known as proanthocyanidins). The different compounds within the same group will be determined by the substitutions in their B ring. The rest of this chapter will only deal with flavonoids, due to their relevance to the present study.

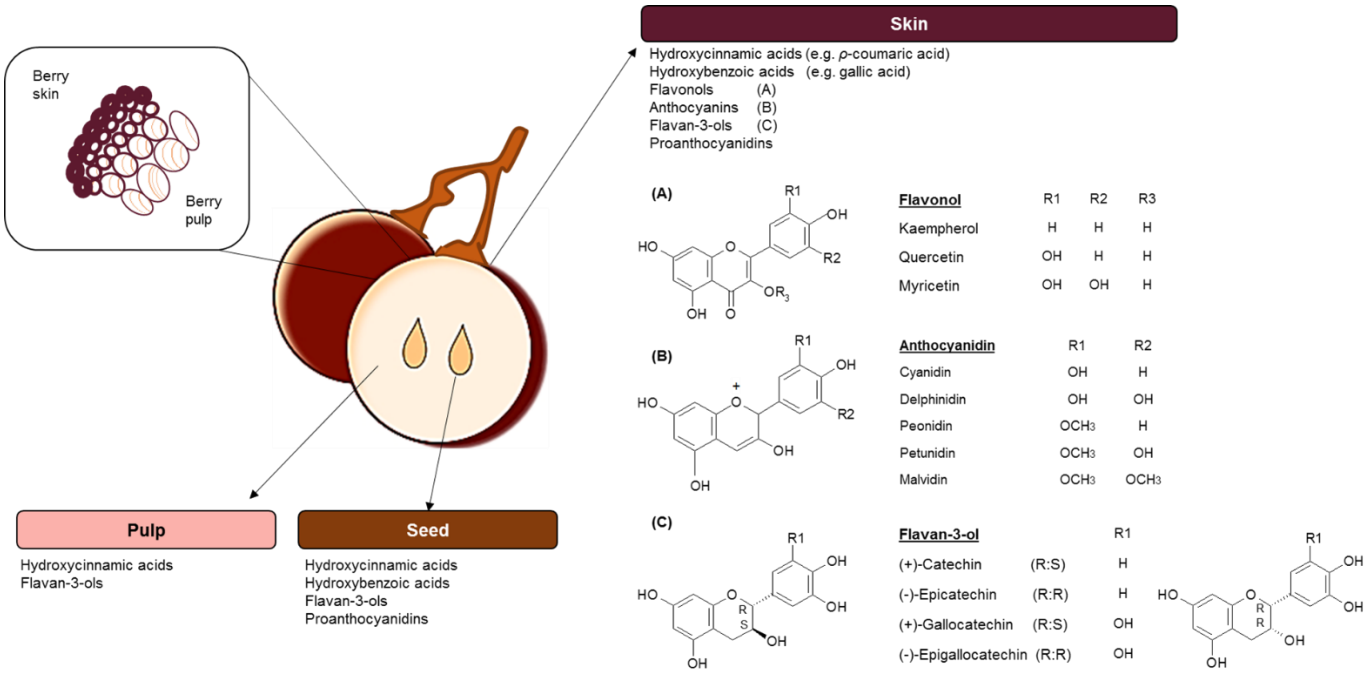

Figure 2.1. Schematic design of the berry structure and the main groups of phenolic compounds in red grapes adapted from literature (Monagas, *et al.*, 2005; Adams, 2006; Castellarin, *et al.*, 2012).

2.2.1 Flavonols

Flavonols are considered important contributors to the colour of red wines, enhancing anthocyanin extraction during the vinification process, as well as playing an important role in the copigmentation phenomenon (Boulton, 2001; Schwarz, *et al.*, 2005). This group of compounds can also influence the bitterness and astringency of red wines (Sáenz-Navajas, *et al.*, 2010; Gonzalo-Diago, *et al.*, 2014).

The most common flavonols found in grapes and wines are kaempferol, quercetin, and myricetin (Figure 2.1A). In grapes berries, these compounds can only be found as their 3-glycosides or glucuronides derivatives, but they are also present in their aglycone form in red wines (Makris, *et al.*, 2006; Monagas, *et al.*, 2006). Several studies have shown a clear relationship between flavonol

biosynthesis in grape berries and sunlight exposure, and thereby the UV radiation, of the grape bunches (Downey, *et al.*, 2003a; Ristic, *et al.*, 2006). Differences in the grape flavonol profile can be found between different grape cultivars (Makris, *et al.*, 2006; Mattivi, *et al.*, 2006; Río Segade, *et al.*, 2008, 2009).

2.2.2 Anthocyanins

Anthocyanins are considered to be one of the most important quality indicators in red wines, as they are the main source of the red colour observed in red grapes and wines (Bridle & Timberlake, 1997; Kennedy, *et al.*, 2006; Barbagallo, *et al.*, 2011). These pigments are accumulated in the grape skins, where they can be found as monomers or free anthocyanins, the exception being the few "teinturier" grape varieties, where anthocyanins are also present in the grape pulp (Adams, 2006).

Chemically, the differences between the individual anthocyanin structures rely on the substitutions and the number of hydroxyl and groups methoxyl attached in the B ring. These different substitutions influence the colour and polarity of the anthocyanins. Thus, the polarity and the blue tones of the molecule increase with the number of hydroxyl groups. Contrary, an increase in methylation in the B ring leads to greater molecular stability and an increase in the red colour (He, *et al.*, 2012a; Casassa, 2017). The most common free anthocyanidins found in red wines are delphinidin, cyanidin, petunidin, peonidin and malvidin (Figure 2.1B) (Ribéreau-Gayon, *et al.*, 2006; Bueno, *et al.*, 2012; He, *et al.*, 2012a). These compounds are generally found in their glycoside forms, mainly as 3-*O*-monoglucoside, with malvidin-3-*O*-glucoside often being the most abundant anthocyanin in red grape varieties (Castañeda-Ovando *et al.*, 2009). Acylated monoglucoside anthocyanins can also be found in grapes and wines. This acylation can influence the anthocyanin colour and increase their stability in the wine matrix (Bakowska-Barczak, 2005). Acylated monoglucose anthocyanins are formed from esterification of the sugar moiety of the anthocyanin with different acids (acetic, p-coumaric or caffeic acid) (Monagas, *et al.*, 2005).

The colour of these anthocyanins depends on several factors and wine conditions, such as pH, temperature and oxygen and sulphur dioxide levels (Torskangerpoll & Andersen, 2005). Changes in pH can alter the equilibrium between the different anthocyanin chemical forms. Thus, the anthocyanins can be found in four chemical states (Figure 2.2): flavylium cation (red colour), quinoidal base (blue to purple), carbinol pseudobase (colourless) and chalcone (pale yellow). The addition of bisulphite bleaches the flavylium cation to a colourless compound (Ribéreau-Gayon, *et al.*, 2006). The colour in young red wines is mainly due to monomeric anthocyanins, as the polymeric forms only contribute to a small percentage of it (Somers & Evans, 1974, 1977). These monomeric pigments are chemically unstable and susceptible to degradation. Several studies have shown how the anthocyanin monomeric fraction, especially acylated forms, decreases as wine ages (Somers, 1971; Mateus *et al.*, 2001; Pérez-Magariño & González-San José, 2004; Versari, *et al.*, 2007;

Versari, *et al.*, 2007; He *et al.*, 2012b; Zeng *et al.*, 2016). In essence, the characteristic red-purple colour in young wines is mainly due to monomeric anthocyanins and the presence of different copigments (Davies & Mazza, 1993; Boulton, 2001).

Copigmentation can be described as the association between anthocyanins with non-coloured compounds. These short-term intra and intermolecular interactions, between anthocyanin and copigments, enhance the wine's colour intensity. Therefore, this phenomenon represents up to 50% of the wine colour after the alcoholic fermentation (Boulton, 2001). These interactions are disrupted over time, increasing the red tones (Somers & Evans, 1979).

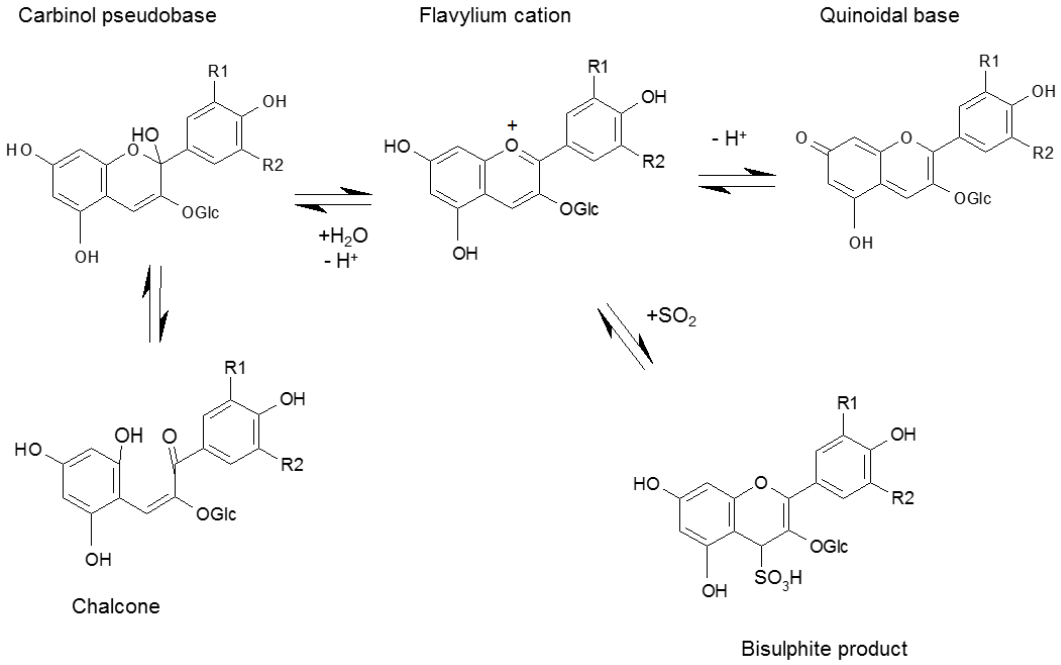

Figure 2.2. Different anthocyanins chemical forms in aqueous solution. Adapted from literature (Ribéreau-Gayon, *et al.*, 2006).

As wine ages, the changes in colour are also influenced by the formation of more complex and stable anthocyanin-derivatives or pigments. As a consequence of this pigment evolution, the most important pigments in aged red wines are pyroanthocyanins and polymeric pigments (Brouillard & Dangles, 1994; Alcalde-Eon, *et al.*, 2006; Boido, *et al.*, 2006; Ribéreau-Gayon, *et al.*, 2006; He, *et al.*, 2012b). The former group can be the result of reactions between free anthocyanins or with yeast by-products and are responsible for the tawny colour in aged red wines (De Freitas & Mateus, 2011). Vitisins were the first pyroanthocyanins identified in wines and are biomarkers associated with the ageing process. Polymeric pigments are the result of the combination between anthocyanins with tannins by direct condensation or mediated by acetaldehyde (Monagas, *et al.*, 2005). The polymerisation reactions between anthocyanins, flavan-3-ols and proanthocyanidins occur at

different rates in the presence of acetaldehyde (Dallas, *et al.*, 1996). Polymeric pigments are more relevant to the mouth-feel of red wines as they are more soluble than proanthocyanidins (He, *et al.*, 2012b).

The aforementioned pigments play an important role in the colour stabilization of red wine, as they are more resistant to the bleaching effects of SO_2 and less sensitive to pH changes (García-Puente Rivas, *et al.*, 2006; Avizcuri, *et al.*, 2016). These pigments also contribute to shift the wine's colour from the red-purple, characteristic of young red wines, to the red-orange nuances appreciated in aged wines (Somers, 1971; Mateus, *et al.*, 2001; Cheynier, *et al.*, 2006).

During maturation of red wine, phenolic molecules continuously evolve to a greater degree of condensation. Several studies have investigated the synthesis and the formation of these new compounds over time, in both model wine solutions and wine. The recent development of new analytical tools has helped to reach a better understanding of these anthocyanin-derivatives compounds found in wines (Harbertson, *et al.*, 2003; Willemse, *et al.*, 2013, 2014).

2.2.3 Flavan-3-ols and condensed tannins

The term tannin includes a wide range of polyphenolic structures. These molecules play an essential role in the winemaking process as they influence the taste and mouth-feel of red wines to a large extent (Gawel, 1998; Santos-Buelga & Scalbert, 2000; Ma, *et al.*, 2014), as well as colour stabilization and wine ageing potential (Versari, *et al.*, 2007). Wine tannins can be classified into two groups: hydrolysable tannins and condensed tannins. The former group is not naturally found in grapes. These phenolic compounds, such as gallotannins and ellagitannins, are extracted into wines from the oak barrels (Cheynier, *et al.*, 2006; Ribéreau-Gayon, *et al.*, 2006; Chira, *et al.*, 2015; Michel, *et al.*, 2016). These hydrolysable tannins can easily be hydrolysed, thereby releasing gallic or ellagic acid in the wine during ageing (Ribéreau-Gayon, *et al.*, 2006). These wood compounds and their derivatives can combine with tannin and anthocyanins, contributing to the colour stability. Vescalagin and castalagin are the two main ellagitannins found in oak (Ribéreau-Gayon, *et al.*, 2006).

Grape-derived flavan-3-ol and condensed tannins are the main source of phenolics contributing to the final wine tannin concentration. Numerous studies have investigated the differences in tannin content and extractability in several grape cultivars and their corresponding wines (Kovac, *et al.*, 1995; Harbertson, *et al.*, 2008; Mattivi, *et al.*, 2009). Grape and wine condensed tannins can be classified according to their mean degree of polymerisation (mDP): oligomers (2<mDP<10) and polymers (>10mDP). Proanthocyanidins (PAs) or condensed tannins (CTs) are the reaction product of polymerisation of different flavan-3-ol elementary units linked by interflavanic bonds (C-C) and occasionally C-O-C bonds (Santos-Buelga & Scalbert, 2000; Monagas, *et al.*, 2005). The most common bonds, also known as B-type, are found between C4-C8 followed by C4-C6 (Monagas, *et al.*, 2005). The flavan-3-ols can also be connected with a second bond (A-type), between the C-2

from the upper subunit and the hydroxyl groups from the C5 or C7 of the lower subunit (Figure 2.3). It was shown that the different links between the monomer units affect the bitterness and astringency perception of these compounds in model wine (Peleg, *et al.*, 1999).

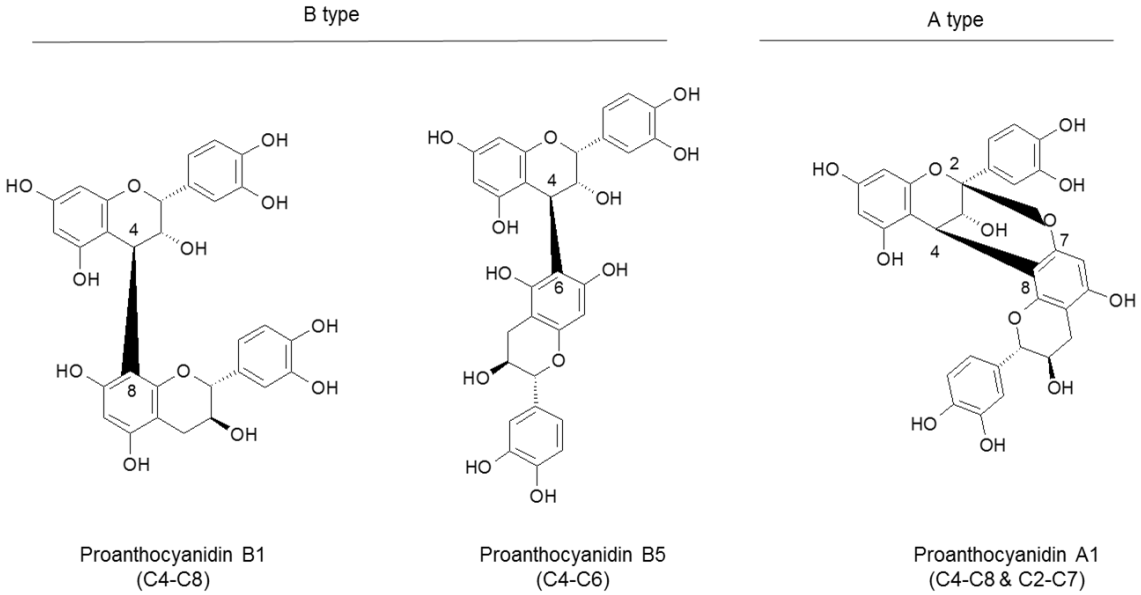

Figure 2.3. Chemical structure of the different type of interflavanic bonds between flavan-3-ols and proanthocyanidin molecules.

Grape skins are the major contributor to the wine phenolic compounds, but unlike anthocyanins, tannins are also extracted from the grape seeds (Meyer & Hernandez, 1970). Grape proanthocyanidins can be subdivided in two groups: procyanidins and prodelphinidins (Cheynier, *et al.*, 2006; Kennedy, *et al.*, 2006; Mattivi, *et al.*, 2009). Procyanidins are found in grape skins and seeds and consists of (+)-catechin and its diasteroisomer, (-)-epicatechin units (Souquet, *et al.*, 1996; Adams, 2006) (Figure 2.1C). Prodelphinidins are exclusively extracted from the berry skins and are composed of (-)-epigallocatechin and (-)-gallocatechin units (Prieur, *et al.*, 1994; Santos-Buelga & Scalbert, 2000; Aron & Kennedy, 2008; Kalili, *et al.*, 2013). The major difference between skin and seed tannins are thus epigallocatechin subunits that have been reported to only occur in grape skins (Souquet, *et al.*, 1996; Mattivi, *et al.*, 2009). Furthermore, seed derived tannins are less polymerised than those extracted from the skins (Prieur, *et al.*, 1994; Kennedy, *et al.*, 2001). Thus, seed tannins are characterised by a higher amount of monomer flavan-3-ols, especially epicatechin-gallate (Downey, *et al.*, 2003a).

Once extracted from the grapes, flavan-3-ol and low molecular weight polymeric tannins, similar to monomeric anthocyanins, are highly reactive. Tannin molecules start a chemical modification from grape crushing leading to the formation of more stable molecules throughout the entire winemaking process (Smith, *et al.*, 2015). Oxygen plays an important role in tannin polymerisation, with other phenolic compounds such as anthocyanin, or between CT creating a larger polymer. CT are more

stable molecules, but become less soluble as their mDP increases, leading to possible precipitation of these phenolic compounds. The mDP and the nature of these compounds, i.e. higher amount galloylation units, can influence the taste and mouth-feel properties of a wine (Chira, *et al.*, 2009).

Accurate phenolic measurements are essential during the winemaking process. Current routine analyses can give an estimation of the total amount of proanthocyanidins in solution. However, the complexity and wide range of phenolics, especially regarding different tannin structures, makes the analyses and further studies of these compounds complex, especially in wine media. The phenolic profile from grape to young and aged wines might differ dramatically, due to the reactivity of phenolic compounds. Current analytical techniques do not allow for the differentiation of polymeric phenolic compounds to a large extend, or the interactions with other wine components, such as the soluble cell wall components extracted into red wines during alcoholic fermentation. Recent developments in analytical chemistry can lead to a deeper understanding of the proanthocyanidin fraction of red wines (Willemse, *et al.*, 2013; Terblanche, 2017). However, less advanced, more accessible techniques are required for the general wine industry (Aleixandre-Tudó, *et al.*, 2017).

2.3 FACTORS AFFECTING GRAPE BERRY PHENOLIC COMPOSITION

2.3.1 Grape composition during ripening

Numerous structural and compositional changes occur during grape berry development. The biosynthesis and accumulation of different phenolic compounds as well as the reactions between these, will determine the grape phenolic profile and influence that of the corresponding wines. Numerous studies have been carried out investigating the impact that grape ripening has on this grape phenolic accumulation and extractability, but results are contradictory. Some of these studies have shown how the accumulation of phenolic compounds starts a few weeks pre-véraison, generally reaching maximum concentrations at véraison, followed by a decrease towards commercial harvest (Kennedy *et al.*, 2000; Downey *et al.*, 2003b; Bautista-Ortín *et al.*, 2012). Contrary to these results, some other authors have also reported a constant amount of grape polyphenols or even an increase in their concentration during ripening (Harbertson, *et al.*, 2002; Canals, *et al.*, 2005; Bordiga, *et al.*, 2011; Bindon, *et al.*, 2014a). As an example, anthocyanin and anthocyanin-derivatives accumulate in grape skins as the sugar levels increase in the fruit, showing a slight decrease in some cases before harvest (Ryan & Revilla, 2003; Fournand, *et al.*, 2006; Río Segade, *et al.*, 2008). These differences might be explained by the effects that the grape cultivar, and the vintage, have on the grape phenolic composition and concentration (Ricardo-Da-Silva, *et al.*, 1992; Obreque-Slier, *et al.*, 2010; Gil-Muñoz, *et al.*, 2011).

Since the nature of phenolic compounds differs among the grape berry structure, the ripeness has a different effect on grape skins and seeds composition and extractability. These changes and interactions between phenolic compounds and other berry components might modulate their extractability (Bindon & Kennedy, 2011; Bindon, *et al.*, 2014b). For example, at lower ripening stages, skin tannin extractability has been shown to be low, increasing during grape maturation (Llaudy, *et al.*, 2008). Hanlin (2009) reported that skin tannin concentration was the highest at fruit set, then followed by a gradual decrease until véraison, remaining relatively constant for a few weeks post-véraison (Adams, 2006; Bordiga, *et al.*, 2011). However, the increase in the cell wall porosity, occurring during ripening, can also create a more encapsulation, enhancing retention of the tannin fraction by the cell wall material (Bindon, *et al.*, 2014b). Conversely, grape seed tannin extractability decreases towards harvest (Kennedy, *et al.*, 2000; Peyrot Des Gachons & Kennedy, 2003; Llaudy, *et al.*, 2008). Grape seeds show structural modifications induced by an intensive lignification of the seeds as the berry ripens, making the inner integument of the seed less accessible (Bautista-Ortín, *et al.*, 2012). Thus, the seed tannin extractability declines over time. Kennedy, *et al.*, (2000) suggested that the browning observed on the seed coat might represent the oxidation of tannin structures during ripening.

Published results are unclear regarding the tannin complexity, concentrations of monomers such as catechin decrease, contrary to the epicatechin or epigallocatechin concentrations (Bindon & Kennedy, 2011; Bordiga, *et al.*, 2011), but no clear trend has been established for larger polymer structures. Results on the grape mDP at different ripening levels have also been contradictory, remaining relatively constant during grape ripening or fluctuating from pre-véraison towards harvest (Downey *et al.*, 2003b; Chira *et al.*, 2009; Obreque-Slier *et al.*, 2010; Bindon & Kennedy, 2011; Bautista-Ortín *et al.*, 2012).

2.3.2 Impact of different vineyard management practices

The fruit composition is influenced by climate and geographical conditions, as well as the vineyard management and viticultural practices. Thus, these factors may influence the phenolic composition of the fruit and ultimately the wine's colour, phenolic composition and sensory attributes (Downey, *et al.*, 2006; Pérez-Lamela, *et al.*, 2007). Different studies have shown the manner in which vineyard factors, *e.g.* sunlight exposure, temperature or plant water status, can modify the vine's microclimate thereby affecting berry growth and composition (Downey, *et al.*, 2006; Ristic, *et al.*, 2006; Teixeira, *et al.*, 2013). In the last few decades new canopy management practices have permitted the manipulation of vine microclimate, modifying the yield and the leaf area exposed to sunlight (Gregan, *et al.*, 2012). These training systems are an alternative to older, conventional training systems and based on the modification from the initial canopy. The leaves and bunch shade exposure will be different, due to the different grapevine canopy architecture and sun exposure, which will in turn influence grape composition (Haselgrove, *et al.*, 2000). Several studies have compared the influence

of different training systems on the vine growth and yield (Reynolds & Heuvel, 2009), as well as the grape and wine phenolic composition, and sensory attributes for both white and red wines (Wolf, *et al.*, 2003; Pérez-Lamela, *et al.*, 2007; Zoecklein, *et al.*, 2008; Mota, *et al.*, 2011; Kyraleou, *et al.*, 2015). For instance, prior to a canopy management strategy, a study of the climate parameters and soil characteristics of the vineyard should be done to determine how efficiently the training system will be to address the producer's requirements. Reduced vigour areas will lead to a more open canopy with a higher sunlight exposure, while a greater vigour will produce a more dense and closed canopy. Thus, vine vigour will also have an important impact on the berry development and on numerous of its phenolic compounds. Cortell *et al.*, (2007) showed how the amount of pigmented polymers increases in berries from low vigour zones. Higher levels of colour, anthocyanins, and bisulphite-resistant pigments were found in wines made from grapes harvested from low vigour areas (Schneider, *et al.*, 1990; Cortell, *et al.*, 2007a,b; Song, *et al.*, 2014). In addition, the interactive effects of some of these vineyard variables and ripening have also been evaluated in terms of grape and wine phenolic composition (Van Noordwyk, 2012; De Beer, 2015). Differences in the colour and phenolic composition of Shiraz wines induced by the above-mentioned factors seem to remain over time during wine ageing (De Beer *et al.*, 2017). These investigations have been conducted in the same vineyard as the present study.

2.4 FACTORS AFFECTING THE CELL WALL STRUCTURE AND COMPOSITION AND ITS ROLE IN PHENOLIC EXTRACTABILITY

As previously described, many different vineyard management practices and ripening affects the vineyard nutritional status and thus the berry development and consequently the grape chemical composition. In addition, phenolic extractability is directly linked to the structure and composition of the grape cell walls. Therefore, all the conformational and compositional changes occurring in the grape pomace, from the vineyard through the fermentation, affect the final colour and phenolic concentration in the wines. Thus, the following section will discuss the changes occurred in the grape cell wall structure and composition during the winemaking process.

2.4.1 Grape skin cell wall structure

Grape berry skin cell walls consist of a framework of proteins and polysaccharides, with the latter consisting of cellulose, hemicellulose, and a pectin-rich fraction (Lecas & Brillouet, 1994). The general plant cell wall structure is divided into three different layers: the middle lamella, the primary, and the secondary cell wall (Raven, *et al.*, 1992). The middle lamella is mainly composed of pectic compounds and structural proteins, connecting the different cells, whereas the primary and the secondary cell walls are mostly formed by groups of polysaccharides and small proteins. Differences

in the cell wall components between the layers, aside from the cell size and distribution, will be crucial to understand phenolic extractability (Vidal, *et al.*, 2001; Doco, *et al.*, 2003). The grape cell wall architecture differs in different tissue levels, with larger cells having thinner cell walls occurring in the grape pulp compared to the cell walls from the grape skin, which are thicker and more compact (refer to the illustration of the cell size in the berry skin and pulp in Figure 2.1) (Ortega-Regules, *et al.*, 2008). In addition, the amount of cell wall material (CWM) is higher in the skin cells. This cell wall morphology and amount of CWM vary between different grape cultivars (Ortega-Regules, *et al.*, 2008).

Grape phenolic compounds can be found in the vacuolar soup, as free forms, but also bound to proteins and polysaccharides (Amrani Joutei, *et al.*, 1994). Different studies have shown the binding capacity between phenolic compounds, especially tannins, and the CWM (Geny, *et al.*, 2003; Le Bourvellec, *et al.*, 2004; Bindon, *et al.*, 2010). Therefore, the CWM is a complex framework whose consequent modifications during ripening and the winemaking process will affect the porosity and the firmness of the grape berry structure (Vicens, *et al.*, 2009; Hanlin, *et al.*, 2010; Bindon, *et al.*, 2012; Bindon, *et al.*, 2014b).

To date, due to their complexity, limited information is available regarding the grape cell wall polysaccharide structure and composition. However, the recent use of new techniques has improved the understanding of the grape berry cell wall structure and their implications on the winemaking process (Gao, *et al.*, 2015, 2016; Zietsman, *et al.*, 2015). Therefore, Gao *et al.* (2016) proposed a new model for the grape berry cell walls (Figure 2.4), as well as different tissue and cell wall polymer interactions.

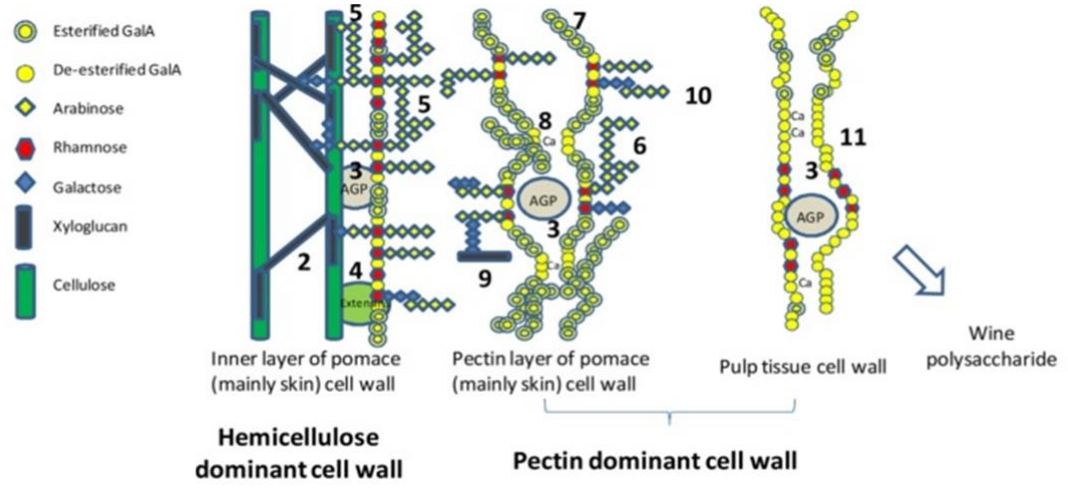

Figure 2.4. Hypothetical model describing the grape berry cell wall structure based on their polysaccharide fraction (Gao, 2016).

2.4.2 Cell wall evolution during ripening

As mentioned before, grapes undergo several reactions which modify the phenolic composition during berry development. Moreover, as the berry ripens, cell wall components evolve as a consequence of several hydrolytic and enzymatic reactions, leading to a de-esterification and depolymerisation of the polysaccharides (Nunan, *et al.*, 1998; Huang, *et al.*, 2005; Vicens, *et al.*, 2009; Zietsman, *et al.*, 2015).

In the past decades, several studies have focused on understanding this process known as berry softening. From véraison, fruit softening is linked to a degradation and solubilisation of the cell wall polysaccharides and an increase in the protein content (Nunan *et al.*, 1998; Huang *et al.*, 2005; Ortega-Regules, *et al.*, 2008; Goulao *et al.*, 2012). The loss of polysaccharides such as xyloglucan affects the berry integrity. These structural changes lead to a breakdown of the cell wall framework increasing the skin porosity and its selectivity and thus leading to greater exposure of the cell wall surface and its active binding sites (Bindon, *et al.*, 2012; Bindon, *et al.*, 2014b; Castro-López, *et al.*, 2016). The high affinity of proanthocyanidins for the CWM is well known, specifically the pectin-rich fraction, by the formation of new cross-linked bridges and hydrophobic interactions that might reduce phenolic extractability during ripening (Cadot *et al.*, 2006; Fournand *et al.*, 2006; Bindon *et al.*, 2010, 2016; Hernández-Hierro *et al.*, 2014; Ruiz-Garcia *et al.*, 2014; Springer *et al.*, 2016). The post-véraison protein increase is possibly not only related to a loss of the cell wall extensibility, but also to a proanthocyanidin retention. However, some studies have found an increase in proanthocyanidin content close to commercial harvest (Bindon, *et al.*, 2013; Bindon, *et al.*, 2014a; Quijada-Morín, *et al.*, 2015). Anthocyanin extractability has also been shown to increase during ripening, also being influenced by the changes occurring in the cell walls (Hernández-Hierro, *et al.*, 2012, 2014).

The conformational changes occurring in the grape seed during ripening are not less important. As the berry ripens, the seed turns browner and its coat becomes more impermeable, thereby becoming less extractable. This browning might be related to the oxidation and polymerisation of phenolics, which can react with other phenolics, as well as proteins or amino acids (Downey, *et al.*, 2003a; Cadot, *et al.*, 2006).

In short, grape phenolic extractability during wine making can be influenced by the grape phenolic concentration and by their interactions with the insoluble CWM, with other phenolics or self-interactions (Castro-López, *et al.*, 2016). However, the cell wall porosity can also restrict the extraction of phenolics (Le Bourvellec, *et al.*, 2012; Bindon, *et al.*, 2014b). Nevertheless, the phenolic retention by the CWM, such as the grape tannins, is influenced by the proanthocyanidin concentration, molecular mass (Renard, *et al.*, 2001; Le Bourvellec, *et al.*, 2004; Bindon, *et al.*, 2013; Bindon, *et al.*, 2014b; Bautista-Ortín, *et al.*, 2016a) and nature (Quijada-Morín *et al.,* 2015). Furthermore, the pore size can therefore restrict the penetration of larger polymers, limiting their encapsulation and hydrophobic interactions with the CWM, thereby facilitating their extraction into the wine (Hanlin, *et al.*, 2010). A recent study has described a possible competition between

anthocyanins and proanthocyanidins for the active binding in the CWM (Bautista-Ortín, *et al.*, 2016b), confirming the speculative role of grape anthocyanins in the tannin extraction (Kilmister, *et al.*, 2014). In this study (Bautista-Ortín *et al.*, 2016b), a lower tannin adsorption was seen when anthocyanins were added into a solution containing CWM and tannins. The increase in the amount of proanthocyanidins remaining in solution might be due to competition between the different phenolics for adsorption with CWM, but could also be due to certain reactions between polyphenols, such as direct condensation. Due to their relevance in the winemaking process, further investigations into the biological and chemical interactions between grape phenolics and the CWM is necessary.

2.4.3 Relevance to winemaking

The grape skin acts not only as a barrier, to protect berry integrity throughout its development, but also influence the phenolic extraction through grape cell walls, affecting the compounds' extraction from the berry to the wine during maceration (Hanlin, *et al.*, 2010). The understanding of the cell wall composition and structural changes during ripening, at harvest and during the winemaking process is very important, especially in red wines, as these conformational changes ensure the extraction of desirable compounds in the wines. Several studies have shown how the phenolic content of red wines is affected by temperature and different winemaking techniques such as cold maceration, skin contact time or the addition of commercial preparations (Zimman, *et al.*, 2002; Sacchi, *et al.*, 2005; Favre, *et al.*, 2014; Lerno, *et al.*, 2015; Smith, *et al.*, 2015). In the past few years various research groups have focused on how various winemaking procedures and particularly the addition of different maceration enzymes, influence the cell wall degradation and the subsequent wine chemical composition (Ducasse, *et al.*, 2010; Apolinar-Valiente, *et al.*, 2015; Gao, *et al.*, 2015; Río Segade, *et al.*, 2015; Zietsman, *et al.*, 2015).

The addition of commercial enzymes helps to accomplish further cell wall de-pectination and enhances the release of compounds such as sugars, organic acids or phenolic compounds into the wine (Romero-Cascales, *et al.*, 2005; Sacchi, *et al.*, 2005; Gao, *et al.*, 2015). Zietsman *et al.* (2015) assessed the impact of commercial enzymes on grape polysaccharides at two ripening levels of Pinotage grapes: ripe (23ºBrix) and overripe grapes (27ºBrix). The authors describe a greater effect of the commercial enzymes on the ripe berries, as their skins were naturally less depolymerised. However, little is known about the interactions between the CWM and specific phenolic compounds and therefore the way the enzymes preparations facilitate their release into wine. Albeit influenced by the CWM, the tissue location might also be a determinant factor in extracting different polyphenols (Quijada-Morín, *et al.*, 2015).

After crushing, soluble cell wall polysaccharides stay in suspension in the grape must and the grape tannins might get bound to the polysaccharides from different tissues or to the yeast cell walls (Gil Cortiella & Peña-Neira, 2017; Watrelot, *et al.*, 2017). Moreover, this soluble pectin content will

increase as the cell wall deconstruction occurs during the alcoholic fermentation (Guadalupe & Ayestarán, 2007; Gao, *et al.*, 2015). The high affinity between certain phenolic compounds and CWM can lead to a retention phenomenon known as adsorption (Ruiz-Garcia *et al.*, 2014; Bindon *et al.*, 2016; Castro-López *et al.*, 2016). Therefore, the presence of certain polysaccharides in wine can enhance or inhibit tannin aggregation (Poncet-Legrand, *et al.*, 2007). This adsorption is favoured by the molecular mass, as well as the galloylation units, of the wine proanthocyanidins (Bautista-Ortín, *et al.*, 2016a). Recent studies have been published regarding the adsorption-desorption phenomena. The additions of commercial enzymes might not only affect the phenolics extraction from grape to wine, but also lowers the adsorption of the extracted proanthocyanidins with the suspended CWM (Bautista-Ortín, *et al.*, 2016a; Castro-López, *et al.*, 2016). However, the type of interactions occurring at different stages of the alcoholic fermentations remain uncertain.

All things considered, the wine proteins, polysaccharides, and polyphenol content play an essential role in red winemaking. They are important not only because of their impact on the sensory properties of the wine, by reducing astringency (Vidal, *et al.*, 2004a,b; McRae & Kennedy, 2011; Quijada-Morín *et al.*, 2014), but also on the colour stability and the subsequent ageing potential. Likewise, these factors must to be taken into account when decisions like fining or clarification are taken in a cellar.

2.5 EVOLUTION OF PHENOLICS COMPOUNDS DURING RED WINE VINIFICATION AND AGEING

2.5.1 Reactions involving anthocyanins and effect on colour

As previously mentioned, elucidating the mechanisms driving anthocyanins degradation and the formation of colour pigments during the winemaking process is essential. The contribution of these compounds to the colour of the wines is highly important, as the primary wine colour perception plays an important role in the quality of a red wine.

Grape and wine anthocyanin levels can vary depending on the cultivar (Romero-Cascales, *et al.*, 2005; Río Segade, *et al.*, 2009), the grape growing conditions, and the winemaking practices (Sacchi, *et al.*, 2005). Grape pigments become more extractable during ripening. Ethanol content leads to a higher anthocyanin and proanthocyanidin extraction, contrary to its negative impact on copigmentation (Hermosín-Gutiérrez, 2003), and thus a greater alcohol potential in wines made from riper berries might explain the increase in colour extraction (Canals, *et al.*, 2005; Casassa, *et al.*, 2009).

Anthocyanin concentrations reach a maximum relatively early during alcoholic fermentation, but decrease towards the end of it (Ribéreau-Gayon, *et al.*, 2006). After this, a decrease of the monomeric forms occurs over time, especially the acylated forms. By the end of the alcoholic

fermentation around 25% of the monomeric forms have already been polymerised, and this increases to around 40% after a year (Monagas et al. 2005; He et al. 2012a; He et al. 2012 b). This decrease in monomeric anthocyanin concentrations is due to the formation of new and more stable pigments by chemical interactions other phenolic compounds, by direct condensation or with the formation of "bridges" mediated by acetaldehyde (Cheynier, *et al.*, 2006; He, *et al.*, 2012b). These compounds show a better resistance to changes in pH and the bleaching effects of SO_2 and thus, a greater colour stability is achieved (García-Puente Rivas, *et al.*, 2006).

Some studies show how around the 50-70% of colour components in an aged wine is due to anthocyanins derived pigments (Somers, 1971; Dipalmo, *et al.*, 2016). Oxygen plays an important role in the reactions involved in the formation of these new polymers as chemical oxidative processes take place in the wines over time. The role of oxygen will be furtherly discussed at a later stage. Additionally, other anthocyanin derived pigments can be formed by condensation reactions between anthocyanins and substrates in the wines such as vinylphenols (pinotin), vinylflavonols or pyruvic acid (vitisins). Thus, the formation of some of these polymer forms, such as the polymeric pigments, is influenced by the wine proanthocyanidin concentration and composition (Timberlake & Bridle, 1977; Bindon, *et al.*, 2014c). However, wine pyroanthocyanins also include pigments such as vitisins A or B, formed by reactions occurring between free anthocyanins or with other yeast by-products at different stages during ageing (Alcalde-Eon, *et al.*, 2006; He, *et al.*, 2012b). The acidic conditions, oxygen and temperature can influence the occurrence of these reactions. Thus, wine pH and the presence of oxygen can favour the reactions mediated by the acetaldehyde. However, cooler temperatures can retard these reactions, as well as limit the formation of excessively larger polymers (He, *et al.*, 2012b).

With the aim of extracting a higher anthocyanin content , a large number of studies have focused on wine colour during winemaking, using either pre-fermentative, fermentative, or post-fermentative extraction techniques (González-Neves, *et al.*, 2015). The most common practices investigated include the use of different maceration times (Zimman, *et al.*, 2002; Marais, 2003a; Casassa & Harbertson, 2014), temperature regimes, increasing the pomace/juice ratio (Marais, 2003b; Bautista-Ortín *et al.*, 2007) or addition of specific products to improve the phenolic extraction such as pectolytic enzymes or oenological tannins (Sacchi, *et al.*, 2005; Soto-Vazquez, *et al.*, 2010; Casassa & Harbertson, 2014). Some of these techniques also target a greater tannin extraction as the proanthocyanidin:anthocyanin ratio also contribute to the formation rate of polymeric pigments over time (Timberlake & Bridle, 1977; Bindon, *et al.*, 2014c; Picariello, *et al.*, 2017).

Cold soaking or cold maceration is a pre-fermentation technique widely used in the wine industry to increase anthocyanin concentration when applied to red must. Nevertheless, cold maceration does not favour the extraction of proanthocyanidins and thus, does not improve the formation of polymeric pigments (Sacchi, *et al.*, 2005; Soto-Vazquez, *et al.*, 2010; Mihnea, *et al.*, 2016). Some studies have found little impact of the cold maceration on the final anthocyanin concentration in the wine (Parenti,

et al., 2004). However, the addition of pectolytic enzymes during cold soaking seems to positively correlate with anthocyanin, copigments and polymeric pigments extraction (Ortega-Heras *et al.*, 2012).

In the same way, yeast selection can also be a determining factor in the wine colour (Morata, *et al.*, 2016). It is well known that anthocyanins can react with certain yeast cell wall components and precipitate, while some yeast might increase acetaldehyde production and thus polymer formation (Hayasaka, *et al.*, 2007).

In general, longer skin contact times during fermentation lead to increased anthocyanin and polymeric pigments concentrations after a year (Auw, *et al.*, 1996; Gómez-Plaza & Cano-López, 2011). Contrary to this, other studies have shown a loss of anthocyanin content during extended maceration, but an increase on the solubility and extractability of tannins (Scudamore-Smith, *et al.*, 1990; Casassa, *et al.*, 2009) , as the seed tannins are extracted even after fermentation (Llaudy, *et al.*, 2008). However, the affinity between certain phenolic compounds and the CWM might also lead to a rebinding effect during extended skin contact (Bindon, *et al.*, 2010; Hanlin, *et al.*, 2010).

Colour changes can also occur during malolactic fermentation (Burns & Osborne, 2013), while certain ageing conditions such as temperature, SO_2, and O_2 levels or closure can also have an influence on the formation of these polymeric forms and colour stability (Alcalde-Eon, *et al.*, 2006; García-Puente Rivas, *et al.*, 2006; Hopfer, *et al.*, 2013; Arapitsas, *et al.*, 2014). The general trend found during ageing involves a decrease in the free and SO_2-bound pigments with an increase in the polymeric forms, especially large polymeric pigments (LPP) (Avizcuri, *et al.*, 2016). Maturation at cooler temperatures can retard the formation of new polymeric pigments (He *et al*. 2012b). Araptisas *et al*. (2014) compared the colour evolution of different commercial wines in cellar and “house” conditions, taking into account differences between room temperature and humidity. The change from purple to brick colour as the wine age is directly linked with the formation of pigments over time. After 2 years, the pyroanthocyanins pigments represent around 50% of the colour in red wines (Dipalmo, *et al.*, 2016). However, the concentration of polymeric pigments has been found to decrease over time in wines aged for a long time (McRae, *et al.*, 2012), which is probably due to precipitation.

2.5.2 Reactions involving tannins

As described above (section 2.2.3), wine tannins with different structures, as well as mean degree of polymerisation (mDP), can be extracted from the grape skin and grape seeds. Studies have shown the different extraction of these compounds in different solvent mixtures leading to a better understanding of their extraction and contribution of skin and seed tannins to the final wine (González-Manzano, *et al.*, 2004; Mattivi, *et al.*, 2009; Downey & Hanlin, 2010; Bindon, *et al.*, 2014b;

Bosso, *et al.*, 2016). Apart from the grape tannins, hydrolysable tannins can be extracted from the oak barrels during maturation, further influencing the chemical and sensory properties of the wine.

Contrary to anthocyanins that are extracted during the early stages of the fermentation, tannin levels increase linearly during the course of fermentation (Ribéreau-Gayon, *et al.*, 2006; Kennedy, 2008). However, skin tannins follow a sigmoidal extraction reaching a plateau (Peyrot Des Gachons & Kennedy, 2003; Cerpa-Calderón & Kennedy, 2008), while the seed tannins show a linear extraction (Hernández-Jiménez, *et al.*, 2012) as the seed coat needs hydration to become extractable (Cadot, *et al.*, 2006). A recent study by Yacco *et al.*, (2016) shows an increase in the tannin size during maceration, reaching a plateau and decreasing towards the end of the fermentation. An increase in (-)-epicatechin-3-*O*-gallate concentrations, with a decrease in (-)-epigallocatechin concentrations with the maceration time show the importance of seed phenolics as the fermentation progresses. As a result, the authors hypothesised not only about the type or concentration of the tannins being extracted during fermentation, but also their reactivity. The interactions and precipitation of these compounds with certain yeast cell walls or other grape compounds might explain the smaller wine tannin size at the end of fermentation (Mekoue Nguela, *et al.*, 2016).

From crushing, grape tannins are chemically modified and undergo continual evolution over time leading to the formation of new and more stable molecules (Smith, *et al.*, 2015). Reactions such as polymerisation, condensation with anthocyanins, or the formation of complexes between tannins and proteins or polysaccharides depends on the type of tannins and their concentration in the wine. The ageing potential of a wine seems to be influenced by the anthocyanin/tannin ratio (Singleton & Trousdale, 1992; Ribéreau-Gayon, *et al.*, 2006; Pascual, *et al.*, 2016; Picariello, *et al.*, 2017), but this needs further clarification.

Several winemaking techniques aim to control the final wine tannin concentration (Kovac, *et al.*, 1990; Canals, *et al.*, 2005; Sacchi, *et al.*, 2005; Smith, *et al.*, 2015). An increase in wine tannin content may favour the formation of pigmented tannins and therefore increase wine colour stability. However, due to their role in bitter taste and astringent mouth-feel, the winemaker might be interested in limiting tannin extraction to avoid an excessive bitterness or astringency in the final wines. As in the case of anthocyanins, the use of different maceration times, temperature, the yeast selection (Bautista-Ortín, *et al.*, 2007) or the additions of enzymes (Fernández, *et al.*, 2015) or exogenous tannins (Harbertson, *et al.*, 2012; Versari, *et al.*, 2013) may play an important role in the tannin extractability (Favre, *et al.*, 2014). As an example, increasing the maceration time has increased the final wine tannin content, as the seed derived tannins are known to become more extractable (Busse-Valverde, *et al.*, 2011; Hernández-Jiménez, *et al.*, 2012; Casassa, 2017). However, some studies have shown a limited effect (Álvarez, *et al.*, 2006) and even a decline in the tannin levels with longer maceration, probably due to adsorption or precipitation (Yacco, *et al.*, 2016).

On the other hand, different techniques are applied to reduce the amount of tannins, especially grape seed tannins, by adsorption, precipitation, or sedimentation. The use of fining agents that will bind the solubilised tannins (Maury, *et al.*, 2001; Poncet-Legrand, *et al.*, 2007; Oberholster, *et al.*, 2013) or specific yeast strains or yeast lees with a high affinity for the tannins (Rodrigues, *et al.*, 2013) has been reported. Early seed removal during fermentation has also been shown to reduce the bitterness in the final wine (Canals, *et al.*, 2008; Lee, *et al.*, 2008; Guaita, *et al.*, 2017).

Different wine ageing conditions such as temperature, humidity, and type of barrel will also influence the wine composition (Hopfer, *et al.*, 2013; Arapitsas, *et al.*, 2014, 2016; Bimpilas, *et al.*, 2015). Short term storage leads to the formation of larger polymers and to an increase in the proanthocyanidins concentrations (García-Falcón, *et al.*, 2007). However, Chira *et al*., (2012) analysed the tannin content and mDP of several vintages of Bordeaux wine. The authors concluded that the oldest wines, from the late 1970s, had a lower amount of tannins and a mDP of 1.8 while the youngest, (2002 to 2005) showed values of mDP between 5.5 and 7.6. Changes occurring in the tannin structure during ageing might also change their solubility, leading to precipitation (Smith, *et al.*, 2015; Casassa, 2017).

The formation of new tannins structures by reactions such as condensation, mediated by acetaldehydes, polymerisation, or oxidation can thus take place during wine ageing (Monagas, *et al.*, 2005; Cheynier, *et al.*, 2006). A number of these reactions depends on the presence of oxygen during barrel and bottle ageing, which will be discussed in the following section.

2.6 WINE OXIDATION: EFFECT ON PHENOLICS

The entire winemaking process, and especially wine maturation, is influenced by a large number of chemical reactions mediated by oxygen. Both, enzymatic and chemical reactions are mediated by the presence of oxygen in the juice and wine respectively. At lower doses, the impact of oxygen can be beneficial, increasing colour stability and improving wine taste and structure (Waterhouse & Laurie, 2006; Gambuti, *et al.*, 2013). Thus, in the last decade, the controlled addition of oxygen and its impact on the colour and phenolic composition of red wines has been under investigation.

When dissolved in wine, oxygen can react with wine polyphenols, but also with other substrates such as ascorbic acid, ethanol, or tartaric acid (Danilewicz, 2003; Du Toit, *et al.*, 2006a). As a consequence, a series of oxidation secondary products such as quinones, acetaldehyde, and ketoacids are formed (Waterhouse & Laurie, 2006). Primarily, the oxidation of polyphenols results in the formation of quinones and H_2O_2 catalysed by metals such as iron and copper in wine (Figure 2.5). Subsequently, these quinones react with other molecules, especially with electron-rich A-ring flavonols forming new bonds between two phenolic compounds (Danilewicz, 2003, 2007). The

formation of a larger tannin polymer occurs when both nucleophiles reacting with each other are already part of condensed tannins (Atanasova, *et al.*, 2002; Waterhouse & Laurie, 2006; Gómez-Plaza & Cano-López, 2011). Recent studies have reported the identification of new oxidation biomarkers (Arapitsas, *et al.*, 2012). For the first time the hypothetical reaction of a gallic acid quinone with the A-ring of a flavonol has been recently shown (Mouls & Fulcrand, 2015). The addition of SO_2 acts by removing the H_2O_2 and recycling the quinones back to its phenol form (Du Toit, 2006). The sulphur dioxide concentration and its forms found in wine, free or bound, are also important for the efficiency of inhibiting wine oxidation (Tao, *et al.*, 2007; Carrascon, *et al.*, 2015; Ferreira, *et al.*, 2015; Danilewicz, 2016; Waterhouse, *et al.*, 2016).

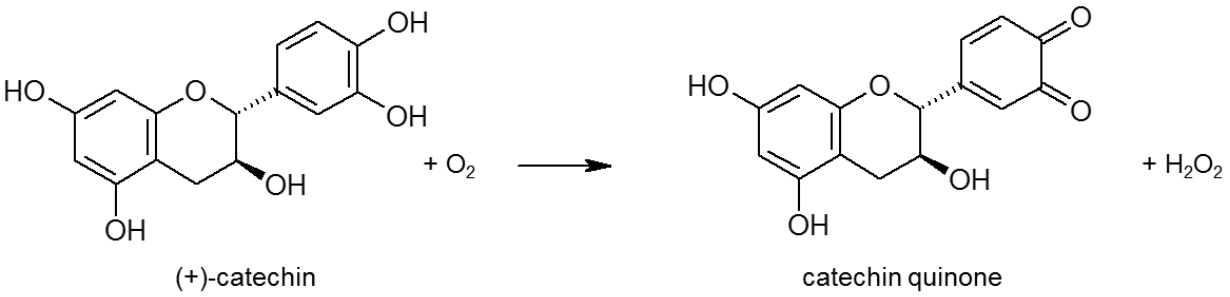

Figure 2.5. Oxidation of (+)-catechin in wine (Danilewicz, 2003).

Equally important is the role played by acetaldehyde in the formation of new oxidation derived pigments as described by Timberlake & Bridle (1976) and later on shown in red wine by Fulcrand *et al.*, (1996). Formed as an oxidation by-product from ethanol, the acetaldehyde reacts first with the flavanol at position C6 or C8, and can combine with other flavan-3-ols, anthocyanins or procyanidins at position C8, thereby creating ethyl-linked products (Figure 2.6) (Timberlake & Bridle, 1976). Additionally, it can participate in the formation of vitisins by direct reactions with malvidin-3-glucoside (Bakker, *et al.*, 1997) or new ethyl-bridges between anthocyanins (Atanasova, *et al.*, 2002; Waterhouse & Laurie, 2006). The high alcohol content in wines allows for the formation of acetaldehyde as an oxidation by-product, even in the presence of SO_2 (Dallas, *et al.*, 1996) and thus, the formation of these new compounds lead to greater colour stability. The use of sulphur dioxide as an antioxidant will also be relevant to the final wine composition over time (Danilewicz, 2007, 2016; Waterhouse, *et al.*, 2016). Theoretically 4 mg/L of SO_2 react with 1 mg/L of O_2 (Du Toit, *et al.*, 2006a) in wine. However, an excessive concentration of SO_2 has a suppressive effect on the formation of polymeric pigments (Tao, *et al.*, 2007). Some of these derived pigments are known to be more resistant to the bleaching effect of SO_2. Ketoacids, especially pyruvic acid, are also involved in the formation of vitisins and pyroanthocyanins (Waterhouse & Laurie, 2006).

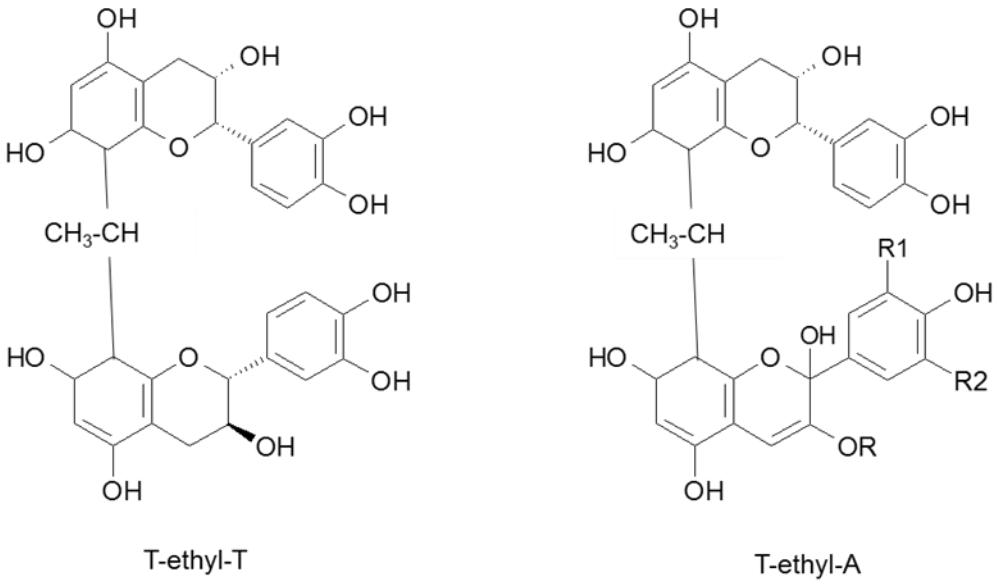

Figure 2.6. Condensation products mediated by the presence of acetaldehyde. Tannin-ethyl-tannin and tannin-ethyl-anthocyanin molecules.

Barrel ageing comes at a high cost to the winemaking industry. As a consequence of this, a lot of research has been done on the impact of cheaper alternatives to barrel ageing. Methods such as wine ageing on yeast lees or the use of microoxygenation (Gómez-Plaza & Cano-López, 2011) at different stages of the winemaking have evaluated the impact on these methods on wine phenolics (Durner, *et al.*, 2010; McRae, *et al.*, 2015; Waterhouse, *et al.*, 2016). However, oxygen intake should be controlled to avoid an excessive production of acetaldehyde and a subsequent over-polymerisation and precipitation (Castellari, *et al.*, 2000; Du Toit, *et al.*, 2006a; Ribéreau-Gayon, *et al.*, 2006), as well as the production of characteristic off-flavours and aromas related to oxidation. Microoxygenation has thus been widely studied by different authors trying to mimic the effect of barrel ageing (Atanasova, *et al.*, 2002; Castellari, *et al.*, 2004; Sánchez-Iglesias, *et al.*, 2009; Geldenhuys, *et al.*, 2012), but Cano-López *et al.*, (2010) was the first group to simultaneously compare both processes, microoxygenation and barrel ageing. The authors observed similar results after 3 months but dissimilarities after 6 months, proving the role of the wood phenolics extracted into the wine. Oxygen dosages used in microoxygenation were also often based on the amount of oxygen previously thought to diffuse into wine, which has been shown to be different with recent research (Del Alamo-Sanza & Nevares, 2014). Different studies have shown a clear impact of microoxygenation on the wine colour and phenolic composition (Quaglieri, *et al.*, 2017). Thus, microoxygenation modifies the wine chromatic profile by a decrease in the wine monomeric anthocyanin, favouring the formation of polymeric pigments and ethyl-bridged compounds (Tao, *et al.*, 2007; Cano-López, *et al.*, 2008; Cejudo-Bastante, *et al.*, 2011; Gambuti, *et al.*, 2017). These polymers, more resistant to the bleaching effect from the SO_2, contribute to the colour stability. Additionally, this technique also promotes the formation of larger proanthocyanidin polymers (Du

Toit, *et al.*, 2006b; Anli & Cavuldak, 2012). In a similar way the hydrolysable tannins extracted from the oak are also affected by oxidative processes and they should be considered as an active part of the final wine chemical and sensory properties (Perez-Prieto, *et al.*, 2003; Michel, *et al.*, 2016).

The effect of oxygen depends on the wine phenolic concentration and composition, thereby influencing the evolution of the wine's colour and phenolic composition (Jorgensen, *et al.*, 2004; Du Toit, *et al.*, 2006a; Jordão, *et al.*, 2006). In essence, skin procyanidins seem to degrade faster than the phenolics extracted from the seeds (Jorgensen, *et al.*, 2004; McRae, *et al.*, 2015) in the presence of oxygen. Furthermore, the ratio between wine anthocyanins and tannins also influence oxidative polymerisation reactions over time (Picariello, *et al.*, 2017; Carrascón, *et al.*, 2018).

In addition, oxygen transfer rate and timing are also determining factors in the wine oxidative process. Singleton (1987) proposed that a slower oxidation was better as it permits a better polymerisation whereas a faster oxidation lead to the conversion of all the phenols into quinones, leading to an oxidised wine. Similarly, the loss of free SO_2, flavan-3-ol monomers and the conversion rate of anthocyanins in new derived pigments was highly influenced by the oxygen levels (Wirth, *et al.*, 2010). Similar results were found by other research groups (Castellari, *et al.*, 2000; Arapitsas, *et al.*, 2012; Geldenhuys, *et al.*, 2012; Gambuti, *et al.*, 2013, 2017). Moreover, an early oxygen addition to the wine might lead to lower tannin levels in the final wine (McRae, *et al.*, 2015). This could be due to the premature formation of new tannin derived pigments, thereby limiting the tannin polymerisation (Arapitsas, *et al.*, 2012). Another possibility might be that more oxidised tannins are less susceptible to depolymerisation, becoming less soluble and thus precipitating. Stronger interaction can be formed between oxidised tannins in the grape solids (McRae, *et al.*, 2015). A lower oxygen effect of microoxygenation was observed when applied after malolactic fermentation (Durner, *et al.*, 2010; Gómez-Plaza & Cano-López, 2011; Arapitsas, *et al.*, 2012).

2.7 SENSORY ROLE OF PHENOLICS IN WINE

All the variables and conditions (the vineyard management, winemaking practices and oxygen exposure or storage conditions) previously described can affect the evolution of a wine and its corresponding sensorial perception (Pérez-Magariño & González-San José, 2006; Chira *et al.*, 2009; Gambuti *et al.*, 2013; Arapitsas *et al.*, 2014; Sáenz-Navajas *et al.*, 2014; Smith *et al.*, 2015).

Wine quality is a complex term to define as it is equally influenced by several intrinsic (Verdú Jover *et al.*, 2004; Sáenz-Navajas, *et al.*, 2013) and extrinsic factors (Sáenz-Navajas, *et al.*, 2013; Lick, *et al.*, 2017). Phenolic compounds together with other wine compounds greatly contribute to the formation of a specific wine matrix which can be linked to wine quality with it ultimately being

accepted or rejected by the consumer (Sáenz-Navajas, *et al.*, 2012; Baker & Ross, 2014; Sáenz-Navajas *et al.*, 2015; Watrelot *et al.*, 2016).

As previously described, the formation of new polymeric phenolic structures in red wine leads to better colour stability, important to the limpidity and the primary visual perception of a wine (Sarni-Manchado *et al.*, 1996; He *et al.*, 2012b; Alcalde-Eon *et al.*, 2014). Additionally, a wide range of polyphenols contribute to the taste and mouth-feel of red wines, with bitterness and astringency being two of the main attributes. Differences in phenolics concentration, as well as in their chemical structure, mDP and galloylation have an impact on the intensity of astringency/bitterness (Peleg, *et al.*, 1999; Cliff, *et al.*, 2007; Ma, *et al.*, 2014).

Bitterness is a gustatory sense strongly associated with the interaction between lower molecular weight polyphenols and the specific bitter taste receptors located at the back of the tongue (Gawel, 1998; Peleg *et al.*, 1999; Hufnagel & Hofmann, 2008; Gonzalo-Diago *et al.*, 2014; Ma *et al.*, 2014). The bitter sensation seems to be negatively correlated with the mDP (Arnold & Noble, 1978; Pascual, *et al.*, 2016). Flavan-3-ols are major contributors to the bitterness in red wines, especially (-)-epicatechin, which is more bitter and has a longer duration than (+)-catechin (Rossi & Singleton, 1966; Kallithraka & Bakker, 1997; Peleg, *et al.*, 1999). In essence, phenolic compounds extracted from the grape seeds, characterised by a lower mDP and higher percentage of galloylation units, are perceived as more bitter compared to skin or stem procyanidins.

On the other hand, astringency is a mouthfeel sensation based on the interaction between the different wine proanthocyanidins with the salivary proteins (Cala, *et al.*, 2010; Dinnella, *et al.*, 2010; McRae & Kennedy, 2011; Ferrer-Gallego, *et al.*, 2015). It is generally described with terms such as “puckering” or “dryness” but it also contributes to the “fullness” and texture of the wine. Contrarily to bitterness, astringency is positively correlated with the mDP and molecular weight (Peleg, *et al.*, 1999; Hufnagel & Hofmann, 2008; Chira, *et al.*, 2009) and thus primarily driven by the presence of polymeric proanthocyanidins in the wine (Gawel, 1998; Sáenz-Navajas, *et al.*, 2012).

The perception of astringency can change over time as it is generally accepted that wines become softer during ageing (McRae, *et al.*, 2012). The decrease in astringency perception of a wine can be associated with a decline in tannin concentration over time, due to the fining effect some soluble proteins or polysaccharides have on phenolics in wine, polymerisation reactions and the subsequent precipitation, or by depolymerisation (Cheynier, *et al.*, 2006; McRae & Kennedy, 2011). The association with anthocyanins or newly derived pigments can also be associated with a “softening” of the wine (Brossaud, *et al.*, 2000; Ribéreau-Gayon, *et al.*, 2006; Oberholster, *et al.*, 2009).

In recent years, some research groups have focused their work on analysing the contribution of specific phenolic compounds to bitterness and astringency in wine (Ferrer-Gallego *et al.*, 2010, 2014; Sáenz-Navajas *et al.*, 2010; Sáenz-Navajas, *et al.*, 2012; Gonzalo-Diago *et al.*, 2014; Sáenz-Navajas *et al.*, 2017) and possible synergistic effects (Sáenz-Navajas, *et al.*, 2012). In the latest chemo-

sensory study by Sáenz-Navajas *et al.*, (2017) wines were fractionated and subsequently sensorialy evaluated. The results show the surprising impact of oligomeric anthocyanins to the bitterness, dryness and persistency of the wine. Additionally, the authors showed different reactivity with the salivary proteins from flavonoid fractions with similar mDP, agreeing with the hypotheses of intramolecular bonding changes that reduce the structural flexibility of the polymer to interact with the salivary proteins (McRae & Kennedy, 2011).

Furthermore the temperature, pH, and ethanol content of a wine also play a significant role in the wine taste and mouth-feel perception (Villamor, *et al.*, 2013). A decrease in pH is generally associated with an increase between the tannin-protein interactions (Kallithraka *et al.*, 1997; Vidal, *et al.*, 2004; Fontoin *et al.*, 2008), thereby increasing the astringency. The ethanol content is negatively correlated with astringency, as it can modify phenolic solubility leading to tannin precipitation, and also contributing to an increase in the bitter taste (Fontoin, *et al.*, 2008; McRae & Kennedy, 2011). The perception of astringency is thought to decrease over the course of time, partly due to loss of the insoluble polymers forming during ageing (Gambuti, *et al.*, 2013). However, according to our knowledge, there is only one published study describing the evolution of wine's bitterness and astringency perception during storage (Sun, *et al.*, 2011).

2.8. CONCLUSION

The importance of grape phenolics in the colour and phenolic composition of their corresponding wines is well known. The relevance of the grape berry cell wall structure and composition in the extractability of these compounds from the grapes into the wines is also beginning to be understood. However, limited information is available about the interactions between phenolic compounds and grape polysaccharides and structural proteins. A better understanding on these interactions, in fresh grapes and fermented pomace, can generate valuable information for the wine industry with regards to the release of specific phenolic compounds. The initial phenolic composition, in young wines, will probably also influence subsequent reactions and the phenolic stability over time. The role that oxygen plays in this development also needs further attention. However, due to the complexity of polymeric tannin and anthocyanin derived moieties, the development of novel analytical techniques is needed to carry out further studies.

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Chapter 3

Research results

Phenolic and sensorial evolution during bottle storage of Shiraz wines with different initial phenolic profiles

Chapter 3: Phenolic and sensorial evolution during bottle storage of Shiraz wines with different initial phenolic profiles.

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ABSTRACT

The aim of the research was to evaluate the colour and phenolic evolution of Shiraz red wines made from the same vineyard, but with different initial phenolic profiles. Several variables were initially evaluated in the first vintage, but grape ripeness was shown to be the most determining factor on the taste and mouth-feel of the wines. In the second vintage wines made from four different ripening levels were aged up to 18 months and periodically analysed (every six months) during this time. The results showed how the nature and concentration of phenolic compounds in young wines can influence the wine evolution during ageing. The colour and phenolic composition between wines made from grapes from the highest sugar level and the rest of the treatments were similar after completing the alcoholic fermentation (AF). However, the differences between the wines, especially in the polymeric fraction, became more noticeable in the course of time. From the results, a larger amount of polymeric forms was found in the wines made from the ripest berries, and subsequently a larger formation of polymeric pigments. These differences in the wines' chemistry also influenced in the taste and mouth-feel evaluation of the wines.

3.1. INTRODUCTION

The use of different winemaking procedures (Marais, 2003; Smith, *et al.*, 2015), as well as the environmental factors and different vineyard management techniques (Wolf, *et al.*, 2003), are well known parameters that can directly or indirectly influence the colour, taste and mouth-feel of a red wine.

The colour in young red wine is mainly due to free anthocyanins, extracted from the grape skins. However, as the wine ages, the monomeric forms decrease as a consequence of the formation of new pigmented polymeric compounds (He, *et al.*, 2012a,b). Subsequently, the increase in these polymeric forms leads to greater wine colour stability. Initially, the formation of these new compounds can be explained by different reactions involving self-association or by the interaction between

anthocyanins and tannins (Somers, 1971; Singleton & Trousdale, 1992; Remy, *et al.*, 2000; Monagas, *et al.*, 2005). Hence, tannins also contribute to the wine colour and are extracted not only from the grape skins as is the case with anthocyanins, but also from the grape seeds (Prieur, *et al.*, 1994; Hernández-Jiménez, *et al.*, 2012). Wine tannins will be directly influenced by the amount of grape skin and seed tannins, as these differ in nature and polymer length, affecting their taste and mouth-feel properties (Brossaud, *et al.*, 2000). The initial grape phenolic composition is therefore of great importance to the wine producer. Different grape phenolic profiles and content between cultivars has been extensively reported by several research groups (Ryan & Revilla, 2003; Pérez-Magariño & González-SanJosé, 2004; Pérez-Lamela, *et al.*, 2007; Obreque-Slier, *et al.*, 2013). In addition to this varietal effect, the soil characteristics and different growing conditions, such as irrigation techniques or canopy management, can influence the vine nutrient status, thereby affecting the vine development and thus, the grape and its wine phenolic composition. As a relevant example to the present study, altering the canopy has been shown to change the yield and the bunch light exposure, thereby affecting berry development and the subsequent phenolic accumulation (García-Falcón, *et al.*, 2007; Reynolds & Heuvel, 2009; Río Segade, *et al.*, 2009). Similarly, the vine vigour has been shown to influence the pigment content in grapes and its corresponding wines (Cortell, *et al.*, 2007a,b).

Moreover, the harvest date and the sugar level are both crucial factors as the different groups of phenolic compounds are synthetized and accumulated at different rates during the berry ripening (Kennedy, *et al.*, 2000, 2001; Adams, 2006; Fournand, *et al.*, 2006). Likewise, the compositional and structural changes occurring in the grape cell walls will modulate the phenolic extractability (Nunan, *et al.*, 1998; Bindon & Kennedy, 2011; Cagnasso, *et al.*, 2011). Hence, many studies have analysed the impact of ripening on the phenolic composition of grapes, skins and seeds (Kennedy, *et al.*, 2000; Harbertson, *et al.*, 2002; Canals, *et al.*, 2005; Obreque-Slier, *et al.*, 2010; Bordiga, *et al.*, 2011; Gil-Muñoz, *et al.*, 2011; Asproudi, *et al.*, 2015; Quijada-Morín, *et al.*, 2016), and in young wines (Cadot, *et al.*, 2012; Bindon, *et al.*, 2014a; Pace, *et al.*, 2014). Similarly, it has been broadly discussed how the affinity of certain phenolic compounds with the cell wall material (CWM) can affect the phenolic extractability, the concentration of phenolic compounds extracted into the wine can therefore decrease during ripening (Fournand, *et al.*, 2006; Bindon, *et al.*, 2010, 2016; Hernández-Hierro, *et al.*, 2014). However, some other studies have found an increase in proanthocyanidins and other phenolics close to commercial harvest (Bindon, *et al.*, 2013; Quijada-Morín, *et al.*, 2015). These changes, altogether, will influence the initial phenolic profile and the subsequent chemistry and sensorial evolution of the wines.

From crushing, a large number of biological interactions and chemical reactions leads to a completely different phenolic profile from grapes to wines. From a chemical perspective, several direct or mediated condensation reactions between the different groups of phenolics are occurring over time (Timberlake & Bridle, 1976; Wang, *et al.*, 2003; Monagas, *et al.*, 2005). Wines experience

a general decrease in colour mainly as a consequence of the decrease in monomeric anthocyanins levels and the formation of new polymeric anthocyanin derivatives (Monagas, *et al.*, 2006). In the course of time, a decrease in flavan-3-ol is also observed (Gómez-Gallego, *et al.*, 2013) as new and larger proanthocyanidins are formed (He, *et al.*, 2008). However, this polymer length of the proanthocyanidin concentration in wines can also experience a decrease over time (McRae, *et al.*, 2012). Whether differences in colour and phenolic composition observed between different young red wines are decreasing (Pérez-Magariño & González-SanJosé, 2004) or persisting over time (De Beer, *et al.*, 2017) requires attention. Duration and storage conditions also have a very important impact on the colour and phenolic evolution of wine (Arapitsas, *et al.*, 2014).

There are different studies evaluating the impact of different vineyard treatments (Mota, *et al.*, 2011; Van Noordwyk, 2012; Song, *et al.*, 2014; De Beer, *et al.*, 2017) and ripening (Cadot, *et al.*, 2012; Bindon, *et al.*, 2014a) on the colour and phenolic composition, as well as their influence on the sensory properties, of grapes and young red wines. However, limited information is available regarding the phenolic and sensorial evolution of red wines over time made from different training systems, especially from various ripeness degrees, but from the same vineyard (Pérez-Magariño & González-SanJosé, 2004; Llaudy, *et al.*, 2006, 2008).

In the present study, the main goal was to assess the colour, phenolic and sensorial evolution of small-scale red wines made from the same vineyard, but with different initial grape phenolic levels. Our target was to highlight the importance of different phenolic profiles in young wines and how these evolve during wine aging. For that, a sensorial descriptive analysis was also carried during two consecutive seasons, 2014 and 2015, as well as phenolic profiles followed over time. The first harvest season, 2014, was used as an exploratory study to evaluate the effect of certain vineyard related factors on these. From the results obtained in 2014, the following harvest season (2015) focused on the impact of grape ripeness on the grape and wine phenolic content and their subsequent evolution over time. These results generate new data to supplement the little information available in literature on the phenolic and senorial evolution of red wines made from different initial phenolic levels.

3.2. MATERIALS AND METHODS

3.2.1. Vineyard characteristics

The present study was conducted during two consecutive harvest seasons (2014 and 2015) at Welgevallen experimental farm of the Department of Viticulture and Oenology of Stellenbosch University (GPS coordinates: 33°56'25.0"S 18°51'56.4"E), a well-characterised vineyard with a North-South row direction.

As mentioned, the first season was an exploratory study, thus, in 2014, Shiraz grapes (SH9C clone) on a 101-14Mgt rootstock were harvested from two different training systems, Vertical Shoot Positioning (VPS) and Smart-Dyson (SD). Additionally, the study included two other vineyard parameters, vine vigour and grape ripening, which might affect the grape phenolic composition at harvest and therefore the wine chemistry. In 2014, as described in Table 3.1, two different training systems were thus studied, (VSP Figure 3.1A) and SD (Figure 3.1B), in two different vigour areas. The vine vigour was visually assesed dividing the vineyard block in two zones: high vigour (HV) and low vigour (LV). Part of the VSP training system Shiraz had previously been converted (during the 2011/2012 growing season) into SD training system. Each of the four treatments were harvested at two different grape maturity stages (ripe -R- and overripe -OR). A similar amount of vines were randomly harvested per row within the vineyard block for each training system, vigour and ripening.

In 2015 we decided to focuss on more ripening levels and due to logitical limitations decided to only use grapes harvested at four grape ripening levels from the VSP training system (low vigour area) (Table 3.1).

Table 3.1. Experimental layout. In 2014, grapes were harvested from two different training system, vigour zones and grape ripening levels. The following season (2015), grapes were harvested at four ripening levels. R= Ripe, OR= over ripe.

Treatments	2014	2015
Training system	VSP , SD	VSP
Vigour	HV , LV	LV
Ripening	R, OR	21°Brix, 23°Brix, 24°Brix & 25°Brix

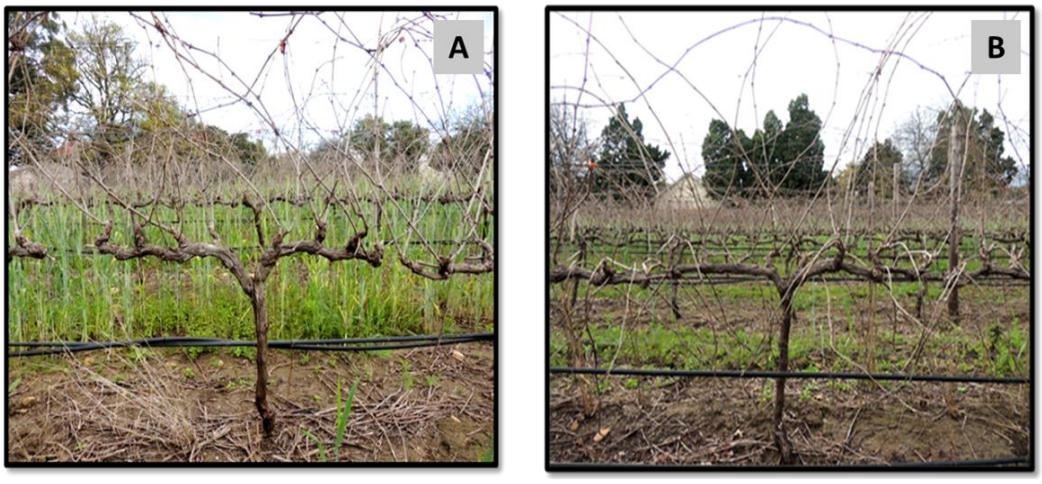

Figure 3.1. Training systems used for the study in 2014 season. A) Vertical Shoot Positioning (VSP). B) Smart-Dyson (SD).

3.2.2. Winemaking procedures

All the small-scale wines were made in triplicate following the standard winemaking procedure at the experimental cellar of the Department of Viticulture and Oenology at Stellenbosch University. In order to reduce vineyard variability, grape bunches from the same vineyard treatment were mixed in the cellar and subsequently separated into 40 kg triplicates used per fermentation. Prior to yeast incoculation, 30 mg/L SO_2 were added to the destemmed grapes and the total acidity was adjusted to 6.0 g/L using tartaric acid (natural L-(+)-tartaric). All musts were co-inoculated with 0.3 g/L of *Saccharomyces cerevisiae* D21 (Lallemand) and 24 hours later, once alcoholic fermentation (AF) had started, with 0.01 g/L *Oenococcus oeni* VP41 (Lallemand). All the alcoholic fermentations were carried out in plastic buckets at 25ºC. Punch-downs were manually performed three times per day and 0.3 g/L of DAP (diammonium phosphate) was only added two days after the beginning of the fermentation. The progression of fermentation was monitored by using a Ballingmeter and fermented until dryness (residual sugar <4 g/L). All grape skins were pressed in a basket press after fermentation, the press and free run wine combined and the wines stored in steel canisters at 20ºC until completion of the malolactic fermentation, monitored with the use of WineScan FT 120 (FOSS Analytical, Hillerød, Denmark). Once MLF was completed, the wines were racked off and 60 mg/L of SO_2 added. All the wines underwent subsequent cold stabilization for three weeks at -4ºC before the total SO_2 was adjusted to 60 mg/L and bottled in green 750 mL bottles under screw tops. The bottled wines were stored at 15ºC until chemical analysis and sensory evaluation were performed.

3.2.3. Colour and phenolic measurements

3.2.3.1. Spectrophotometric analysis

Grape phenolics were extracted following the method described by Bindon, *et al.*, (2014b). Briefly, 50 g of fresh berries were softly crushed by hand in order to simulate the phenolic extraction occurring during alcoholic fermentation better than the traditional hard extractions methods (Bindon *et al.* 2014b). After 40 hours shaking in a hydroalcoholic solution (15% ethanol, 10g/L of tartaric acid and a pH of 3.4) at 30ºC, the extract was used to measure the grape phenolic composition. This same extraction procedure was applied to every treatment during both seasons, 2014 and 2015. Grape tannin content was then detemined from the grape extracts by the methyl cellulose precipitation (MCP) method (Sarneckis, *et al.*, 2006) and the results were expressed (in catechin equivalents) in mg/g of berry. Grape anthocyanin and total phenolics (TP) levels were also measured from the grape extracts (Somers & Evans, 1974).

Different colour and phenolic parameters - colour density (CD), modified colour density (MCD), copigment content, SO_2 resistant pigments, total red pigments (TRP) and total phenolics (TP) - were measured by spectrophotometric analysis using Boulton indexes on the wines (Somers & Evans, 1974; Boulton, 2001).

Wine samples were initially analysed right before bottling, once the malolactic fermentation and cold stabilisation were completed and this was considered the time 0 (T0) of bottling storage. Afterwards the wines were analysed once every 6 months. A new bottle was opened for every set of analysis. In 2014, wines samples were analysed up to 24 months in bottle for the colour and phenolic content. Sensorial evaluation of the wines was performed after 6 and 12 months of storage.In 2015, the wine samples were analysed up to 18 months of storage and a a new sampling stage was added, after the completion of alcoholic fermentation (AF). In 2015, the sensory evaluation of the wines was also performed after 6 and 12 months of storage.

3.2.3.2. HPLC analysis

Monomeric and polymeric phenolic compounds were individually quantified from time 0 every 6 months (from T0 up to 24 months in 2014, and from AF to 18 months in 2015) using HPLC based on the methods by (Peng, *et al.*, 2001, 2002). Grape phenolic extraction was performed in 70 % methanol solution using 50 homogenised berries (for each fermentation repeat) using an IKA Ultra-Turrax T 18b (IKA Labortechnik, Staufen, Germany) homogeniser. The grape homogenates were extracted for 45 minutes. Once centrifuged (5 min at 8000rpm), the grape extract was injected into the HPLC.

The separation was carried out on a polystyrene/divinylbenzene reverse-phase chromatographic column (PLRP-S. 150 cm x 4.6 mm, Agilent). The mobile phases used were a 1.5% v/v orthophosphoric acid solution in de-ionised water (mobile phase A) and an acetonitrile solution (mobile phase B). The injection volume was 20µL. The linear gradient used for the two phases was the following: from 0 min to 73 min (solution A: 95% and B: 5%), from 73 min to 78 min (A: 75.2% and B: 24.8%); and staying constant at A: 50% and B: 50%for the remainder of the run. The flow rate was 1mL/min at a constant temperature of 35°C. The methods allows for the quantification at four different wavelengths, 280 nm for flavan-3-ol and polymeric phenols, 320 nm for hydroxycinnamic acids, at 360 nm for flavonols and 520 nm for the anthocyanins and pigments. To simplify the large set of data, certain individual compounds were grouped namely the sum of total hydroxycinnamic acids, total flavonols and the total glucosylated-anthocyanins, total acetylated-anthocyanins and total coumaroylated-anthocyanins. Wine samples were centrifuged for 5 min at 8000 rpm and the supernatant was injected.

3.2.4. Sensory analysis

All wines from both seasons, 2014 and 2015, were evaluated over time in order to assess the aging effect from a sensory perspective. The wines were subjected to a Descriptive Analysis (DA) after 6 and 12 months storage in bottles. For every sensory evaluation, all wines were previously screened

by wine experts from the Department of Viticulture and Oenology at Stellenbosch University. To reduce the number of samples, experts selected two out of the biological triplicates to be evaluated by a sensory panel. The DA method is used to describe quantitatively the perceived sensory differences between different samples (Lawless & Heymann, 2010). Sensory evaluations were performed by a group of panellists (9 to 12) who were trained on red wine sensory aroma attributes, as well as taste and mouth-feel attributes. We tried to keep most members of the panel in all four tastings to keep consistency assessing the sensory changes over time. A wide list of aroma descriptors was generated by the panellists during the first training session. A second and third session were meant to standardise the panellists using fresh products, as well as standards from *Le nez du vin* (Jean Lenoir, Provence, France), to reach a consensus for the final descriptors list. The list of the different attributes and standards used (for 2014 and 2015 wines) is shown in the Appendix Table 3.1 and 3.2. From the third session, the panellist were trained and familiarised with the actual wines. Training of the panel required periods between four to five weeks in a 2 hour session three times a week.

Wine samples were served in standard ISO dark wine tasting glasses, with each glass containing 25 mL of wine. The panellist rated the most relevant aroma attributes on a line scale from 0 to 100. All taste and mouth-feel attributes were rated in a 0 to 100 line scale. Each sample was coded with a 3-digit random code and served in a complete randomised order (Lawless & Heymann, 2010). Panellists performed the analysis in individual booths, with each booth being fitted with a data collecting system (Compusense® five, Version 5.2, Compusense Inc., Guelph, Ontario, Canada). The testing area was light- and temperature-controlled (21°C).

3.2.5. Statistical analysis

All analyses were carried out using Statistica 13.2 (TIBCO Statistica software, Palo Alto, CA, USA). Mixed model repeated measures ANOVAs were used and Fisher's least significant difference (LSD) corrections were used for post-hoc analyses. Significant differences were judged on a 5% significance level ($p \leq 0.05$). PanelCheck software (V.1.4.0, Nofima Mat, Norway) was used to weigh the panellists' performance for the wine sensory evaluation and to generate STATIS biplots based on covariance. The distribution of certain chemical and sensory datasets were analysed with Principal Component Analysis (PCA) using SIMCA 14.1 software (Sartorium Stedim Biotech - Malmö, Sweden).

3.3. RESULTS AND DISCUSSION

The main purpose of the present study was to evaluate the colour and phenolic changes occurring during ageing in red wines made from grapes with different initial phenolic profiles harvested from

the same vineyard. We will thus not endeavour to explain differences in grape phenolic content from viticulture observations/data. As previously mentioned, 2014 was an exploratory study to investigate the evolution of different red wines with a different initial phenolic content. Firstly, the results from the exploratory study will be described, followed by a more detailed and descriptive analysis of the work performed during 2015, where more ripening levels were investigated.

3.3.1. Phenolic and sensorial evolution of 2014 wines

3.3.1.1. Grape chemical composition 2014

The grape chemical composition was clearly affected by the different vineyard treatments and ripening, as shown in Tables 3.2 and 3.3. When looking individually at the different grape phenolic parameters, the training system did not have a major effect on the grape extracts' total phenolic content (TP), whereas it significantly influenced the tannin and anthocyanin concentration (Appendix Table 3.3). Thus, the grape tannin concentration was significantly higher in VSP-HV treatments when compared to the equivalent SD treatments. On the other hand, the grape anthocyanin concentration was highly influenced by the grape ripening and the training system, but not the vigour (Appendix Table 3.3). Therefore, at the earliest ripening stage (R), VSP grapes showed a higher anthocyanin concentration (for both HV and LV) when compared to the SD grapes (Table 3.2). However, whilst this concentration remained constant during ripening for SD grapes, it significantly decreased in VSP grapes, reducing the training system differences found at R (Table 3.2). The vigour thus seemed to play a greater role in the TP content. However, the differences in TP in terms of the vigour were only significant for VSP at R, and in the case of SD grapes at the OR stage (Table 3.2). Contrary to the findings described by Cortell *et al.*, (2007a), the vigour did not influence the anthocyanin concentration in the grape extracts. Regarding the impact of the grape ripening on the grape tannins, significant variation was only seen in the case of VSP-HV (tannin concentration decreased). These findings agree with several authors who suggested that grape tannins remained constant (Harbertson, *et al.*, 2002) or either decrease during ripening (Downey, *et al.*, 2003; Adams, 2006; Hanlin & Downey, 2009). However, literature is currently contradictory, not only on the grape tannins accumulation patterns, but also their extractability during ripening. The grape softening occurring in the grape skins during ripening seems to be a determining factor affecting the phenolic extractability (Bindon & Kennedy, 2011; Bindon, *et al.*, 2014c). Nevertheless, in the interest of our exploratory study, the different vineyard treatments led to grapes with differences in their phenolic profiles.

Additionally, in Table 3.3, no significant differences were found in the amount of polymeric phenols between the ripening stages in most of the treatments (only increased significantly in VSP-LV). As seen in the spectrophotometric results, the vigour played a role in the anthocyanin and polymeric pigment concentration. Firstly, the concentration of total acetylated anthocyanins was significantly higher in VSP-LV treatments when compared to VSP-HV treatments. These differences were not

found for SD treatments. Regarding the total glucosylated and coumaroylated anthocyanins, the VSP-LV treatment only showed a greater concentration compared to the VSP-HV treatment at the OR stage (Table 3.3). Here again, no significant differences were found for SD treatments for these compounds. On the other hand, the polymeric pigments content showed an increasing trend during grape ripeness (only significant in VSP-LV treatment). The polymeric phenol concentration was higher in the grapes from LV vines (for both training systems) compared to those from HV (although only significant for the last stage of ripening).

3.3.1.2. Colour and phenolic evolution of 2014 wines

All the wine treatments were firstly analysed before bottling (T0). Thus, once bottled, the wines were analysed every 6 months up to 24 months of storage. Firstly, the wine phenolics (tannins, total red pigments -TRP- and total phenolics –TP) were analysed at T0. We evaluated the impact of the different treatments on the amount of tannins, TRP and TP, as these were also analysed in the grape extract. Therefore, the results displayed in Table 3.4 and 3.5 show an impact from the vineyard treatments and ripeness on most of the phenolic analyses performed at bottling. However, from univariate test of significance the role of the different variables was different in grapes and wines, especially for the tannin concentration (Appendix Table 3.3 and 3.4). Contrary to fresh grapes, the wine tannins were strongly influenced by the ripening level, increasing in concentration as the wines were made from riper berries, except for SD-HV treatment (Table 3.4). Additionally, training system and vigour also influenced the tannin content. Whereas no significant differences were found between VSP and SD for the wines made from grapes harvested from the HV area, SD wines showed a greater tannin content in LV when compared to VSP wines. Regarding the wines' TRP, and similar to the results in grape extracts, training system and ripening played a relevant role in their amount. As in fresh grapes, VSP wines showed a greater amount of TRP (not significant for LV wines at R stage). Furthermore, the TRP decreased in concentration during ripening with the exception of VSP-LV wines. Nevertheless, the impact of the vine vigour was perceived in young wines, with a greater TRP content in wines made from LV vines (except for VSP-R) in agreement with the findings from Cortell *et al.*(2007b). In general, LV wines had a significantly higher amount of TP when compared to HV wines, except for VSP (HV and LV) at the earlier ripening stage (R).

Table 3.2. Grape soluble solids (ºBrix), pH and TA and phenolic composition (tannin, anthocyanins and total phenolics) at harvest in 2014. R= Ripe, OR= over ripe, LV= low vigour, HV= high vigour. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

	SD				VSP			
	R		OR		R		OR	
	HV	LV	HV	LV	HV	LV	HV	LV
ºBrix	23.67 ± 0.09 ^{d}	23.17 ± 0.07 ^{de}	26.53 ± 0.09 ^{ab}	26.86 ± 0.12 ^{a}	22.46 ± 0.56 ^{e}	23.80 ± 0.11 ^{d}	25.93 ± 0.28 ^{b}	24.83 ± 0.09 ^{c}
pH	3.80 ± 0.02 ^{e}	4.09 ± 0.10 ^{a}	3.94 ± 0.02 ^{d}	4.26 ± 0.02 ^{b}	3.59 ± 0.05 ^{c}	3.85 ± 0.05 ^{de}	4.08 ± 0.03 ^{a}	4.05 ± 0.01 ^{a}
TA (g/L)	3.85 ± 0.10 ^{ab}	3.39 ± 0.06 ^{b}	4.11 ± 0.08 ^{a}	3.54 ± 0.04 ^{ab}	4.14 ± 0.58 ^{a}	4.11 ± 0.16 ^{a}	4.02 ± 0.06 ^{a}	3,31 ± 0.03 ^{b}
Tannins (mg/g berry)	0.40 ± 0.19 ^{cd}	0.51 ± 0.02 ^{bcd}	0.31 ± 0.10 ^{d}	0.60 ± 0.15 ^{bc}	0.88 ± 0.21 ^{a}	0.67 ± 0.12 ^{ab}	0.65 ± 0.08 ^{b}	0.68 ± 0.01 ^{ab}
Anthocyanin (mg/g berry)	0.40 ± 0.07 ^{bc}	0.40 ± 0.02 ^{bc}	0.27 ± 0.06 ^{c}	0.43 ± 0.05 ^{b}	0.78 ± 0.14 ^{a}	0.89 ± 0.04 ^{a}	0.32 ± 0.04 ^{bc}	0.30 ± 0.11 ^{bc}
TP (AU)	36.53 ± 4.73 ^{b}	41.20 ± 2.35 ^{b}	28.83 ± 3.22 ^{c}	51.93 ± 2.34 ^{a}	37.28 ± 5.35 ^{b}	56.26 ± 3.14 ^{a}	35.81 ± 3.77 ^{b}	37.82 ± 0.89 ^{b}

Table 3.3. Grape phenolic composition in 2014. Individual and group of phenolic compounds (mg/g of berry). R= Ripe, OR= over ripe, LV= low vigour, HV= high vigour. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

	SD				VSP			
	R		OR		R		OR	
	HV	LV	HV	LV	HV	LV	HV	LV
Gallic acid	0.001 ± 0.00 ^{ab}	0.001 ± 0.00 ^{b}	0.002 ± 0.00 ^{ac}	0.001 ± 0.00 ^{ab}	0.002 ± 0.00 ^{cd}	0.002 ± 0.00 ^{ac}	0.002 ± 0.00 ^{d}	0.002 ± 0.00 ^{cd}
Catechin	0.007 ± 0.00 ^{a}	0.008 ± 0.00 ^{abc}	0.009 ± 0.00 ^{bc}	0.008 ± 0.00 ^{ab}	0.010 ± 0.00 ^{c}	0.009 ± 0.00 ^{abc}	0.009 ± 0.00 ^{abc}	0.008 ± 0.00 ^{abc}
Polymeric phenols	1.968 ± 0.10 ^{ac}	2.104 ± 0.23 ^{ac}	2.243 ± 0.03 ^{ab}	2.450 ± 0.13 ^{abc}	2.006 ± 0.09 ^{ac}	1.714 ± 0.403 ^{c}	2.312 ± 0. 12 ^{ab}	2.703 ± 0.03 ^{b}
∑ Hydroxycinnamic. acids	0.007 ± 0.00 ^{a}	0.010 ± 0.00 ^{b}	0.007 ± 0.00 ^{a}	0.007 ± 0.00 ^{a}	0.010 ± 0.00 ^{b}	0.010 ± 0.00 ^{b}	0.007 ± 0.00 ^{a}	0.011 ± 0.00 ^{b}
∑ Flavonols	0.062 ± 0.00 ^{ab}	0.077 ± 0.01 ^{bc}	0.048 ± 0.00 ^{a}	0.077 ± 0.00 ^{bc}	0.083 ± 0.01 ^{c}	0.083 ± 0.00 ^{c}	0.075 ± 0.01 ^{ab}	0.075 ± 0.01 ^{bc}
∑ Glucosylated anth.	0.466 ± 0.05 ^{ab}	0.530 ± 0.04 ^{abc}	0.420 ± 0.03 ^{a}	0.439 ± 0.01 ^{ab}	0.504 ± 0.06 ^{abc}	0.549 ± 0.04 ^{bc}	0.452 ± 0.03 ^{ab}	0.590 ± 0.03 ^{c}
∑ Acetylated anth.	0.235 ± 0.03 ^{ab}	0.272 ± 0.02 ^{bc}	0.203 ± 0.01 ^{a}	0.236 ± 0.00 ^{ab}	0.216 ± 0.03 ^{a}	0.270 ± 0.01 ^{bc}	0.209 ± 0.01^{a}	0.303 ± 0.02 ^{c}
∑ Coumaroylated anth.	0.505 ± 0.05 ^{ab}	0.516 ± 0.06 ^{ab}	0.468 ± 0.03 ^{ab}	0.490 ± 0.02 ^{ab}	0.471 ± 0.05 ^{ab}	0.580 ± 0.02 ^{b}	0.445 ± 0.03 ^{a}	0.570 ± 0.03 ^{b}
Polymeric pigments	0.083 ± 0.00 ^{ac}	0.101 ± 0.01^{ab}	0.093 ± 0.00 ^{a}	0.124 ± 0.01^{b}	0.069 ± 0.01 ^{c}	0.078 ± 0.01 ^{c}	0.081 ± 0.01 ^{ac}	0.113 ± 0.01^{b}

Table 3.4. Tannin concentration (mg/L), Total Red Pigments (TRP) and Total Phenolics (TP) for all 2014 wines at bottling (T0). R= Ripe, OR= over ripe, LV= low vigour, HV= high vigour. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

	SD				VSP			
	R		OR		R		OR	
	HV	LV	HV	LV	HV	LV	HV	LV
Tannins (mg/L)	456.60 ± 113.16^{cd}	745.12 ± 106.22^{bc}	653.25 ± 106.24^{bc}	1182.95 ± 95.31^{a}	260.28 ± 46.12^{d}	316.69 ± 69.80^{d}	807.27 ± 163.76^{b}	645.18 ± 92.49^{bc}
TRP (AU)	23.05 ± 1.23^{b}	32.02 ± 1.12^{a}	14.46 ± 3.48^{c}	21.37 ± 1.39^{b}	33.76 ± 1.18^{a}	36.18 ± 0.63^{a}	25.33 ± 0.25^{b}	34.19 ± 0.43^{a}
TP (AU)	39.58 ± 1.09^{d}	49.99 ± 1.51^{ab}	35.43 ± 2.74^{d}	49.18 ± 1.84^{ab}	44.68 ± 0.98^{c}	47.51 ± 0.44^{abc}	46.58 ± 0.27^{bc}	51.49 ± 0.91^{a}

Table 3.5. Individual and groups of phenolic compounds (mg/L) in 2014 wines at bottling (T0). R= Ripe, OR= over ripe, LV= low vigour, HV= high vigour. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

	SD				VSP			
	R		OR		R		OR	
	HV	LV	HV	LV	HV	LV	HV	LV
Gallic acid	4.62 ± 0.49^{d}	8.35 ± 1.64^{bc}	7.43 ± 0.29^{c}	8.95 ± 1.51^{bc}	4.82 ± 0.11^{d}	4.71 ± 0.29^{d}	11.67 ± 0.47^{a}	10.59 ± 0.50^{ab}
Catechin	30.63 ± 4.19^{ab}	29.99 ± 0.85^{ab}	26.31 ± 0.73^{b}	31.05 ± 3.28^{ab}	34.13 ± 4.01^{b}	30.58 ± 0.55^{ab}	36.73 ± 1.49^{a}	33.56 ± 0.86^{ab}
B1	75.21 ± 7.10^{a}	35.78 ± 0.73^{bc}	36.12 ± 1.33^{bc}	33.93 ± 1.24^{bc}	41.72 ± 4.74^{bc}	55.72 ± 18.78^{ab}	36.65 ± 1.94^{bc}	33.35 ± 1.13^{c}
Polymeric phenols	245.00 ± 36.22^{c}	401.81 ± 71.57^{b}	648.01 ± 46.24^{a}	673.29 ± 18.30^{a}	437.30 ± 1.77^{b}	302.08 ± 17.29^{bc}	706.59 ± 41.87^{a}	724.52 ± 84.19^{a}
∑ Hydroxycinnamic. acids	38.16 ± 3.14^{b}	24.55 ± 7.20^{cd}	29.39 ± 1.85^{bc}	29.30 ± 2.94^{bc}	50.38 ± 1.88^{a}	38,31 ± 2.46^{b}	24.32 ± 2.49^{cd}	18.37 ± 1.98^{d}
∑ Flavonols	31.43 ± 7.57^{abc}	15.50 ± 7.20^{d}	28.25 ± 1.39^{bc}	22.03 ± 4.65^{bcd}	42.59 ± 1.94^{a}	34.18 ± 0.92^{ab}	20.31 ± 1.98^{cd}	14.81 ± 1.52^{d}
∑ Glucosylated anth.	171.16 ± 18.60^{bc}	108.87 ± 30.63^{d}	137.22 ± 4.37^{cd}	133.58 ± 7.48^{cd}	216.21 ± 6.65^{a}	194.97 ± 12.10^{ab}	121.51 ± 5.57^{d}	102.57 ± 9.41^{d}
∑ Acetylated anth.	85.12 ± 9.06^{bc}	48.19 ± 17.11^{d}	64.42 ± 1.34^{cd}	61.45 ± 6.85^{d}	111.18 ± 2.45^{a}	92.59 ± 4.40^{ab}	49.47 ± 2.74^{d}	42.18 ± 3.52^{d}
∑ Coumaroylated anth.	33.92 ± 3.86^{a}	20.68 ± 7.71^{b}	22.58 ± 0.92^{b}	22.85 ± 0.57^{b}	42.62 ± 0.49^{a}	35.70 ± 1.70^{a}	23.47 ± 1.65^{b}	16.86 ± 2.07^{b}
Polymeric pigments	16.51 ± 1.04^{d}	23.18 ± 2.25^{cd}	40.39 ± 3.43^{a}	35.94 ± 4.57^{ab}	30.46 ± 2.57^{abc}	27.02 ± 1.10^{bc}	37.28 ± 3.24^{a}	39.49 ± 5.58^{a}

The analysis of the individual phenolic compounds confirmed the differences between the wines before bottling (Table 3.5). Training system and vigour played a role, but the influence of the grape ripeness was especially prevalent (Appendix Table 3.5). Thus, as shown in Table 3.5, a lower amount of total hydroxycinnamic acids, total flavonols and total monomeric anthocyanins (glucosylated, acetylated and coumaroylated anthocyanins) was observed in VSP-OR wines when compared to the wines from less ripe berries (R). These differences were however in most cases not found in SD wines. On the other hand, a significantly greater amount of polymeric phenols was found in wines made from OR berries (Table 3.5). Similar patterns were observed for the concentration of polymeric pigments. A greater amount of polymeric pigments was found in wines made from OR grapes however, these differences, between R and OR, were not significant in the case of VSP-HV wines (Table 3.5).

These dissimilarities between grape and wine phenolics can be explained by different factors, such as the influence of the grape seeds during fermentation or the role of the cell wall composition on the phenolic extractability. From there, wines were periodically analysed to assess if these initial differences were maintained over time (Figure 3.2 and 3.3). Although ANOVA analysis were performed to assess the statistical impact of the different variables on the overall and the individual wine colour and phenolic composition, PCA plots were also generated to ascertain the cumulative effect from all the different variables on the overall wine composition. Therefore, the PCA loading plot displayed in Figure 3.2E shows the sample distribution according to their colour and phenolic parameters analysed. A clear impact from vigour and grape ripeness was observed in Figure 3.2B and C. LV wines were mainly distributed on the right side of PC1 axis (42.4%), characterised by a greater colour, TRP and TP content and SO_2 resistant pigments. Additionally, in Figure 3.2C, the wines made from riper berries were distributed along the PC2 (18.3%), mainly characterised by a higher amount of tannins but also hue, TP and SO_2 resistant pigments. However, these differences between treatments were found to be reduced over time (Figure 3.2D), contrary to the results found by De Beer *et al.* (2017). In the course of time, as displayed in Figure 3.2D and Figure 3.2E, a lower colour, TRP and copigment values were found in older wines, compared to young wines, whereas the hue and tannin levels increased.

These separations between treatments were also observed when individual phenolics were analysed (Figure 3.3). However, training system and vigour did not seem to influence the distribution of the wine samples according to their different groups of individual phenolic compounds to the same extend than ripening (Appendix Table 3.5). On the other hand, grape ripeness, together with the time of sampling, played a larger

role in affecting the individual phenolic profile of the wines over time (Appendix Table 3.5). Thus, as displayed in Figure 3.3C, the wines from riper berries (OR) were characterised by a higher amount of polymeric forms (Figure 3.3E), both as polymeric phenols and polymeric pigments, and lower values of total hydroxycinnamic acids. During ageing, all the wines distributed to the side of the PC1 (Figure 3.3D & E) characterised by a lower concentration of glucosylated, acetylated and coumaroylated anthocyanins, which are in agreement with literature (Somers & Evans, 1979; Pérez-Magariño & González-SanJosé, 2004; Boido, *et al.*, 2006). Additionally, as displayed in Figure 2D, differences between treatments, found in grapes and young wines, became smaller over time as the wine samples were more closely distributed along the PC1 axis after ageing.

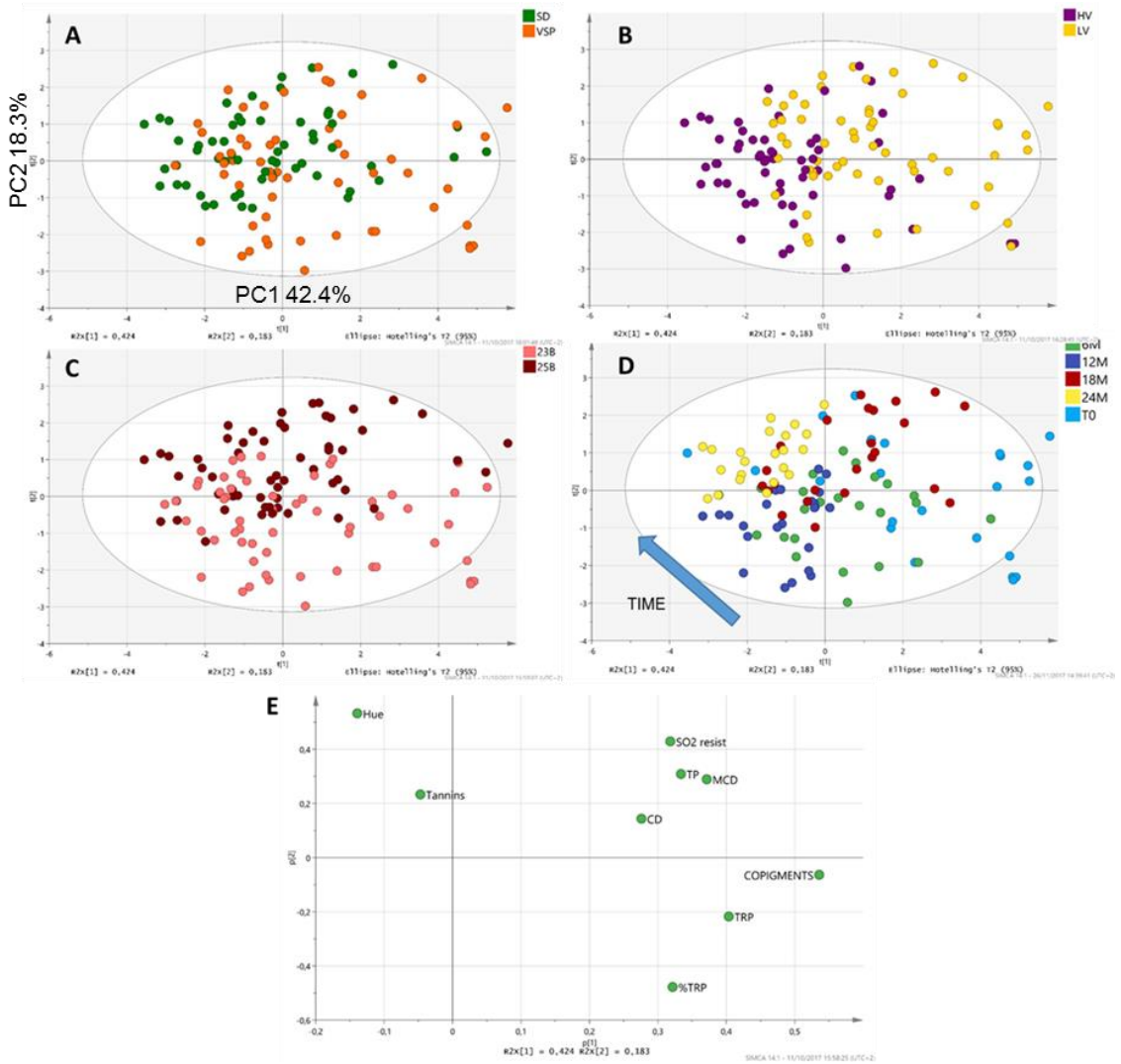

Figure 3.2. Wine sample distribution according to their phenolic content analysed by spectrophotometric methods. A) PCA scores scatter plot coloured according to the training system. B) PCA scores scatter coloured according to the vine vigour. C) PCA scores scatter plot coloured according to grape ripening. D) PCA scores scatter plot coloured according to the sampling stages. E) Loading plot with the colour and phenolic parameters.

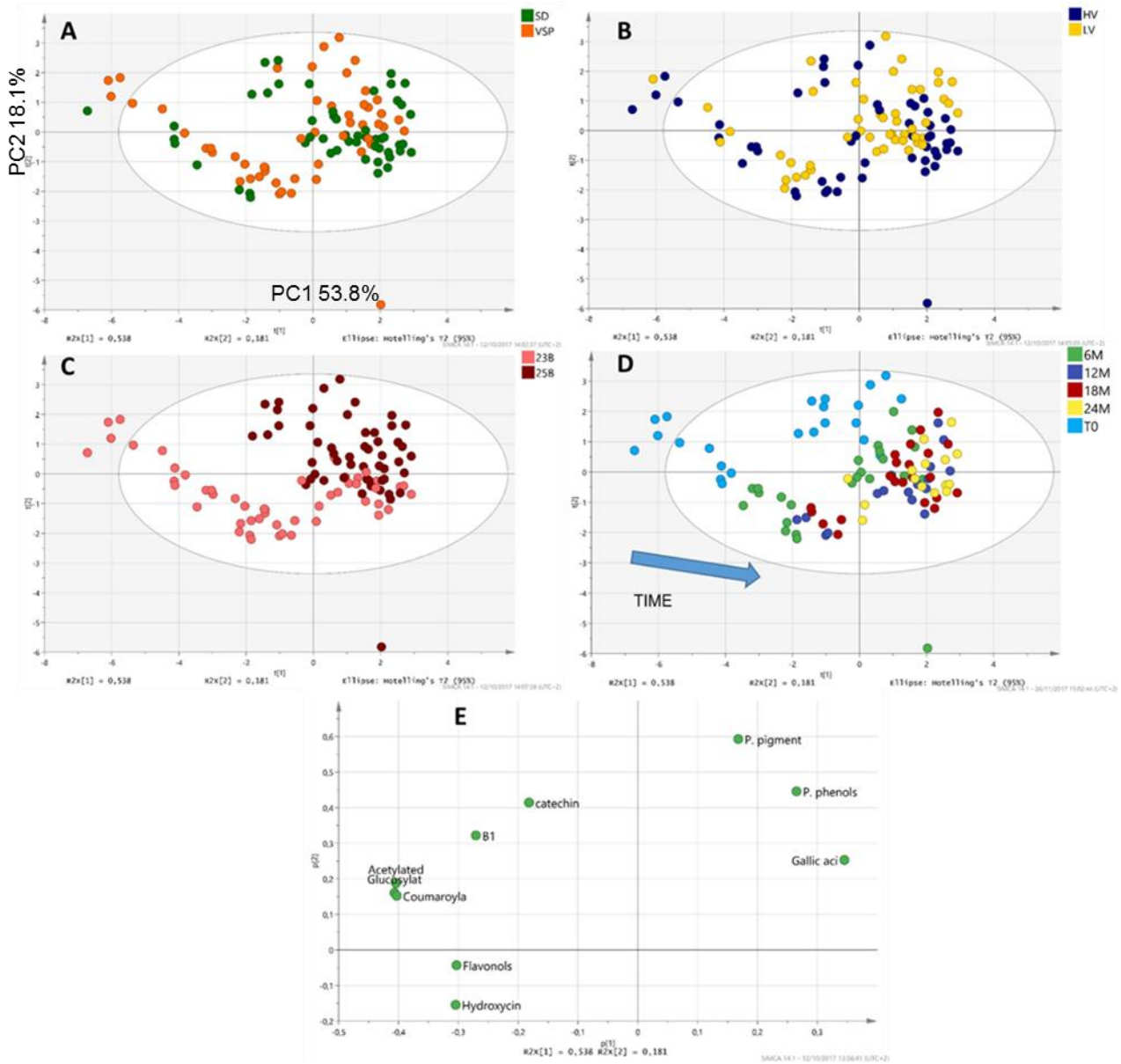

Figure 3.3. Wine sample distribution according to the group of individual phenolic compounds analysed with HPLC. A) PCA scores scatter plot coloured according to the training system. B) PCA scores scatter plot coloured according to the vine vigour. C) PCA scores scatter plot coloured according to grape ripening. D) PCA scores scatter plot coloured according to the sampling stages. E) Loading plot displaying the phenolic composition.

3.3.1.3. Sensory evaluation of 2014 wines

Sensory analysis were performed on the different wine treatments after 6 and 12 months of storage. Due to their link to with the phenolic composition, the assessment of taste and mouth-feel was the main focus of the study. However, the impact of the training system, vigour and grape ripeness on the aroma profile of the wines was also checked. Therefore, based on the significant aroma attributes (Appendix Figure 3.1), VSP wines

tended to be described by vegetative descriptors whereas SD wines were characterised as more floral and raisin character after 6 months (Appendix Figure 3.2A). These general trends remained six months later when VSP and SD wines, distributed along the PC1 axis (83.1%), were frequently characterised by vegetables, pencil shavings or meaty attributes for VSP wines and muscat, dark berries or raisins for SD wines (Appendix Figure 3.2B).

On the other hand, the grape ripeness seemed to play the biggest role in the taste and mouth-feel perception. Thus, as illustrated in Figure 3.4A, after 6 months, the wine samples were separated along the PC1 axis (72%), mainly based on the differences between acidity and the rest of attributes. Thus, most of the 23 ºBrix (R) wines (including all four treatments) were described as being higher in acidity. On the other hand, the wines made from riper berries were often described as more bitter and "fuller" (Appendix Table 3.6 and 3.7. Astringency did not show an increase with the ripening, except for VSP HV wines (Appendix Table 3.6).

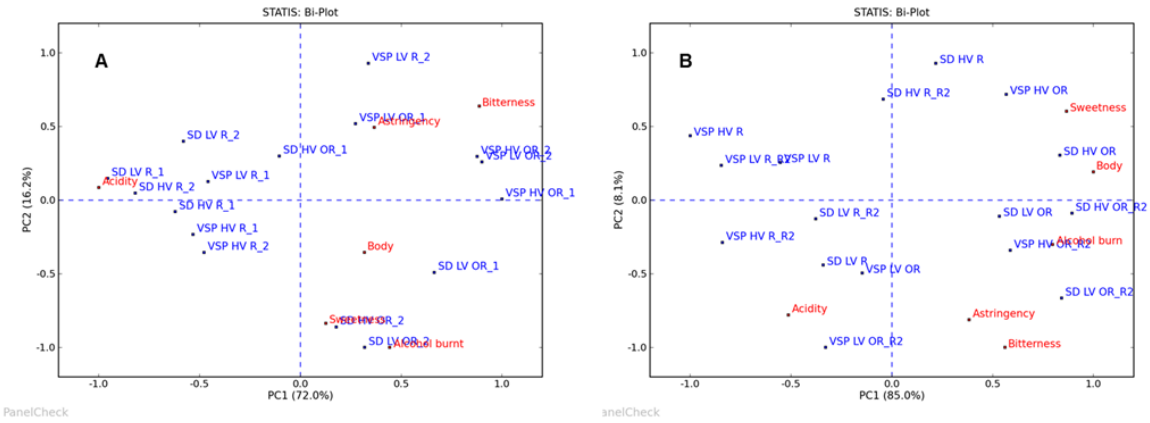

Figure 3.4. Wine sample distribution according to the taste and mouth-feel. A) Wine evaluation after 6 months of bottle storage B) Wine evaluation after 12 months of bottle storage. Wine codes according to training system (VSP, SD), vigour (HV, LV) and ripening (R and OR).

Unfortunately, a considerable number of panellist changed from the first to the second session due to unforeseen circumstances in 2104. We are therefore not able to compare 6 and 12 months with each other statistically. However, the trends remained relatively stable over time. In Figure 3.4B, the grape ripeness level continued being the main driver along the PC1 axis (85%). These findings agree with a previous study on the impact of different training systems on the astringency and bitterness of the wines performed in the same vineyard (Van Noordwyk, 2012; De Beer, 2015). The chemistry results, together with the sensory evaluation led to a further investigation in 2015 of the influence

of grape ripeness on the colour and phenolic composition in young wines and their subsequent evolution over time.

3.3.2. Phenolic and sensorial evolution of 2015 wines

3.3.2.1. Grape chemical composition 2015

In 2015, grapes were harvested at four different ripening levels (21ºBrix, 23ºBrix, 24ºBrix and 25ºBrix) from the VSP training system from the low vigour zone of the vineyard. A higher pH in the grapes at 25 ºBrix, with a decrease in TA levels was observed during the grape ripeness (Table 3.5). Looking at the phenolic composition of the grape extracts, as shown in Table 3.6, no significant differences were found for the grape tannin content between the different ripening stages. These results were similar to what was found in section 3.3.2, for 2014 for the grapes samples collected from the same training system and vigour (VSP-LV) at two ripening levels. However, a significant increase was found from 21ºBrix to 23ºBrix (remaining constant in 24ºBrix and 25ºBrix) in the concentration of anthocyanins, while TP increased significantly from 23 ºBrix to 24 ºBrix (Table 3.6).

Table 3.5. Grape parameters (Brix, pH and TA) at harvest in 2015. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the ripening levels.

	21ºBrix	23ºBrix	24ºBrix	25ºBrix
ºBrix	21.23 ± 0.22 [d]	22.89± 0.12 [c]	24.30 ± 0.29 [b]	25.17 ± 0.15 [a]
pH	3.36 ± 0.02 [a]	3.39 ± 0.02 [a]	3.41 ± 0.01 [a]	3.81 ± 0.03 [b]
TA (g/L)	6.10 ± 0.22 [a]	5.65 ± 0.03 [b]	5.06 ± 0.07 [c]	4.11 ± 0.09 [d]

Table 3.6. Phenolic composition of 2015 fresh grapes from four ripening levels. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the ripening levels.

	21ºBrix	23ºBrix	24ºBrix	25ºBrix
Tannins (mg/g berry)	0.50±0.01 [a]	0.45±0.06 [a]	0.47±0.04 [a]	0.54±0.05 [a]
Anthocyanins (mg/g berry)	0.35±0.01 [a]	0.42±0.04 [b]	0.47±0.02 [b]	0.45±0.02 [b]
Total phenolics (AU)	37.43±1.27 [b]	30.62±5.25 [b]	46.01±1.04 [a]	49.61±1.82 [a]

From the individual phenolics results (Table 3.7), no significant differences were found in the concentration of gallic acid, B1, total flavonols or monomeric anthocyanins (total glucosylated anthocyanins, total acetylated anthocyanins and total coumaroylated). However, an increase was observed in the polymeric compounds analysed. A gradual increase in the polymeric phenol concentration was found during ripening, but only significant when comparing 21ºBrix grapes to 25ºBrix grapes. On the other hand, this increase was only significant from 24ºBrix to 25ºBrix for polymeric pigments. Additionally,

the total amount of hydroxycinnamic acids showed a decrease during ripening (significant from 21ºBrix to 23ºBrix) (Table 3.7).

Table 3.7. Grape phenolic composition in 2015. Individual and groups of phenolic compounds (mg/g of berry) for grapes at four different harvest stages (21ºB, 23ºB, 24ºB and 25ºB). The different letters indicate significant differences (ANOVA, $p < 0.05$) between the ripening *levels.*

	21ºBrix	23ºBrix	24ºBrix	25ºBrix
Gallic acid	0.003 ± 0.00 [a]	0.003 ± 0.00 [a]	0.002 ± 0.00 [a]	0.003 ± 0.00 [a]
Catechin	0.010 ± 0.00 [a]	0.006 ± 0.00 [a]	0.019 ± 0.00 [b]	0.004 ± 0.00 [a]
B1	0.020 ± 0.00 [a]	0.018 ± 0.00 [a]	0.014 ± 0.00 [a]	0.015 ± 0.00 [a]
Polymeric phenols	1.504 ± 0.06 [a]	1.828 ± 0.13 [ab]	1.796 ± 0.08 [ab]	1.971 ± 0.14 [b]
∑ Hydroxycinnamic. acids	0.007 ± 0.00 [a]	0.006 ± 0.00 [b]	0.007 ± 0.00 [b]	0.006 ± 0.00 [b]
∑ Flavonols	0.125 ± 0.08 [a]	0.112 ± 0.01 [a]	0.122 ± 0.01 [a]	0.120 ± 0.01 [a]
∑ Glucosylated anth.	0.457 ± 0.01 [a]	0.442 ± 0.04 [a]	0.459 ± 0.02 [a]	0.416 ± 0.03 [a]
∑ Acetylated anth.	0.179 ± 0.00 [a]	0.160 ± 0.01 [a]	0.183 ± 0.01 [a]	0.164 ± 0.01 [a]
∑ Coumaroylated anth.	0.290 ± 0.02 [a]	0.269 ± 0.02 [a]	0.297 ± 0.02 [a]	0.274 ± 0.03 [a]
Polymeric pigments	0.032 ± 0.00 [a]	0.038 ± 0.01 [a]	0.039 ± 0.01 [a]	0.055 ± 0.00 [b]

3.3.2.2. Colour and phenolic evolution of 2015 wines

In 2015, with the objective of assessing the ripening effect in young wines, the analysis of the colour and phenolic compounds was also done after the alcoholic fermentation (AF). At this stage, only the hue and the MCP tannin concentration were not significantly affected by the grape ripeness (Appendix Table 3.8). Analysing the groups of individual phenolic compounds measured by HPLC, only gallic acid, catechin and B1 were not significantly influenced by the ripening after AF (Appendix Table 3.9). From this stage, storage time also played a significant role in the colour and phenolic evolution.

The evolution, up to 18 months of storage, of the different colour and phenolic parameters measured by spectrophotometric methods is described in Table 3.8. Time and grape ripeness played a role in the wines' colour and phenolic composition (Appendix Table 3.10). Total red pigments (TRP), SO_2 resistant pigments and copigments were highly influenced by time. On the other hand, although time was also significant, the TP content and, especially, the MCP tannin levels, were strongly influenced by the different ripeness degrees (Appendix Table 3.11). Thus comparing AF with after 18 months, a significant decrease was found in the CD, TRP and copigments, with the exception of the copigments in wines made from 21ºBrix grapes (Table 3.8). In

this time, the amount of SO_2 resistant pigment remained relative constant for wines made from 21 and 23ºBrix grapes. Contrary, these pigments decreased in wines made from 24ºBrix grapes, whereas they increased in the wines made from 25ºBrix grapes. These colour and phenolic evolution is also shown in Figure 3.5, where the sample distribution followed a clear pattern over time. Firstly, in Figure 3.5A, the samples were clearly distributed along the PC1 axis (47.9%) according to the grape ripeness. Wines made from 21ºBrix, 23ºBrix and 24ºBrix grapes were found on the negative side of the axis 1, whereas wines made from 25ºBrix grapes were mostly found on the positive axis of PC1. In young wines (AF), wines made from 25ºBrix grapes were found more closely distributed with the wines made from the other grape ripeness levels. Nonetheless, differences were observed from T0 between different wines, especially between the wines made from 25ºBrix grapes and the rest of the treatments. Additionally, along PC2 (27.4%) the wine samples were separated according to the sampling stage. From the loading plot, one can observe a generally decrease in colour and phenolic compounds over time as illustrated by the distribution of the wine samples by a lower amount of TRP, TP, CD copigments and SO_2 resistant pigments.

As occurred in 2014 data, some of the differences found in young wines made from different grape ripeness became smaller over time. As an example, no significant differences were found in the TRP levels between all the wines after the decreases that occurred during the 18 months of storage. Similarly, the copigments concentration appeared to decrease over time, except for the wines made 21ºBrix grapes where the decrease was not significant. Boulton, 2001 also states that the co-pigmentation effect decreases over time. The differences in the concentration of copigments, especially higher in wines made from 24º and 25ºBrix grapes when compared to 21º and 23ºBrix, became statistically insignificant after 12M.

The different wines reached their maximum copigment levels at different stages over time. This may be related to a higher availability of copigment factors in the wine media as the berry ripens. From there, the decrease observed may be related to the dissociation of the copigmentation complexes. This decrease in concentration of copigment, as a results of cofactor availability, may also be linked to the formation of SO_2 resistant pigments over time (Somers, 1971; Bindon, *et al.*, 2014d). In all the wines, the concentration of the SO_2 resistant pigments reached their peak after 6 months of bottle storage, but drastically reduced after this presumably as consequence of precipitation (Table 3.8). Therefore, the lack of compounds available to form more stable pigmented polymers in the wines made from less ripe grapes could lead to the association between free anthocyanins and colourless cofactors to stabilise the colour in young wines.

Table 3.8. Colour and phenolic measurements up to 18 months in bottle for 2015 wines made from four different ripening stages (21ºBrix, 23ºBrix, 24ºBrix and 25ºBrix). The different letters indicate significant differences (ANOVA, $p < 0.05$) between the ripening levels.

		AF	T0	6 M	12 M	18 M
CD (AU)	21ºBrix	15.75 ± 1.80 ^{cde}	8.89 ± 0.86 ^{jk}	12.79 ± 0.68 ^{fghi}	8.96 ± 0.85 ^{jk}	7.88 ± 0.65 ^{k}
	23ºBrix	17.53 ± 2.85 ^{bc}	11.46 ± 0.78 ^{hij}	14.66 ± 1.16 ^{defg}	11.34 ± 0.31 ^{hij}	10.07 ± 0.24 ^{ijk}
	24ºBrix	19.17 ± 1.29 ^{b}	13.83 ± 3.26 ^{efgh}	16.46 ± 1.94 ^{cd}	13.79 ± 0.32 ^{efgh}	12.30 ± 0.60 ^{ghi}
	25ºBrix	24.77 ± 2.71 ^{a}	17.44 ± 1.76 ^{bcd}	23.02 ± 3.91 ^{a}	15.70 ± 2.02 ^{cdef}	17.20 ± 1.00 ^{bcd}
Hue	21ºBrix	0.42 ± 0.02 ^{h}	0.52 ± 0.03 ^{defg}	0.49 ± 0.02 ^{g}	0.54 ± 0.05 ^{def}	0.61 ± 0.02 ^{bc}
	23ºBrix	0.42 ± 0.02 ^{h}	0.50 ± 0.01 ^{e}	0.50 ± 0.02 ^{e}	0.54 ± 0.02 ^{dfg}	0.60 ± 0.03 ^{bc}
	24ºBrix	0.44 ± 0.02 ^{h}	0.50 ± 0.03 ^{f}	0.51 ± 0.02 ^{defg}	0.53 ± 0.04 ^{deg}	0.60 ± 0.01 ^{bc}
	25ºBrix	0.39 ± 0.01 ^{h}	0.64 ± 0.08 ^{ab}	0.60 ± 0.03 ^{c}	0.62 ± 0.01 ^{bc}	0.66 ± 0.03 ^{a}
MCD (AU)	21ºBrix	13.65 ± 0.80 ^{defg}	14.30 ± 0.78 ^{def}	13.73 ± 1.04 ^{def}	8.63 ± 0.24 ^{l}	7.88 ± 0.38 ^{l}
	23ºBrix	14.78 ± 1.40 ^{de}	15.53 ± 0.69 ^{cd}	14.39 ± 0.25 ^{def}	11.43 ± 0.62 ^{gh}	10.07 ± 0.14^{hi}
	24ºBrix	17.65 ± 0.60 ^{bc}	17.96 ± 0.59 ^{b}	13.27 ± 0.36 ^{efg}	13.31 ± 0.28 ^{defg}	12.31 ± 0.34 ^{fgh}
	25ºBrix	19.18 ± 0.03 ^{b}	21.54 ± 1.80 ^{a}	14.99 ± 0.99 ^{de}	15.46 ± 1.13 ^{cde}	17.20 ± 0.58 ^{bc}
TRP (AU)	21ºBrix	31.67 ± 2.87 ^{cd}	28.71 ± 1.59 ^{de}	28.77 ± 2.68 ^{de}	18.64 ± 0.91 ^{g}	16.82 ± 1.72 ^{g}
	23ºBrix	35.45 ± 7.03 ^{bc}	31.96 ± 0.99 ^{cd}	29.26 ± 2.16 ^{de}	21.09 ± 1.26 ^{fg}	18.72 ± 0.33 ^{g}
	24ºBrix	43.68 ± 2.24 ^{a}	40.24 ± 4.38 ^{ab}	36.42 ± 3.56^{bc}	24.68 ± 1.70 ^{ef}	22.01 ± 0.25 ^{fg}
	25ºBrix	39.77 ± 2.28 ^{ab}	26.15 ± 4.81 ^{ef}	25.16 ± 3.85 ^{ef}	17.70 ± 5.35 ^{g}	18.49 ± 4.44 ^{g}
TP (AU)	21ºBrix	41.27 ± 3.83 ^{ghij}	40.44 ± 2.21 ^{ghij}	44.97 ± 3.92 ^{defgh}	36.36 ± 3.65 ^{ghij}	35.34 ± 3.04 ^{j}
	23ºBrix	46.69 ± 7.94 ^{cdefg}	44.40 ± 1.72 ^{efghi}	46.29 ± 8.67 ^{cdefg}	39.36 ± 2.78 ^{ghij}	37.10 ± 0.30^{hij}
	24ºBrix	64.80 ± 3.72 ^{a}	56.96 ± 10.32 ^{ab}	54.03 ± 7.18 ^{bc}	45.54 ± 3.38 ^{defgh}	43.31 ± 0.72 ^{fghij}
	25ºBrix	52.85 ± 2.24 ^{bcde}	51.46 ± 8.03 ^{bcdef}	53.15 ± 1.27 ^{bcd}	44.22 ± 3.77 ^{fghi}	45.47 ± 7.51 ^{cdefgh}
SO_2 resistant pigments (AU)	21ºBrix	2.64 ± 0.09 ^{i}	4.57 ± 0.41 ^{ef}	6.14 ± 1.04 ^{cd}	2.02 ± 0.23 ^{i}	2.38 ± 0.11 ^{i}
	23ºBrix	2.84 ± 0.61 ^{ghi}	5.87 ± 0.41 ^{d}	7.26 ± 1.33 ^{c}	2.5 ±0.02 ^{i}	3.06 ± 1.84 ^{ghi}
	24ºBrix	8.83 ± 0.27 ^{b}	7.26 ± 0.91 ^{c}	10.86 ± 0.57 ^{a}	2.76 ± 0.53 ^{hi}	3.87 ± 0.41 ^{fg}
	25ºBrix	3.92 ± 0.60 ^{fgh}	8.43 ± 1.03 ^{b}	11.20 ± 0.94 ^{a}	5.50 ± 0.71 ^{de}	6.14 ± 1.27 ^{cd}
Copigments (AU)	21ºBrix	14.57 ± 4.22 ^{defg}	18.70 ± 1.72 ^{bcd}	24.14 ± 9.02 ^{abc}	15.29 ± 3.63 ^{defg}	10.13 ± 0.54 ^{g}
	23ºBrix	18.68 ± 4.84 ^{bcd}	24.73 ± 5.16 ^{ab}	17.85 ± 0.38 ^{cde}	14.97 ± 3.11 ^{defg}	12.01 ± 0.68 ^{efg}
	24ºBrix	27.64 ± 2.18 ^{a}	23.16 ± 0.62 ^{abc}	18.30 ± 1.03 ^{cde}	10.20 ± 3.28 ^{g}	13.53 ± 0.38 ^{defg}
	25ºBrix	28.14 ± 9.10 ^{a}	16.77 ± 1.24 ^{def}	14.94 ± 2.65 ^{defg}	8.99 ± 1.46 ^{g}	11.46 ± 1.63 ^{fg}
Tannins (mg/L)	21ºBrix	699.26 ± 100.94 ^{def}	713.46 ± 129.51 ^{de}	332.13 ± 30.35 ^{h}	478.87 ± 112.61 ^{gh}	485.00 ± 9.99 ^{gh}
	23ºBrix	609.28 ± 122.88 ^{efg}	521.23 ± 92.75 ^{fg}	601.93 ± 93.80 ^{efg}	617.34 ± 62.41 ^{efg}	532.90 ± 57.30 ^{fg}
	24ºBrix	715.21 ± 168.68 ^{de}	582.22 ± 75.81 ^{efg}	592.60 ± 83.97 ^{efg}	811.19 ± 44.34 ^{cd}	716.14 ± 62.85 ^{de}
	25ºBrix	860.56 ± 117.23 ^{bcd}	1133.56 ± 231.00 ^{a}	835.69 ± 150.20 ^{cd}	908.29 ± 115.38 ^{bc}	1023.85 ± 42.78 ^{ab}

Colour density (CD), total red pigments (TRP),total phenolics (TP).

However, these decreases in pigment concentration did not always affect the amount of TP. The amount of TP remained relatively constant in the wines made from 21ºBrix berries. On the other hand, the rest of the wines (made from 23ºBrix, 24ºBrix and 25ºBrix grapes) showed a decrease over time, being not significant in the case of the wines made from 25ºBrix grapes.

Regarding the tannin levels, the trend was found that that levels were higher in wines made from riper grapes (Appendix Table 3.11) as suggested by Bindon *et al.* (2013), but this was only significant when wines AF made from 23ºBrix and 25ºBrix grapes were compared. The ANOVA and LSD test showed constant MCP tannins levels, from AF to after 18M, in all the wines, except for the wines made from 21ºBrix grapes (Table 3.8). Nonetheless, and contrary to what we found for the TRP, the smaller differences found in MCP tannin levels at AF became larger over time. Thus, a greater concentration was found in wines made from 24ºBrix and especially 25ºBrix grapes after 18 months (Table 3.8). These differences may be related to the formation of larger polymers. However, although it remains uncertain, the tannin size and polymer conformation may affect the tannin precipitation with MCP, as it was shown to occur with the BSA tannin precipitation method (Harbertson, *et al.*, 2014).

These results disagree with the study by Bindon *et al.* (2013) as the tannins levels found in wines made from the last harvest were not always significantly higher. On the other hand, the study suggested a higher tannin value as the harvest advanced, which manifest especially after prolonged ageing.

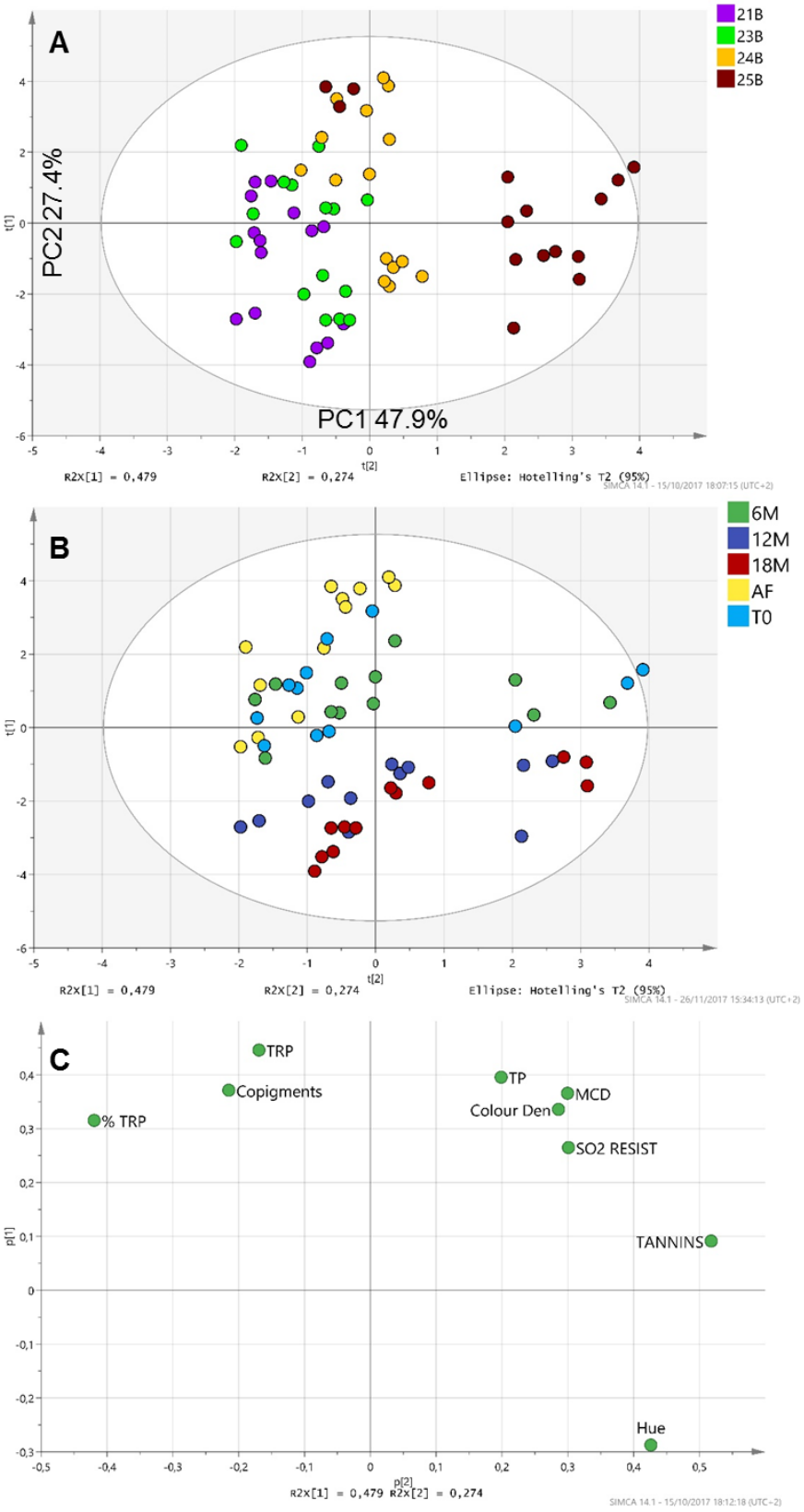

Figure 3.5. Sample distribution according to the colour phenolic content analysed by spectrophotometric methods. A) PCA scores scatter plot coloured according grape ripeness. B) PCA scores scatter plot coloured according to time. C) Loading plot with the colour and phenolic parameters.

3.3.2.3. Individual phenolic compounds of 2015 wines

The HPLC results of the individual phenolic compounds (Table 3.9) shows the effect of grape ripeness and aging on the evolution of the individual phenolic compounds (Appendix Table 3.12). A similar wine sample distribution according to the evolution of the colour and phenolics as determined spectrophotometrically was also found for individual phenolic compounds (Figure 3.5 and 3.6). In Figure 3.6B, the wine samples distributed along the PC1 (52.7%) according to the time of sampling. As expected, as the wines aged, they were characterised by a lower free anthocyanin content, with an increase in polymer fractions. These results agree with the findings of Pérez-Magariño & González-San José (2004). However, and similarly to the spectrophotometric results , wines made from 25ºB grapes showed a different evolution when compared to the other three ripening stages, especially from MLF and onwards.

These differences were mainly explained by the concentration of polymeric phenols and polymeric pigments in the wines. Both parameters were especially influenced by the grape ripeness (Appendix Table 3.13). In short, a larger concentration of polymeric phenols and polymeric pigments was found in the wines made from 25ºBrix grapes, already significant at AF. Thereby, these results show a greater levels of larger molecules during grape ripening, contrary to the findings from some authors (Obreque-Slier, *et al.*, 2010; Bautista-Ortín, *et al.*, 2012). The decrease observed in 25ºB wines, after 12 months of storage, is probably due to over polymerization reactions and the subsequent precipitation of insoluble compounds.

On the other hand, the amount of polymeric pigments in young wines (AF) was not significantly higher than wines made from 25 ºBrix compared to those made of 23ºBrix and 24ºBrix grapes (Table 3.9). Only with the course of time, the wines made from 25ºBrix grapes experienced a significant increase in polymeric pigments during bottle storage. This greater formation of polymeric pigments is probably linked to the quicker degradation of free anthocyanins (glucosylated, acetylated and coumaroylated anthocyanins) occurring in the wines made from 25ºBrix grapes (Table 3.9 and Appendix Figure 3.3). These trends may be explained by a greater proanthocyanidins concentration and therefore higher availability to react with the monomeric anthocyanins and form these polymeric pigments (Singleton & Trousdale, 1992; He, *et al.*, 2012a), in the wines made from the ripest grapes.

Table 3.9. Evolution of the individual and groups of phenolic compounds (mg/L) up to 18 months of storage for 2015 wines made from four different ripening stages (21ºBrix, 23ºBrix, 24ºBrix and 25ºBrix). The different letters indicate significant differences (ANOVA, $p < 0.05$) between the ripening levels.

		AF	MLF	6 M	12 M	18 M
Gallic acid	**21ºBrix**	6.74 ± 0.50 ^{fg}	5.65 ± 0.36 ^{h}	7.48 ± 0.50 ^{ef}	8.89 ± 0.39 ^{d}	9.74 ± 0.50 ^{cd}
	23ºBrix	6.74 ± 0.61 ^{fg}	6.03 ± 0.31 ^{gh}	7.74 ± 0.33 ^{e}	9.42 ± 0.33 ^{cd}	10.11 ± 0.26 ^{bc}
	24ºBrix	7.02 ± 0.52 ^{ef}	5.95 ± 0.47 ^{gh}	7.39 ± 0.65 ^{ef}	9.88 ± 0.09 ^{bcd}	10.31 ± 0.78 ^{abc}
	25ºBrix	7.46 ± 0.32 ^{ef}	9.15 ± 0.14 ^{d}	9.80 ± 0.63 ^{cd}	10.88 ± 0.71 ^{ab}	11.05 ± 0.85 ^{a}
Catechin	**21ºBrix**	7.15 ± 1.57 ^{ef}	5.38 ± 0.88 ^{f}	17.76 ± 0.89 ^{abc}	13.37 ± 0.42 ^{abcde}	8.38 ± 0.96 ^{def}
	23ºBrix	9.09 ± 4.04 ^{def}	6.92 ± 2.47 ^{ef}	11.47 ± 6.37 ^{cdef}	12.45 ± 0.29 ^{abcdef}	7.63 ± 0.09 ^{ef}
	24ºBrix	18.37 ± 11.74 ^{ab}	9.11 ± 2.66 ^{def}	9.55 ± 3.44 ^{def}	15.03 ± 0.03 ^{abcd}	8.48 ± 0.63 ^{def}
	25ºBrix	8.3 ± 2.13 ^{def}	19.18 ± 0.77 ^{a}	12.30 ± 5.60 ^{bcde}	4.68 ± 2.46 ^{f}	9.15 ± 0.49 ^{def}
B1	**21ºBrix**	10.19 ± 1.54 ^{ef}	11.04 ± 1.23 ^{def}	19.05 ± 2.47 ^{b}	18.46 ± 0.70 ^{bcd}	17.49 ± 0.58 ^{bcd}
	23ºBrix	14.83 ± 3.06 ^{bcdef}	10.40 ± 3.48 ^{ef}	17.14 ± 2.21 ^{bcde}	15.28 ± 1.24 ^{bcdef}	14.72 ± 0.60 ^{bcde}
	24ºBrix	14.72 ± 10.19 ^{bcdef}	12.06 ± 5.27 ^{cdef}	19.31 ± 3.68 ^{b}	19.99 ± 0.01 ^{ab}	19.01 ± 1.06 ^{bc}
	25ºBrix	16.85 ± 2.99 ^{bcde}	27.42 ± 4.23 ^{a}	17.7 ± 8.19 ^{bcd}	8.23 ± 0.07 ^{f}	15.5 ± 4.36 ^{bcdef}
Polymeric phenols	**21ºBrix**	401.52 ± 31.99 ^{fghi}	280.67 ± 16.91 ^{k}	311.19 ± 23.65 ^{jk}	331.05 ± 4.80 ^{ijk}	311.78 ± 23.83 ^{jk}
	23ºBrix	445.58 ± 83.09 ^{ef}	349.21 ± 17.75 ^{hijk}	362.47 ± 34.56 ^{ghij}	367.91 ± 20.97 ^{fghij}	387.72 ± 18.68 ^{fghij}
	24ºBrix	481.06 ± 34.07 ^{e}	402.26 ± 22.20 ^{fghi}	436.78 ± 18.01 ^{efg}	430.05 ± 37.86 ^{efgh}	443.91 ± 7.60 ^{ef}
	25ºBrix	587.63 ± 45.80 ^{d}	738.32 ± 88.32 ^{ab}	812.88 ± 115.76 ^{a}	649.19 ± 1.08 ^{cd}	704.44 ± 43.23 ^{bc}
∑ Hydroxycinnamic acid	**21ºBrix**	32.71 ± 0.66 ^{kl}	41.51 ± 3.74 ^{ghi}	47.64 ± 1.03 ^{cdef}	47.68 ± 0.59 ^{bcdef}	51.27 ± 0.91 ^{bc}
	23ºBrix	31.55 ± 0.96 ^{l}	36.51 ± 2.32 ^{jk}	37.87 ± 0.81 ^{ij}	39.70 ± 2.02 ^{hij}	43.95 ± 0.87 ^{efgh}
	24ºBrix	43.34 ± 1.60 ^{fgh}	47.14 ± 1.68 ^{cdef}	49.45 ± 0.90 ^{bcd}	52.98 ± 0.93 ^{ab}	56.77 ± 1.50 ^{a}
	25ºBrix	32.98 ± 0.35 ^{kl}	45.14 ± 1.80 ^{defg}	46.30 ± 1.91 ^{def}	49.09 ± 1.25 ^{bcde}	51.55 ± 2.44 ^{bc}
∑ Flavonols	**21ºBrix**	82.55 ± 1.69 ^{e}	84.99 ± 6.94 ^{cde}	83.46 ± 9.80 ^{de}	48.10 ± 10.62 ^{g}	51.49 ± 5.02 ^{g}
	23ºBrix	82.32 ± 7.96 ^{e}	89.47 ± 1.55 ^{bcde}	87.95 ± 4.66 ^{cde}	51.78 ± 0.54 ^{g}	57.04 ± 0.49 ^{fg}
	24ºBrix	96.52 ± 1.28 ^{abc}	102.21 ± 1.27 ^{ab}	96.29 ± 3.38 ^{abcd}	56.96 ± 0.42 ^{fg}	67.82 ± 1.99 ^{f}
	25ºBrix	104.74 ± 3.18 ^{a}	86.42 ± 5.74 ^{cde}	82.12 ± 1.48 ^{e}	49.56 ± 4.42 ^{g}	48.96 ± 2.00 ^{g}
∑ Glucosylated anthocyanins	**21ºBrix**	199.87 ± 11.97 ^{e}	257.14 ± 6.25 ^{bcd}	212.32 ± 10.59 ^{e}	126.26 ± 2.33 ^{fg}	109.21 ± 2.63 ^{fgh}
	23ºBrix	207.94 ± 15.92 ^{e}	272.96 ± 1.84 ^{bc}	226.52 ± 2.92 ^{de}	132.35 ± 8.83 ^{fg}	122.43 ± 1.73 ^{fg}
	24ºBrix	289.75 ± 7.38 ^{ab}	321.26 ± 3.46 ^{a}	247.78 ± 5.97 ^{cd}	146.25 ± 10.77 ^{f}	134.78 ± 3.23 ^{f}
	25ºBrix	250.77 ± 9.20 ^{cd}	137.06 ± 24.97 ^{f}	96.03 ± 28.02 ^{gh}	77.56 ± 15.85 ^{hi}	51.39 ± 17.94 ^{i}
∑ Acetylated anthocyanins	**21ºBrix**	87.15 ± 4.61 ^{e}	105.37 ± 2.62 ^{c}	83.20 ± 4.49 ^{e}	48.18 ± 0.87 ^{fgh}	38.46 ± 1.94 ^{ghi}
	23ºBrix	88.79 ± 7.90 ^{de}	111.00 ± 0.49 ^{bc}	90.47 ± 0.87 ^{de}	52.97 ± 1.62 ^{fg}	42.94 ± 0.89 ^{fghi}
	24ºBrix	124.61 ± 3.43 ^{ab}	131.65 ± 0.33 ^{a}	102.00 ± 3.11 ^{cd}	57.37 ± 2.14 ^{f}	48.67 ± 1.65 ^{fgh}
	25ºBrix	111.55 ± 3.64 ^{bc}	50.56 ± 10.45 ^{fgh}	37.15 ± 10.40 ^{hi}	30.01 ± 5.84 ^{ij}	17.97 ± 4.64 ^{j}
∑ Coumaroylated anthocyanins	**21ºBrix**	28.80 ± 1.53 ^{e}	39.34 ± 0.83 ^{bc}	29.74 ± 2.26 ^{de}	16.43 ± 0.49 ^{fgh}	12.95 ± 1.19 ^{fghi}
	23ºBrix	29.35 ± 1.84 ^{de}	38.23 ± 0.62 ^{bc}	29.23 ± 1.20 ^{de}	15.15 ± 1.24 ^{fghi}	12.74 ± 0.67 ^{ghi}
	24ºBrix	50.24 ± 1.89 ^{a}	48.80 ± 1.70 ^{a}	34.34 ± 1.54 ^{cd}	18.70 ± 2.15 ^{fg}	14.40 ± 0.70 ^{fghi}
	25ºBrix	41.65 ± 2.25 ^{b}	18.27 ± 3.82 ^{f}	12.31 ± 3.65 ^{hi}	8.91 ± 1.48 ^{ij}	5.65 ± 1.66 ^{j}
Polymeric pigments	**21ºBrix**	21.91 ± 0.30 ^{efg}	12.99 ± 2.39 ^{g}	14.43 ± 1.60 ^{g}	16.17 ± 1.72 ^{efg}	14.85 ± 1.11 ^{g}
	23ºBrix	24.21 ± 4.48 ^{def}	15.44 ± 1.31 ^{fg}	17.79 ± 2.00 ^{efg}	20.56 ± 2.11 ^{efg}	19.56 ± 0.87 ^{efg}
	24ºBrix	24.90 ± 2.47 ^{def}	18.98 ± 0.65 ^{efg}	21.42 ± 1.89 ^{efg}	26.24 ± 0.24 ^{def}	25.53 ± 0.59 ^{de}
	25ºBrix	33.02 ± 6.02 ^{cd}	45.74 ± 2.76 ^{ab}	53.38 ± 18.89 ^{a}	39.23 ± 4.28 ^{bc}	50.77 ± 12.51 ^{a}

Additionally, an increase in gallic acid was observed over time (Table 3.9). Although the difference between wine treatments in young wines (AF) were not significant, after MLF, a greater concentration of gallic acid was found in the wines made from 25ºBrix grapes. With the course of time (after 12 months), this difference became insignificant when compared to 24ºBrix. Gallic acid is indirectly related to the wine colour and polymeric pigment formation (Liu, *et al.*, 2016). This increase, observed in all our wines, may come as a result of the dissociation of certain compounds. It is known that gallic acid can be found as the galloyl unit from galloylated proanthocyanidins, but it can also act as a cofactor in the wine (Boulton, 2001; Liu, *et al.*, 2016). Firstly, the liberation of gallic acid could come from the breakdown or hydrolysis of galloylated proanthocyanidins. However, this release have not been proved in wine (Prieur, *et al.*, 1994). Secondly, the gallic acid could be released by the hydrolysis of wine copigments. Thus, the drop in wine copigments from AF to MLF, and especially prevalent in 25ºB wines (Table 3.8), may be linked to the increase in gallic acid concentrations and the increase in polymeric pigments levels during the same period (Table 3.9).

Other phenolic compounds, such as hydroxycinnamic acids and flavonols, were greatly influenced by the time as well as the grape ripeness (Appendix Table 3.13). Similarly to gallic acid, the increase in the total concentration of hydroxycinnamic acids may be explained by the hydrolysis of these copigments, thereby liberating the acids into the wine. As described in literature, these molecules play an essential role not only in the concentration of copigmentation complexes, but also in the formation of pyroanthocyanins over time (Darias-Martín, *et al.*, 2002; Gómez-Gallego, *et al.*, 2013) during the aging of the red wines (Hermosín-Gutiérrez, *et al.*, 2005). After 18 months, the greatest total hydroxycinnamic acid concentration was found in the wines made from 24ºBrix grapes, followed by those from 21º and 25ºBrix grapes. Thus, these results show no clear trend between the increase of grape ripening and the higher hydroxycinnamic acid concentrations over time.

Conversely, the concentration in total flavonols declined over time (Table 3.9). The concentration of these compounds was initially higher in wines made from 24º Brix and 25º Brix grapes. However, their loss with the course of time was quicker in wines made 25ºBrix grapes. By the end of MLF, the flavonol content had significantly dropped in wines made from 25º Brix grapes. On the other side, wines made from the other three grape ripening levels (21ºBrix, 23ºBrix and 24ºBrix) only experienced a significant loss after 12 months of storage.

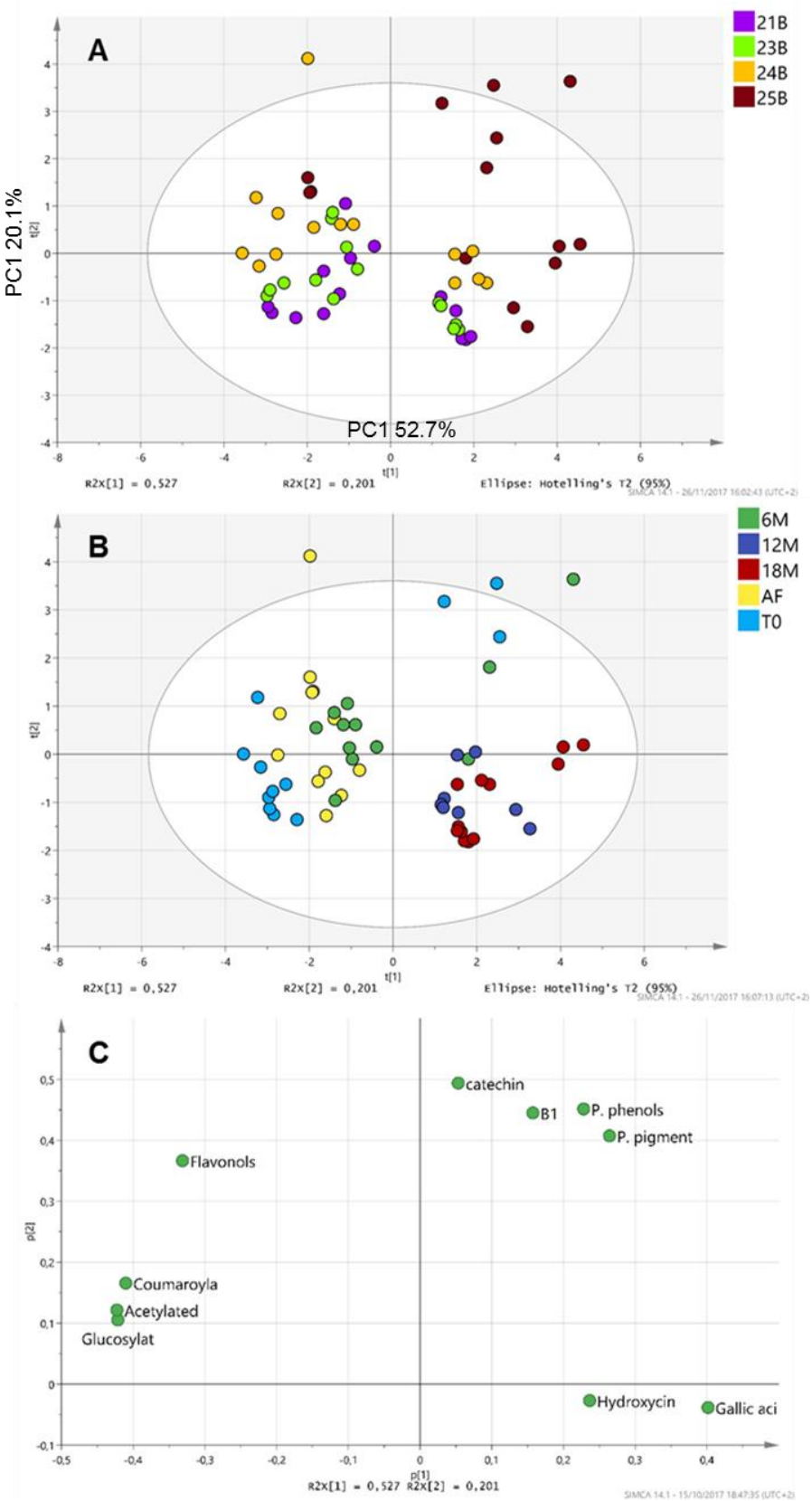

Figure 3.6. Sample distribution according to the groups of individual phenolic compounds measured with the HPLC. A) PCA scores scatter plot coloured according grape ripeness. B) PCA scores scatter plot coloured according to time. C) Loading plot with the group of individual phenolic compounds.

Altogether, the HPLC results described the influence of grape ripeness on the release of specific phenolic compounds which may be more difficult to extract at lower commercial harvest stages. The changes occurring on the grape skin cell walls during grape ripeness may be linked to the release of these molecules. In short, the grape maturity leads to the liberation of larger molecules which may be involved on the formation, of more stable compounds. From there, the colour and phenolic composition in young wines will influence the evolution of the corresponding wines during aging. A general loss of phenolics was observed in all the wines. The loss of wine colour is linked to the degradation of monomeric free anthocyanins and the formation of polymeric pigments. This loss in colour has been extensively reported in literature (Somers & Evans, 1979; He, *et al.*, 2012a). This decrease was already found after 6M of storage (Table 3.9) and remained relatively stable from 12M to 18M. The increase in polymeric phenol levels is also linked to the reactions occurring during wine ageing, as new compounds can be formed from condensation reactions between anthocyanins and tannins (Timberlake & Bridle, 1976; Rivas-Gonzalo, *et al.*, 1995). These compounds only showed a significant increased with time in the wines made from 25ºBrix grapes. Then, the phenolic compounds extracted from riper grapes into wines were more probably susceptible to react and form larger polymers, thereby creating a different ageing trend when compared to the wines made from 21ºBrix, 23ºBrix and 24ºBrix grapes.

3.3.2.4. Sensory evolution of 2015 wines

As in 2014, the aroma and taste and mouth-feel was evaluated for all 2015 wines after 6 and 12 months of storage. Firstly, the wines made from the riper berries (25ºBrix) showed a different aromatic profile, associated with spicy (black pepper, coriander seeds, meaty) descriptors compared to the rest of the wines, described by more green (herbaceous and cooked vegetables) and fruity (cherry, red berries) attributes (Appendix Table 3.5A). This trend remained over time (Appendix Figure 3.5B).

Concerning the taste and mouth-feel, as previously seen in 2014 sensory results, the grape ripening played a significant role. All attributes evaluated by the panellists were significantly different and highly influenced (except the acidity) by the grape ripeness (Appendix Table 3.14). Figure 3.7 illustrates the differences in the intensity for the taste and mouth-feel attributes evaluated after 6 months (Figure 3.7A) and 12 months (Figure 3.7B). Firstly, a clear trend is observed from the wines made from less ripe grapes (made from 21º Brix grapes), described as being more sour, less astringent or bitter and with a lower body (significant when compared to wines made from 25ºBrix grapes), compared

to wines made from riper grapes. The ANOVA and LSD test analysis did not show significant changes in the alcohol burn, astringency, bitterness and body (except for wines made from 21ºBrix grapes where the body of the wines significantly increased) of all the wines from 6 to 12 months (Figure 3.7). On the other hand, the acidity showed a significant decrease in in the case of wines made 21º Brix grapes, but it increased in wines made from 25ºBrix grapes. The opposite trend was observed for the sweetness taste of the wines. Whilst its perception decreased in the wines made from the riper berries (25ºBrix), it significantly increased in the wines made from 21 and 23ºBrix grapes. (Figure 3.7B).

Overall, similar results were reported in literature with higher astringency and bitterness perception (Cadot, *et al.*, 2012; Bindon, *et al.*, 2014a), but also with a greater body structure (Van Noordwyk, 2012) in wines made from riper grapes. Some authors have found a decrease in the astringency as the berry ripens, due to a more harder extraction of compounds from the seeds (Llaudy, *et al.*, 2008). Astringency is mainly driven by the tannin concentration, and especially due to their polymeric forms (McRae & Kennedy, 2011) whereas bitterness is related to the presence of galloylated compounds (mainly extracted from the seeds) and with flavan-3-ols and flavonols (Peleg, *et al.*, 1999; Hufnagel & Hofmann, 2008; Sáenz-Navajas, *et al.*, 2010) but recently has also been linked to the presence of anthocyanin-derived pigments (Sáenz-Navajas, *et al.*, 2017). The larger concentrations of tannins, gallic acid, polymeric phenols or polymeric pigments in the wines made from 25ºBrix grapes, played a role in the greater astringent perception and bitter taste of these wines. The intensity of astringency in our wines correlated with the amount of MCP tannins (R^2=0.69) and the concentration of polymeric phenols (R^2=0.92), which correlates with that of Mihnea, 2016. However, after 6 and 12 months, the concentration of MCP tannins was not significantly higher (ANOVA and LSD test) in wines made from 25ºBrix grapes when compared with the ones made from 24ºBrix grapes (Table 3.8). This may be linked to the degree of polymerization and complexity of the tannins. Unfortunately, none of the current methods used for this project allowed us to further investigate the proanthocyanidins in terms of their complexity.

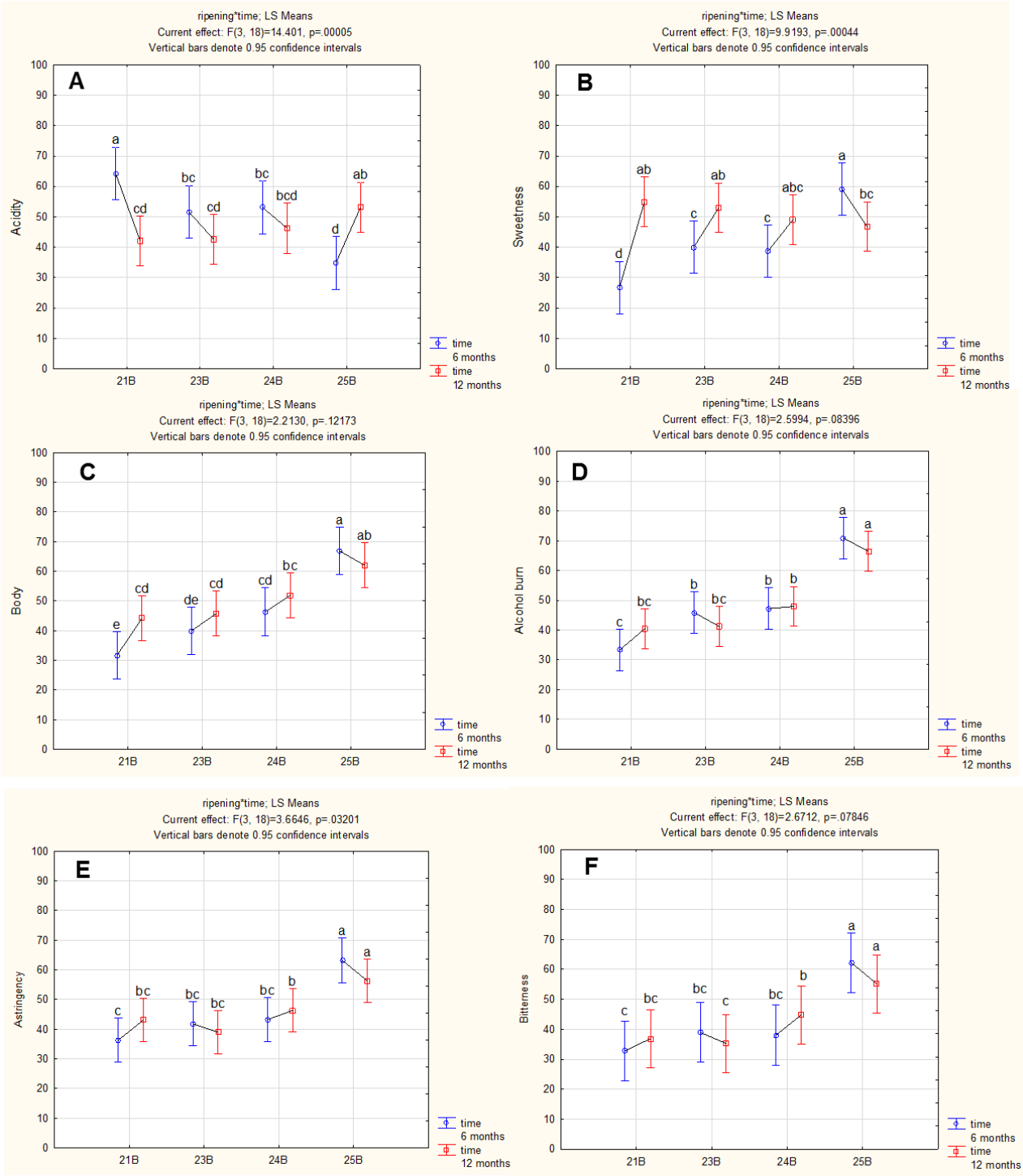

Figure 3.7. Evolution of the taste and mouth-feel attributes intensity (0-100) over time. A) Acidity, B) sweetness C) body, D) alcohol burn E) astringency F) bitterness. The different letters represent significant differences (ANOVA, $p < 0.05$) between the different wines (made from four grape ripeness) and their own evolution over time.

3.4. CONCLUSION

These results confirmed the influence of different stages of grape ripeness on the wine chemical composition, especially on larger molecules. However, slight differences in ºBrix content between VSP and SD in 2014 might have affected the phenolic composition and sensory properties of the wines. In both seasons, a general loss of colour and monomeric anthocyanins was found over time. This loss in phenolic content served to reduce the differences observed in the young wines' treatments in 2014. In parallel, the formation of polymeric phenols and polymeric pigments was also found in all wine treatments and vintages. In 2015, the wines made from 25ºBrix grapes showed a different ageing trend when compared to the rest. The concentration of polymeric phenols and polymeric pigments in these wines was especially higher after ageing. In short, we have shown how different phenolic profiles in young wines, as a result of the natural changes occur during grape ripeness, will also influence the evolution from young to aged wines. Thus, a greater availability of tannins in solution (represented by polymeric phenols) in wines made from 25ºBrix grapes at AF, led to a larger formation of polymeric pigments over time. These results could vary with the use of different vineyard blocks. However, the variability shown within the same vines brings a clear link between extractability of phenolic compounds and ageing potential. The extractability of specific phenolic compounds can therefore be influenced by different factors, such as climatic conditions or vineyard management, affecting the wine ageing potential of the wines. In conclusion, this study supplements the little information available in literature linked to the evolution of colour and phenolic compounds in aged wines (Pérez-Magariño & González-SanJosé, 2004, 2006).

The differences observed during grape ripening, especially between 24ºBrix and 25ºBrix, may be directly related to the structural modification occurring in the grape cell wall architecture. A deeper study on the cell wall structure can help to reach a better understanding of the diffusion of these compounds into the wines. This study could be relevant for the wine industry targeting to unravel the extraction of specific phenolic compounds with a positive impact on the colour stability, as well as in the taste and mouth-feel properties of the wines. The following Chapters will discuss the impact of grape ripening and fermentation on the grape berry phenolic and cell wall polysaccharide composition.

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Chapter 4

Research results

Investigating the relationship between the cell wall polysaccharide structure and grape phenolic compounds in Shiraz.
Part I: vintage and ripeness effect

Chapter 4: Investigating the relationship between the cell wall polysaccharide structure and grape phenolic compounds in Shiraz. Part I: vintage and ripeness effect.

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ABSTRACT

Phenolics compounds evolve during the entire winemaking process and play an important role in colour stability and sensory properties of red wine. In young wines, the phenolic composition is often related to the degree of extractability of the grape phenolic compounds. This study evaluated the skin berry cell wall composition and how these influences grape and wine phenolics at three ripeness levels (21ºBrix, 23ºBrix, and 25ºBrix) and over two consecutive vintages (2015 and 2016). The vintage effect was highly significant, especially in the pectin fraction of the grape cell walls, and the concentration of certain phenolics. The climatic differences between seasons might have influenced the structural differences in their cell wall composition. Firstly, a higher grape and wine phenolic content, especially in polymeric phenols, was found in 2015. Additionally, grape berry cell walls, especially at earliest stages of ripening, were found to be more intact in 2015 compared to 2016 which were more degraded. Thus, a possible relationship was found between berry intactness, especially pectin-rich components, and the corresponding phenolic extractability during the winemaking.

4.1. INTRODUCTION

It is widely accepted that wine phenolic compounds are quality indicators, especially in red wines. Anthocyanins are the main compounds responsible for the colour in grapes and young red wines. On the other hand, proanthocyanidins play a role in the colour stabilisation over time and the taste and mouth-feel properties of red wines (Ribéreau-Gayon, *et al.*, 2006). The composition of the

grapes is the main variable influencing the amount of wine phenolics, however, their high reactivity leads to a continuous evolution, characterised by different chemical and biological interactions and the subsequent formation of new compounds over time (Monagas, *et al.*, 2005; Garrido & Borges, 2013). Several research studies have evaluated the impact of ripeness (Pérez-Magariño & González-SanJosé, 2004; Hanlin & Downey, 2009; Bindon & Kennedy, 2011; Bindon, *et al.*, 2014a; Bindon, *et al.*, 2014b; Hernández-Hierro, *et al.*, 2014; Zietsman, *et al.*, 2015) and different grape phenolic extractability techniques, such as different skin contact times or the addition of enzymes, on the release of phenolic compounds (Sacchi *et al.*, 2005; Gao *et al.*, 2015; Smith *et al.*, 2015; Gao, *et al.*, 2016). Anthocyanins are extracted from the grape skins (Ribéreau-Gayon, *et al.*, 2006; He, *et al.*, 2012). These compounds generally accumulate in the grape skins, becoming more extractable during the course of ripening (Hernández-Hierro, *et al.*, 2012, 2014). However, a possible decrease in concentration has been reported in the later stages of ripening and in over-ripe grapes (Ryan & Revilla, 2003; Fournand, *et al.*, 2006; Pérez-Magariño & González-SanJosé, 2006). On the other hand, the accumulation and extractability of grape proanthocyanidins show different trends during grape maturation. Tannins can be found as free molecules in the vacuoles or bound to cell wall proteins or polysaccharides (Amrani Joutei, *et al.*, 1994). Furthermore, the differences in the nature of skin-derived and seed-derived tannins influence their affinity for cell wall material (CWM) and their extractability (Geny *et al.*, 2003; Hanlin *et al.*, 2010; Bindon & Kennedy, 2011; Bindon, *et al.*, 2014b; Quijada-Morín *et al.*, 2015). Recent studies have shown that the skin proanthocyanidins are likely to become more extractable as the grapes are harvested later (Bindon, *et al.*, 2013), whereas the seed tannin extractability appears to decrease during ripening (Kennedy, *et al.*, 2000; Peyrot Des Gachons & Kennedy, 2003; Bautista-Ortín, *et al.*, 2012). Additionally, it has been demonstrated that the proanthocyanidin extraction is also influenced by the anthocyanin content (Kilmister, *et al.*, 2014; Bautista-Ortín, *et al.*, 2016a).

The loss of berry firmness is characteristic of the fruit softening process occurring during the ripening process (Brummell, 2006). This fruit softening is linked to the degradation and solubilisation of berry skin and pulp tissue cell wall polysaccharide, by the action of pectolytic enzymes, together with an increase in its protein content (Nunan, *et al.*, 1998; Huang, *et al.*, 2005; Ortega-Regules, *et al.*, 2008; Goulao, *et al.*, 2012). The polysaccharides in the CWM can be divided into those that are either water or hydroalcoholic soluble (Vicens, *et al.*, 2009). All these molecules are distributed in two main tissues, a pectin-rich fraction and a hemicellulose-rich fraction. The former is characterized by a pectin layer rich in galacturonic acid (GalA) content, consisting mostly of homogalacturonan (HG) and rhamnogalacturonan-I (RG-I) (Gao, *et al.*, 2015). The principal changes occurring during fruit ripening are associated with a general decrease in GalA, linked to the de-pectination process and changes in the methyl esterification of the HG (Ortega-Regules, *et al.*, 2008). These structural changes in the cell wall framework, such as the progressive loss of arabinan and galactan side chains of RG-I may increase the skin porosity and affect pectin solubilisation (Brummell, 2006; Bindon, *et al.*, 2010). Additionally, the loss in xyloglucan content during ripening also affects the

firmness and subsequently the berry integrity, although in some studies these were found to be not significant (Ortega-Regules, *et al.*, 2008; Vicens, *et al.*, 2009; Bindon, *et al.*, 2013). All these compositional changes can play a role in the phenolic extractability.

From crushing, the grape berry cell walls firstly experience a physical breakdown, followed by a de-pectination linked to the release of phenolic compounds during the alcoholic fermentation. These phenolic compounds can interact with other phenolics or with the polymers of the cell walls. During this skin maceration, some cell wall polysaccharides and proteins are released into the wines (Guadalupe & Ayestarán, 2007; Gao, *et al.*, 2015). Therefore, a strong association between different groups of phenolics and berry cell wall components can potentially influence their extractability during alcoholic fermentation and final concentration in the wine (Bindon, *et al.*, 2014b). This association occurs by hydrophobic interactions and hydrogen bonds. The strong affinity between tannins and the cell wall pectin layer, especially to HG and RG-I (Watrelot, *et al.*, 2013, 2014) has been reported in apples and grapes (Renard *et al.*, 2001; Ruiz-García *et al.*, 2014). Additionally, some studies have described this greater exposure of the cell wall surface as an increase in the number of active binding sites (Bindon, *et al.*, 2010, 2012; Castro-López, *et al.*, 2016), potentially leading to the retention of the proanthocyanidins, which is highly influenced by the tannin molecular weight (Renard, *et al.*, 2001; Le Bourvellec, *et al.*, 2004; Bindon, *et al.*, 2012) and nature (Quijada-Morín, *et al.*, 2015). The higher the degree of polymerisation (DP) in grape proanthocyanidins, the greater the number of reactive sites allowing for increased interaction between pectins and proanthocyanidins (Le Bourvellec, *et al.*, 2004; Watrelot, *et al.*, 2017). In the same manner, the degree of methyl-esterification in HG also increases the affinity of the CWM for the proanthocyanidin molecules (Watrelot, *et al.*, 2013).

Different winemaking practices, such the use of commercial enzymes, can increase the cell wall de-pectination, thereby enhancing the release of phenolics compounds into the wine during alcoholic fermentation (Guadalupe & Ayestarán, 2007; Zietsman, *et al.*, 2015; Gao, *et al.*, 2016). Parameters, such as the presence of chelating agents or pH, can influence the concentration of these polysaccharides released into the wines (Gil Cortiella & Peña-Neira, 2017). In short, the final concentration of cell wall proteins and polysaccharide diffused are important to the wine colloidal stability as these can enhance or inhibit tannin self-aggregation and bind to other components in solution (Riou, *et al.*, 2002; Poncet-Legrand, *et al.*, 2007; Watrelot, *et al.*, 2017). Additionally, recent studies have been showing the adsorption-desorption phenomena between phenolic compounds and CWM. The higher molecular mass proanthocyanidins, as well as molecules with a higher percentage of galloylation, show a higher preference to be retained by the CWM (Bindon, *et al.*, 2010; Bautista-Ortín, *et al.*, 2016b).

Given the importance of the grape skin polysaccharides, the present work aims to establish a link between the grape berry cell wall structure and the grape phenolic composition during ripeness and how the relationship between both factors will influence the phenolic extractability. To date, limited

information is available regarding the polysaccharide composition of the grape berry during ripening and especially its relationship with the grape phenolics (Quijada-Morín, *et al.*, 2015). The use of new techniques, such as Comprehensive Microarray Polymer Profiling (CoMPP) have been recently reported in grape and wines to indirectly evaluate their polysaccharide profile (Gao *et al.*, 2015; Zietsman *et al.*, 2015, 2017; Gao, *et al.*, 2016). The work form Zietsman and co-workers (2015) in Pinotage grapes showed a clear difference in the composition of the grape cell walls components due to different ripeness levels. The author found that the cell wall structure in riper berries was less affected by the addition of commercial enzymes, as they were naturally more depectinated. In addition, Gao *et al.*, (2015) used the same technique in Cabernet Sauvignon to describe the impact of the use of different commercial enzymes on the cell wall deconstruction and the subsequent release of phenolic compounds during the winemaking process. From there, the present work focused in Shiraz with the aim of investigating the link between grape berry cell wall composition and the release of phenolic compounds into the wine. The study included the vintage effect, as it was performed during two consecutive seasons (2015 and 2016), and the impact of the progress of ripening, using ºBrix as the ripeness indicator in the same vineyard. For this purpose, we analysed the changes occurring in the cell walls of fresh skins and pomace (at a monosaccharide and polysaccharide level) and the phenolic composition of fresh grapes and their corresponding wines made from Shiraz grapes harvested at three different ripeness levels.

4.2. MATERIALS AND METHODS

4.2.1. Grapes and winemaking procedures

Shiraz grapes (SH9C clone) were harvested at three ripeness levels (21ºBrix, 23ºBrix, and 25ºBrix) during two consecutive harvest seasons (2015 and 2016) from the Welgevallen experimental farm (GPS coordinates: 33°56'25.0"S 18°51'56.4"E), vineyard of the Department of Viticulture and Oenology of Stellenbosch University. The same vines, with a Smart-Dyson trellising system on a 101-14Mgt rootstock, were used during both vintages. Vines from the same rows were alternatively harvested for the three ripeness levels. With the objective to reduce the vineyard variability, approximately 120 kg of grapes (per ripeness level) were randomly harvested from six different rows, mixed in the cellar and finally split into three repeats. The winemaking process followed is described in Chapter 3.

4.2.2. Skin cell wall preparation

Fresh berries were collected from three ripeness levels during both seasons. The skins were manually separated from the flesh and seeds. The same procedure was applied to fermented pomace. Afterwards, the skins were ground to a fine powder under liquid nitrogen using a Retsch Mixer Mill (30 round/minute, 30 second, Retsch, Haan, Germany). The ground material was then boiled in 100 % ethanol for 20 minutes to ensure the deactivation of any possible endogenous enzymatic activity. After centrifugation (3 min, 3000 rpm), the pellet was then washed with the following solvent mixtures: 100 % ethanol, methanol:chloroform (1:1), methanol:acetone (1:1), and acetone, to generate an alcohol-insoluble residue (AIR) (Gao, *et al.*, 2015). The dry AIR powder was then re-suspended in water, frozen, and freeze-dried. The samples were stored at room temperature prior to the subsequent analysis. Between four to five AIR samples were prepared per biological triplicate.

4.2.3. Monosaccharide analysis of cell wall samples

The degradation occurring in the cell wall was analysed by the measurement of the following nine major cell wall monosaccharides: arabinose (Ara), fucose (Fuc), rhamnose (Rha), xylose (Xyl), mannose (Man), galacturonic acid (GalA), galactose (Gal), glucose (Glu), and glucuronic acid (GlcA). Thus, AIR samples from fresh grapes and fermented pomace of each fermentation were analysed as described in Gao *et al.* (2015), following a modified method based on that of York *et al.*,(1986), using gas chromatography coupled with mass spectrometry (GC–MS) to determine cell wall monosaccharides. The AIR samples were hydrolysed into monosaccharides using 2 M trifluoroacetic acid (TFA) for 2 h, at 110ºC and then converted to their methoxy derivatives using methanol/methanol HCl (16 h, 80ºC), followed by silylation with hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine (HMDS/TMCS/pyridine) (3:1:9, Sylon HTP kit, Sigma-Aldrich, MO, USA). The separation and analysis of each of these derivatives were performed using a gas chromatograph (Agilent 6890 N, Agilent Technologies, CA, USA) coupled to an Agilent 5975 MS mass spectrometry fitted with a polar (95% dimethylpolysiloxane) ZB-Semivolatiles Guardian GC column (30 m, 0.25 mm ID, 0.25 µm film thickness). The analysis were performed under the following conditions: the oven temperature was maintained at 70 °C for 2 min, followed by an increase to 76 °C at a rate of 1 °C/min and then increased to 300 °C at 8 °C/min. The final oven temperature was held for 5 min (Zietsman, *et al.*, 2015).

4.2.4. Comprehensive Microarray Polymer Profiling (CoMPP) analysis of cell wall fractions

A semi-quantitative approach was used to analyse the polysaccharide fraction of the grape berry cell walls. For this purpose, comprehensive microarray polymer profiling (CoMPP) was performed on the

AIR samples, thereby profiling the polysaccharide composition in the pectin- and hemicellulose-rich fractions of the grape pomace. Following the procedure described in Moller *et al.* (2007), approximately 10 mg of AIR samples were sequentially extracted using the solvents CDTA (diamino-cyclo-hexane-tetra-acetic acid) for the pectin fraction and NaOH to extract a hemicellulose-rich fraction. The nitrocellulose arrays printed (in triplicate) with the different fractions were probed individually with different antibodies (mAbs) and carbohydrate-binding module (CBMs) that recognise cell walls polymers. A mean spot signal was calculated and the highest mean signal in the dataset was set to 100 and the rest of the dataset was normalised accordingly.

4.2.5. Colour and phenolic measurements

4.2.5.1. Spectrophotometric analysis

Grape phenolic analyses were performed following the soft “wine-like” extraction method described in Chapter 3. The same extraction procedure was applied to every treatment analysed during both vintages, 2015 and 2016. The same colour and phenolic analysis reported in Chapter 3 were also performed in this work. Three grape “soft extractions” were performed for each biological ferment of each ripeness level.

4.2.5.2. HPLC analysis

Monomeric and polymeric forms were individually quantified for fresh grapes and wines after completing the alcoholic fermentation (AF). The grape extraction procedure for HPLC analysis described in Chapter 3 was also used in this case. The individual quantification of phenolic compounds was performed with the HPLC method also described in Chapter 3. Data processing was performed with Agilent ChemStation software (Agilent Technologies) using the following phenolic standards: gallic acid, (+)-catechin, caffeic acid, p-coumaric acid, quercetin-3-glucoside, quercetin from Sigma-Aldrich Chemie,(Steinheim, Germany), and malvidin-3-glucoside from Extrasynthese (Lyon, France).

4.2.6. Statistical analysis

Most statistical analyses were carried out using Statistica 13.2 (TIBCO Statistica software, Palo Alto, CA, USA). Mixed model repeated measures ANOVAs were used and Fisher's least significant difference (LSD) corrections were used for post-hoc analyses. Significant differences were judged on a 5% significance level ($p \leq 0.05$). The distribution of certain chemical and sensory datasets were

analysed with Principal Component Analysis (PCA) using SIMCA 14.1 software (Sartorium Stedim Biotech - Malmö, Sweden).

4.3. RESULTS

4.3.1. General grape parameters at harvest

Table 4.1 summarises the main grape parameters (ºBrix, pH, TA, and berry weight) at the three ripeness levels investigated during 2015 and 2016. These parameters showed typical grape maturation trends, *i.e. a* decrease in acidity and an increase in the pH, with an increase in the total soluble solids (ºBrix) of the grapes. A similar weight loss during ripening was found for both seasons. However, in 2016, a longer period was required to reach 25 ºBrix compared with 2015 grapes.

The harvest season in 2016 in Stellenbosch area was characterised by a drier season than in 2015, preceded by a warm summer with a lower than usual rainfall (VinPro, 2015, 2016). The mean maximum temperatures, as well as the mean of the minimum temperatures, between December and March were higher in 2015/2016 when compared to the same period in the previous season, 2014/2015. Additionally, during this period the maximum monthly temperatures registered 39.4, 41.8 and 41.1ºC in the vineyard for December 2014, January 2015 and February 2015 respectively. However, in the following vintage during the corresponding months these temperatures were 46.6, 43.4 and 43.2ºC respectively. The high temperatures in December 2015 might have affected not only the phenolic biosynthesis, affecting their accumulation during ripening, but also the cell wall integrity. However, the temperatures warmed up in the end of February and March 2015, leading to an earlier last of harvest compared to 2016, as it is shown in Table 4.1 (from 23ºBrix to 25ºBrix).

Table 4.1. Grape parameters analysed at harvest of fresh Shiraz grapes at three different ripeness stages (21ºBrix, 23ºBrix and 25ºBrix) during two different vintages (2015 and 2016).

	2015			2016		
	21ºBrix	**23ºBrix**	**25ºBrix**	**21ºBrix**	**23ºBrix**	**25ºBrix**
Harvest date	16-Feb	27-Feb	04-Mar	16-Feb	29-Feb	14-Mar
ºBrix	21.70 c	23.33 b	25.37 a	20.80 d	23.03 b	25.00 a
	0.10	0.22	0.18	0.15	0.26	0.15
pH	3.44 a	3.63 b	3.92 c	3.40 a	3.55 b	3.91 c
	0.01	0.02	0.02	0.03	0.02	0.09
TA (g/L)	5.51 a	4.70 b	4.78 b	5.59 a	5.56 a	4.73 b
	0.11	0.04	0.09	0.13	0.07	0.29
Berry weight (g)	1.24 a	1.16 ab	1.04 b	1.21 a	1.09 ab	1.05 b
	0.05	0.09	0.06	0.03	0.03	0.03

4.3.2. Influence of the vintage and ripeness on the monosaccharide composition of the grape skin cell walls

Figure 4.1 illustrates the content in mol % of the nine major monosaccharides in the skins of the fresh grapes harvested at three different ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) during two consecutive vintages. From our results no clear relationship was observed between grape ripeness (from 21ºBrix to 25ºBrix) and the overall monosaccharide composition (Appendix Table 4.1). Other studies have focused on the evolution of the grape berry cell walls from véraison (Nunan, *et al.*, 1998; Vicens, *et al.*, 2009). In this study, the three ripeness levels were during the commercial harvest, when the berry is developed. However, the combination between ripeness and vintage was found to be significant (Appendix Table 4.1). Arabinose (Ara), xylose (Xyl), galacturonic acid (GalA) and glucose (Glu) were affected by the grape ripeness and vintage, especially Ara and GalA. In 2015, GalA decreased from 47% to 38% (from 23ºBrix to 25ºBrix), as a part of this de-pectination process occurring during ripening. On the other hand, in 2016, the proportion of GalA remained constant. Therefore, these results suggest a vintage effect according to the GalA data. This higher amount in GalA levels in 2015 can be linked to a firmer berry, with a more intact pectin layer. Thus, the grape pomace in 2015 was more intact, especially when comparing the earliest ripeness levels (21 ºBrix grapes).

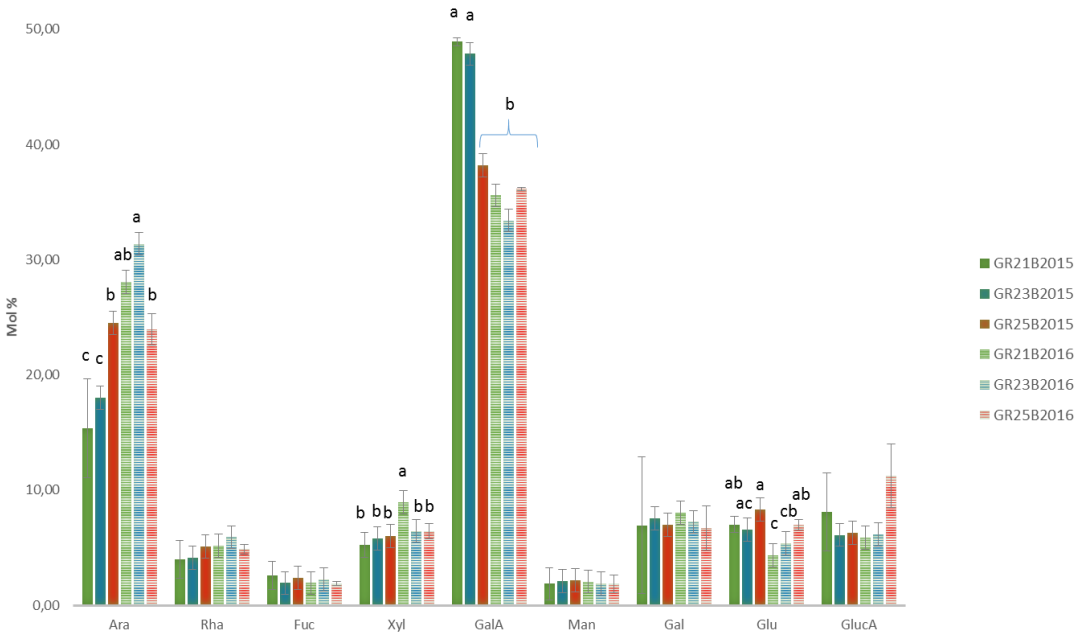

Figure 4.1. Monosaccharide composition (mol %) of fresh Shiraz grapes at three different ripeness stages (21ºBrix, 23ºBrix and 25ºBrix) during two different vintages (2015 and 2016). Different letters illustrate the significant differences (ANOVA, LSD test) between the three ripeness and two vintages within the individual monosaccharides. The nine major monosaccharides are displayed: Ara (arabinose), Rha (rhamnose), Fuc (fucose), Xyl (xylose) GalA (galacturonic acid), Man (mannose), Gal (galactose), Glu (glucose), GlucA (glucuronic acid).

Ara was found to be the second major monosaccharide for both seasons. It can be generally found as side chain residues part of rhamnogalacturonan I (RG-I) in the pulp and skin tissue or as part of the arabinogalactan-proteins (AGPs) (Vidal, *et al.*, 2001; Gao, *et al.*, 2015). Moreover, a greater % of Ara is related to a higher proportion of hairy region (RG-I) and a higher exposure of the hemicellulose fraction, due to a more depectinated pectin layer (Gao *et al.*, 2016). The % of Ara was significantly higher in 2016 compared to 2015. A higher arabinose and galactose content in coffee leaves has been reported due to these plants being under heat stress (Lima, *et al.*, 2013). However, the trends during ripening were different between the two vintages. In 2015, the mol % of Ara increased during ripening, being only significant from 23ºBrix to 25ºBrix (18% to 24 mol %) as illustrated in Figure 4.1. Since the results are expressed in relative mol %, this can be explained by the decrease in GalA in the de-pectination process. The opposite trend was observed from 23ºBrix to 25ºBrix in 2016. Ripeness had a greater impact in 2015 than 2016 (Appendix Table 4.2 and 4.3) which might be related with the differences in the climatic conditions at an early stages of berry development. This lower GalA content and the higher proportion in Ara also led to a greater degree of de-pectination, and thus softer and more degraded grape berries, in 2016.

4.3.3. Influence of the vintage and ripeness on the polysaccharide composition and structure of grape skin cell walls

The polysaccharide composition of the fresh grape skins and the pomace's skins were analysed after extracting the cell wall material with CDTA, yielding a pectin rich extract, and with NaOH, that extracts mostly hemicelluloses. Different polysaccharide polymers were measured by using sets of monoclonal antibodies (mAbs) and carbohydrate binding molecules (CBMs) which reacted towards certain cell wall polysaccharides and proteins (epitopes) in these extracts. Therefore, CoMPP provides different, additional information to complement the monosaccharide results and interpret the cell wall structure by the relative abundance of the cell wall polysaccharides and proteins due to a greater exposure to their specific antibodies or solubilisation within the extraction medium. As described in section 4.3.2, the differences in the skin polysaccharides were overall greater between vintages than the ripeness. The overall CoMPP results supported the monosaccharide data indicating more intact grape berry cell walls in 2015. From the CoMPP results, ripeness has an impact on the polymers of the grape berry cell walls (Appendix 4.4 and 4.5).

Firstly, as expected, homogalacturonan (HG) and rhamnogalacturonans (RG-I) were the main group of polysaccharides found in the pectin fraction (CDTA extraction). The higher levels of GalA in 2015 grapes were confirmed by the slightly higher signal intensity of some of the HG epitopes (LM18, LM19, LM20, 2F4) in riper berries. In Figure 4.2 one can see the separation of their three ripeness levels along the PC1 in Figure 4.2A1/B1 (47%), and Figure 4.2A2/B2 (50%) is shown. The distribution of some HG epitopes (LM18, LM19, 2F4), was associated with the progression of de-

pectination during ripening for both seasons. However, HG showed variable trends for the ripening within and between the seasons (2015 and 2016).

These small variations, as is the case occurred with JIM7, are better explained when looking at their relative abundance in the heatmap displayed in Figure 4.3. The antibody JIM7 (which react with highly esterified HG) progressively decreased (in the CDTA fraction) from 21ºBrix to 25ºBrix in 2015 whereas it increased in 2016. The degree of esterification influences the affinity and binding between polysaccharides and to proanthocyanidins, and it affects the skin porosity (Hanlin, *et al.*, 2010; Watrelot, *et al.*, 2013). These results in 2016 could be interpreted as an easier extraction with the CDTA due to a greater solubility of the specific epitopes/polysaccharides while the grape skins of 2015 seemed to have a more intact HG layer. This is also illustrated by the relative higher abundance of Ca^{2+} cross-linked HG (mAb 2F4) indicating a firmer middle lamella in the grape cell walls in 2015. The increase in exposure in these Ca^{2+} cross-linked HG in the 2016 grapes is the most obvious pattern correlating with the ripeness. The cell walls of riper grapes is more porous (Bindon, *et al.*, 2012; Bindon, *et al.*, 2014b) probably due to pectin degradation by the plant endogenous enzymes.

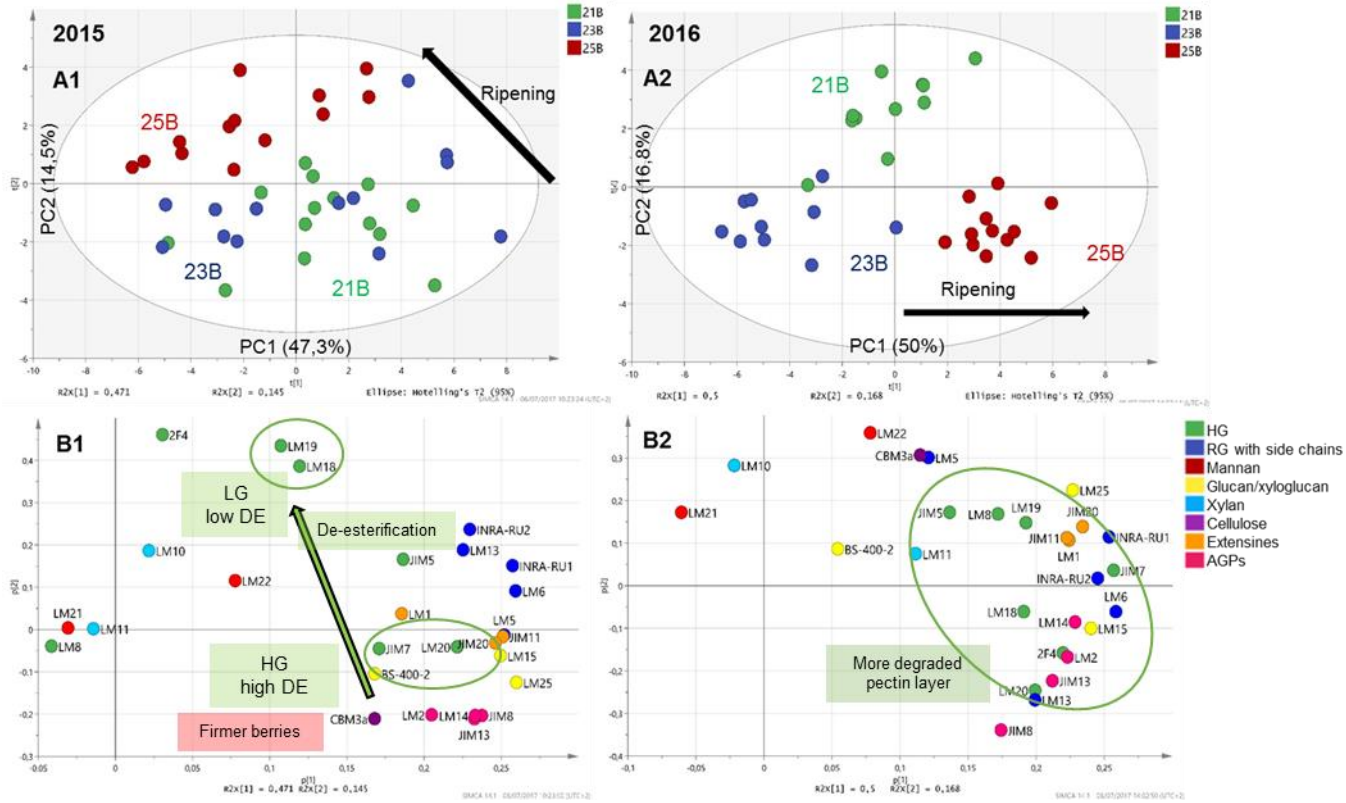

Figure 4.2. PCA plot and Loading plot of the CDTA extract (pectin-rich fraction) in three different ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) of Shiraz grapes during two consecutive vintages (2015 and 2016). In the PCA, the samples are coloured according to the ripeness levels. In the loading score plots, the different polysaccharides and proteins epitopes are coloured by groups.

	Year	Ripening	HG partially/de-esterified (mAb JIM5)	HG partially esterified (mAb JIM7)	HG partially/de-esterified (mAb LM18)	HG partially/de-esterified (mAb LM19)	HG partially esterified (mAbLM20)	HG Ca2+ crosslinked (mAb 2F4)	Xylogalacturonan (mAb LM8)	Backbone of rhamnogalacturonan I (mAb INRA-RU1)	Backbone of rhamnogalacturonan I (mAb INRA-RU2)	(1→4)-β-D-galactan (mAb LM5)	(1→5)-α-L-arabinan (mAb LM6)	Linearised (1→5)-α-L-arabinan (mAb LM13)	(1→4)-β-D-(galacto)/(gluco)mannan (mAb LM21)	(1→4)-β-D-(gluco)mannan (mAb LM22)	(1→3)-β-D-glucan (mAb BS-400-2)	Xyloglucan (XXXG motif) (mAb LM15)	Xyloglucan/unsubstituted β-D-glucan (mAb LM25)	(1→4)-β-D-xylan (mAb LM10)	(1→4)-β-D-xylan/arabinoxylan (mAb LM11)	Cellulose (crystalline) (mAb CBM3a)	Extensin (mAb LM1)	Extensin (mAb JIM11)	Extensin (mAb JIM20)	AGP (mAb JIM8)	AGP (mAb JIM13)	AGP (mAb LM14)	AGP, β-linked GlcA (mAb LM2)
CDTA GRAPES	2015	21B	47	82	30	28	8	22	0	34	22	10	28	9	11	0	0	0	8	0	0	5	7	5	5	9	24	0	13
	2015	23B	44	79	29	28	7	24	0	34	24	8	28	10	5	0	0	0	8	0	0	0	8	5	5	8	22	0	13
	2015	25B	44	75	32	31	7	27	0	33	24	6	27	10	13	0	0	0	6	0	0	0	7	0	0	5	16	0	10
	2016	21B	46	82	21	28	0	11	0	37	26	7	26	0	9	0	0	0	9	0	0	14	5	0	7	0	18	5	8
	2016	23B	47	86	23	25	0	13	0	32	20	5	24	0	8	0	0	0	0	0	0	9	0	0	0	0	20	0	8
	2016	25B	45	90	29	29	0	22	0	39	30	5	32	8	0	0	0	0	9	0	0	10	5	0	8	10	31	7	16
NaOH GRAPES	2015	21B	0	0	0	5	0	0	0	15	9	15	16	5	22	0	5	51	52	0	0	24	29	13	18	7	20	0	0
	2015	23B	0	0	0	0	0	0	0	16	10	14	16	6	18	0	9	50	52	0	0	24	30	12	20	6	19	0	0
	2015	25B	5	0	0	9	0	0	0	15	10	12	15	6	22	0	7	46	48	0	0	21	26	10	17	5	16	0	0
	2016	21B	0	0	0	12	0	0	0	20	14	18	19	6	22	0	8	62	72	6	0	53	16	5	15	0	17	6	0
	2016	23B	6	0	14	14	0	0	0	13	14	14	17	7	18	0	0	81	64	0	0	50	22	9	18	6	20	5	0
	2016	25B	0	0	0	0	0	0	0	17	20	12	18	7	14	17	5	76	66	7	6	55	29	8	21	6	17	5	0
										0		10		20		30		40		50		60		70		80		90		100

Figure 4.3. Heat map of the epitope abundance (0-100) in CDTA and NaOH extracts (CoMPP results) of fresh Shiraz grapes at three different ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) during two consecutive seasons (2015 and 2016). Values lower than 5 are indicated as 0.

Furthermore, the NaOH extract also indicated a more intact cell wall in 2015. As expected, the rhamnogalacturonans (RG-I) were the second major constituent of the pectin layer. However, the RG-I epitopes were extracted not only with the CDTA but also with the NaOH (Figure 4.3). The signals associated with RG-I (INRA-RU1, INRA-RU2, LM5, LM6) in the NaOH extract showed a RG-I coating layer strongly associated with xyloglucans and cellulose microfibrils. Moreover, a higher proportion of xyloglucans (LM15 & LM25) and cellulose (CBM3a) was found in the NaOH extract in 2016. The greater abundance of these epitopes presumably comes as a consequence of less intact RG-I coating layer, confirming a de-pectination level in the grape pomace from 2016 (Figure 4.3). Looking at the evolution during ripeness, as illustrated in Figure 4.4, the PCA plots of the NaOH extract show a clear trend between the three ripeness levels, especially for 25ºBrix, separated along their respective PC1. The separation along the PC1 is driven by an increase in the exposure of the inner layers during grape ripeness.

The protein results supported the polysaccharide data (Figure 4.3). We found a similar abundance in the structural proteins epitopes, represented by extensins (LM1, JIM11 and JIM20) and arabinogalactan-proteins (AGPs) (JIM8, JIM13, LM14 and LM2) in the CDTA extract. The AGP's recognized by JIM13 shows a small ripeness effect in the 2016 data. The amount of AGPs epitopes in the NaOH was similar between the two vintages and there was a small increase with ripeness in the epitope for extensins (mAbLM1 and JIM20) in 2016.

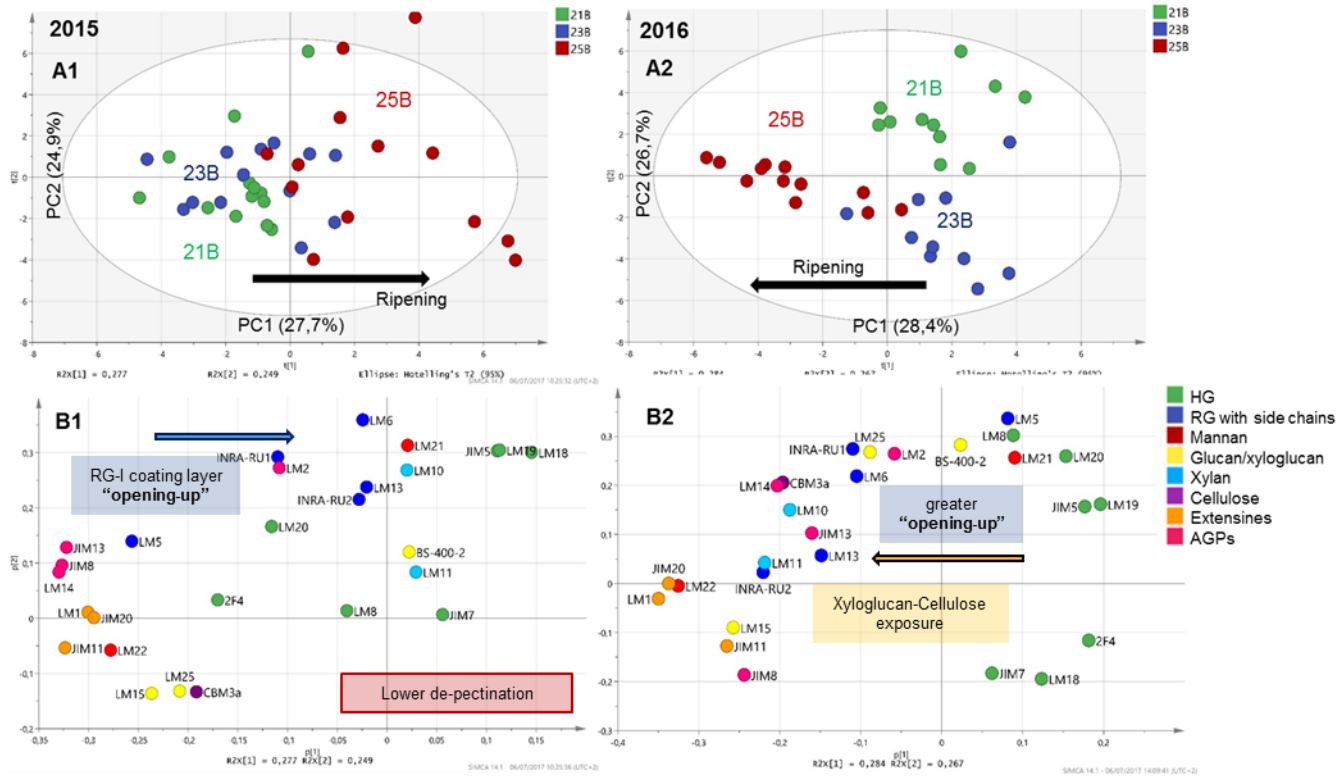

Figure 4.4. PCA plot and loading plots for the NaOH extract (hemicellulose-rich fraction) in three different ripeness (21ºBrix, 23ºBrix and 25ºBrix) of Shiraz grapes during two consecutive vintages (2015 and 2016). In the PCA plots, the samples were coloured according to the ripeness levels. In the loading score plots, the different polysaccharides and proteins epitopes are coloured by groups.

4.3.4. Influence of the vintage and ripeness on the phenolic composition of fresh grapes

The grapes were the main source of phenolics in the wines, as no commercial tannin or oak additions were made to the wines. The chemical profiling of the fresh grapes was therefore correlate grape cell wall structural changes with the wine phenolics. Grape phenolic concentrations normally increase during grape ripening, but can decrease in concentration in over ripe grapes (Adams, 2006). The climatic conditions can also affect their biosynthesis and accumulation patterns (Ristic, *et al.*, 2006; Sun, *et al.*, 2017). From our results, a strong and significant vintage effect was also found for the grape phenolic composition (Appendix Table 4.6 and Appendix Table 4.7). Therefore, the phenolic accumulation patterns during ripening were different between 2015 and 2016.

Overall, from the "soft" wine-like extraction (Table 4.2), the vintage was found to be a determining factor (Appendix Table 4.6). However, similar results were found, between the vintages, regarding the amount of total phenolics (TP). The content in grape TP increased from 21ºBrix to 23ºBrix and afterwards did not change significantly. However, the concentration of anthocyanins and tannins during ripening was different between 2015 and 2016. The levels of anthocyanins were similar between the both seasons at 21ºBrix and 23ºBrix. In contrast, in 2015, the concentration of free anthocyanins decreased in the final ripening phase (25ºBrix) whereas it increased in 2016 (Table 4.2). The increase in anthocyanin extractability has been widely reported in literature (Gil-Muñoz, *et*

al., 2011). However, similar to what was found in our 2015 results, the levels of anthocyanins can also experience a decrease in overripe grapes (Ryan & Revilla, 2003). Regarding the grape tannin, no significant variation was found in their concentration during ripening in 2016, This is contrary to the results found in 2015 where an increase in the grape tannin concentration was observed from 23ºBrix to 25ºBrix. These results in 2016 agree with findings previously reported by some authors (Harbertson, *et al.*, 2002; Canals, *et al.*, 2005). Nevertheless, the conditions of the "soft extraction" do not favour the extraction of phenolics from seeds. The tannin concentration might thus be underestimated as it has been shown that the seed coat requires a hydration period to become more easily extractable (Cadot, *et al.*, 2006).

Table 4.2. Grape phenolics extracted from the "soft berry extraction" for the three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) in fresh Shiraz grapes 2015 and 2016. The concentrations of tannin and anthocyanin are expressed in mg/g of berry.

	2015						2016					
	21ºBrix		23ºBrix		25ºBrix		21ºBrix		23ºBrix		25ºBrix	
Tannins mg/g of berry	0.37	a	0.41	a	0.65	b	0.51	ab	0.59	b	0.47	ab
	0.04		0.01		0.09		0.04		0.12		0.07	
Anthocyanins mg/g of berry	0.37	a	0.37	a	0.26	b	0.31	ab	0.37	a	0.49	c
	0.02		0.01		0.01		0.02		0.04		0.03	
TP (AU)	21.84	d	40.45	bc	36.64	c	35.24	c	45.41	ab	50.61	a
	0.77		1.81		2.91		2.45		1.64		4.03	

TP: total phenolics (AU)

Nevertheless, the overall individual phenolics results (Table 4.3) also showed a strong vintage effect (Appendix Table 4.7), affecting the concentration of the individual phenolic compounds, but also confirmed some patterns, such as the decrease in anthocyanins in 2015, observed with the "soft extraction" (Table 4.2). The vintage effect was clearly observed when comparing the grapes at 21ºBrix from both seasons. At that stage, the concentration of the individual phenolic compounds (except catechin) was significantly lower in 2016 than in 2015. From then, different trends were observed during ripening with a general lower variation in 2016. This was clearly observed in the concentration of B1 (Table 4.3). In 2015, B1 showed a significant decrease during grape ripening (from 21ºBrix to 25ºBrix). However, in 2016, levels did not change significantly during ripening.

Table 4.3. Individual and groups of phenolic compounds µg/g for the three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) in fresh Shiraz grapes 2015 and 2016.

	2015						2016					
	21ºBrix		23ºBrix		25ºBrix		21ºBrix		23ºBrix		25ºBrix	
Gallic acid	2.41	a	2.03	b	1.49	c	1.45	c	0.98	d	1.29	cd
	0.24		0.07		0.05		0.11		0.02		0.06	
Catechin	7.81	b	6.94	b	16.97	a	16.86	a	8.96	ab	7.90	b
	0.34		0.88		5.15		3.97		1.31		0.97	
B1	23.94	a	17.19	ab	13.45	b	13.01	b	9.03	b	10.21	b
	1.29		0.40		1.48		3.60		2.70		4.39	
Polymeric phenols	1521.39	ab	1766.92	a	1410.14	bc	1022.51	d	1160.52	cd	923.75	d
	113.86		173.70		4.68		23.75		141.38		20.34	
∑ Hydroxycinnamic acids	14.24	a	15.25	a	7.69	ab	5.61	b	9.34	ab	10.02	ab
	4.46		4.05		0.05		0.29		0,00		0.82	
∑ Flavonols	213.97	a	187.56	ab	158.85	bc	120.87	cd	127.85	cd	107.80	d
	10.90		22.09		12.48		8.52		5.84		15.49	
∑ Glucosylated anthocyanins	508.48	a	430.14	abc	325.03	cd	257.75	d	443.41	ab	395.83	bc
	17.62		62.66		32.13		21.66		19.21		39.50	
∑ Acetylated anthocyanins.	205.49	a	197.33	a	155.98	ab	103.80	b	176.48	a	166.26	a
	7.99		32.45		11.86		10.63		8.72		18.29	
∑ Coumaroylated anthocyanins	361.81	a	349.94	a	250.28	ab	172.49	b	249.77	ab	271.56	ab
	43.78		76.52		4.88		23.27		8.04		14.91	
Polymeric pigments	36.18	bc	47.96	ab	50.13	a	18.80	d	31.65	c	23.72	cd
	2.71		6.64		1.34		1.39		5.60		3.93	

Similar results were found in the concentration of gallic acid and polymeric phenols during ripening. The gallic acid content decreased with the course of ripening in 2015 whereas in 2016, although a decreasing trend was also observed, did not change significantly from 21ºBrix to 25ºBrix. Regarding the amount of polymeric phenols, their concentration in fresh grapes reached its peak at 23ºBrix (in both seasons) decreasing towards 25ºBrix, but these changes were also not always significant. However, the variation in the grape polymeric phenol concentration was not significant in 2016.

The levels of the total hydroxycinnamic acids was also significantly higher in 2015 when compared to 2016 at the earliest ripeness stage (21ºBrix) (Table 4.3). However, with the course of time, a decrease was observed in 2015 and the differences between the two vintages got reduced. Regarding the flavonol concentration, a lower amount was also found in 2016. Once again, the changes occurring in flavonol concentration during ripening were not significant in 2016 (Table 4.3), whereas a decrease was observed for the previous vintage (2015).

Lower concentrations of anthocyanin and polymeric pigments were also observed in the 21ºBrix fresh grapes of 2016 (Table 4.3). However, a decreasing tendency was observed in 2015 for the

concentration of glucosylated-anthocyanins, acetylated-anthocyanins and coumaroylated-anthocyanins during ripening (Table 4.3). This decrease was only found to be significant in the amount of glucosylated anthocyanins and it could explain the decrease in anthocyanins concentrations found with the "soft extraction" (Table 4.2). In contrast, the concentration of anthocyanins in 2016 showed an increase during ripening (not significant in the case of coumaroylated anthocyanins). This decrease found, from 23ºBrix to 25ºBrix in 2015 grapes, could partly be explained by the formation of polymeric pigments during ripening (Table 4.3). The concentration of polymeric pigments was also significantly lower in 2016.

4.3.5. Influence of vintage and ripeness on the phenolic composition of the wines

Different colour and phenolic parameters were measured in the young wines. The phenolic composition in red young wines can be potentially related to the grape phenolics (Du Toit & Visagie, 2012). The differences in the wine phenolic composition can also be influenced by different alcohol levels (increasing with the grape ripeness), as previously reported by Canals *et al.* (2005). In combination, grape ripeness and vintage, had a significant impact on the phenolic profile of the wines (Cagnasso, *et al.*, 2011).

Table 4.4. Colour and phenolic measurements in wines after alcoholic fermentations in 2015 and 2016 made from for the three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix).

	2015						2016					
	21ºBrix		23ºBrix		25ºBrix		21ºBrix		23ºBrix		25ºBrix	
CD (AU)	16.10	b	27.33	a	24.70	a	11.35	c	17.84	b	17.64	b
	1.15		0.38		1.08		0.36		0.76		1.64	
Hue	0.41	a	0.41	a	0.46	c	0.41	a	0.44	b	0.44	b
	0.00		0.00		0.01		0.00		0.01		0.01	
MCD (AU)	13.94	b	21.38	a	21.51	a	8.28	c	15.12	b	16.73	b
	0.54		1.48		1.34		0.50		0.58		0.83	
TRP (AU)	29.26	c	42.32	a	39.47	ab	24.17	d	37.50	b	37.82	b
	0.90		1.84		1.66		0.35		0.98		0.18	
TP (AU)	38.95	b	55.14	a	55.80	a	39.40	b	51.62	a	53.37	a
	1.22		2.69		2.61		1.33		1.28		0.06	
Copigments (AU)	16.05	bc	23.18	ab	30.98	a	11.38	c	18.13	bc	13.99	c
	0.49		1.22		6.60		0.24		0.52		1.47	
SO_2 resistant pigments (AU)	2.45	b	4.29	a	4.83	a	1.52	c	2.45	b	2.33	b
	0.08		0.19		0.26		0.20		0.17		0.14	
Tannins (mg/L)	543.25	b	816.91	a	1021.35	a	311.57	c	584.99	b	842.03	a
	24.03		54.01		139.01		39.62		11.86		55.53	

CD: Colour Density (AU), MCD: Modified Colour Density (AU). TRP: Total Red Pigments (AU). TP: Total Phenolics (AU).

In terms of grape ripeness, colour and other phenolic characteristics determined spectrophotometrically were often significantly lower in wines made from 21ºBrix grapes (Table 4.4).

However, no significant differences were found between wines made from 23ºBrix and 25ºBrix grapes within the same vintage, except for hue in 2015 and tannins in 2016, which increased.

The vintage effect was not reflected in the amount of TP of the wines (Table 4.4). However, the overall results often showed higher colour density, copigments (only significant when the wines made from 25ºBrix were compared), SO_2 resistant pigments, and tannins values (except for 25ºBrix) in 2015 wines (Table 4.4). In both seasons, the concentration of SO_2 resistant pigments content in wines was higher in the wine made from riper berries (Table 4.4). Their concentration in wines can partly be explained by the polymeric pigments levels found in grapes with the HPLC (R^2=0.68). Additionally, the amount of TRP was significantly higher in 2015 compared to 2016, except at 25ºBrix. Also, the concentration of wine copigments was higher as the wines were made from riper berries in 2015, whereas their levels were constant in 2016. The higher content of copigments, SO_2 resistant pigments and TRP contributed to the greater colour density (CD) and modified colour density (MCD) (where the pH effect is negated) in 2015.

The results of the individual wine phenolic compounds determined with HPLC supported the abovementioned spectrophotometric data. The vintage effect clearly influenced the concentration of gallic acid and hydroxycinnamic acids (significantly higher in 2015). A greater amount of B1 (Table 4.5) was found in wines from 2016 compared to 2015, which was not observed in fresh grapes. On the other hand, as found in fresh grapes, the amount of polymeric phenols was significantly higher in 2015 (Table 4.5). The HPLC results also showed the impact of ripeness on the increase of the polymeric phenol extractability with the increase in ºBrix in 2015. The concentration of these compounds in fresh grapes declined from 23ºBrix to 25ºBrix. However, their concentration in wines made from 25ºBrix grapes was higher than those made from 23ºBrix.

Moreover, although a positive correlation was also found between wine MCP tannins and wine polymeric phenols (R^2=0.61), differences in wine MCP tannin content between vintages in wines made from 25ºBrix grapes were not significant different (Table 4.4). The possibility of a greater amount of wine tannins bound to polysaccharides in solution which may react with methylcellulose may explain these differences. An increase in the tannin concentration measured by the BSA method (Bovine Serum Albumin) has been reported when polysaccharides were added to the wine (Watrelot *et al.*, 2017). A cleavage of the hydrogen bonds between phenolics and polysaccharides could occur with the acidic conditions of the HPLC method. Thus, we can't exclude the possibility that the tannins precipitating with MCP in 2016 are bound to other components.

The concentration of hydroxycinnamic acids was also significantly higher in wines of 2015 (Table 4.5), in all ripeness stages, compared to the wines of 2016. In contrast to this and despite the differences in fresh grapes, similar concentrations of wine flavonols were found between the two vintages (except in wines made from 25ºBrix). In 2015, the flavonols seemed to become more extractable during ripening.

Concerning the anthocyanin and polymeric pigment content, from the results in wine (Table 4.5) and as previously discussed in fresh grapes, the vintage effect seems to have a lower impact on the wine monomeric anthocyanins (glucosylated, acetylated, and coumaroylated anthocyanin), compared to the ºBrix accumulation during grape ripening. The anthocyanin levels were fairly similar between the two vintages. In both seasons, the wines made from 21ºBrix grapes, had the lowest concentration in any of the three anthocyanin forms (Table 4.5). From there, in 2015, these compounds reached their peak in concentration in the wines made from 23ºBrix grapes, but decreasing again in terms of glucosylated anthocyanin and acetylated anthocyanins in wines made from 25ºBrix grapes. However, in 2016, the concentration in wine anthocyanins was did not change significantly between wines made from 23ºBrix and 25ºBrix grapes. On the other hand, the polymeric pigments were strongly influenced by the vintage. As for polymeric phenols, a greater amount of wine polymeric pigments was found in 2015.

Table 4.5. Individual and groups of phenolic compounds (mg/L) in finished wines (after alcoholic fermentation - AF) in wines made from three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) in 2015 and 2016.

	2015						2016					
	21ºBrix		**23ºBrix**		**25ºBrix**		**21ºBrix**		**23ºBrix**		**25ºBrix**	
Gallic acid	5.96	d	8.09	ab	7.28	c	6.13	d	7.42	bc	8.61	c
	0.17		0.33		0.18		0.10		0.40		0.21	
Catechin	6.11	c	8.78	b	11.24	a	10.14	ab	10.07	ab	9.70	ab
	1.01		0.60		1.17		0.13		0.26		0.10	
B1	14.24	d	17.56	cd	25.18	c	47.51	b	62.19	a	64.47	a
	1.24		2.10		1.08		1.61		5.04		2.96	
Polymeric phenols	375.69	c	643.24	b	711.43	a	182.76	d	329.84	c	372.98	c
	16.35		27.22		18.52		9.88		19.91		20.45	
∑ Hydroxycinnamic acids	34.28	c	48.26	a	42.69	b	17.95	e	17.95	e	23.64	d
	0.56		2.62		2.77		0.30		1.26		0.56	
∑ Flavonols	83.95	b	99.63	a	102.64	a	83.49	b	93.82	ab	82.57	b
	4.48		0.96		4.85		1.70		7.23		3.62	
∑ Glucosylated anthocyanins	188.81	b	261.52	a	205.30	b	153.18	c	238.14	a	237.87	a
	7.22		9.33		10.31		0.79		12.09		7.83	
∑ Acetylated anthocyanins.	83.85	c	114.21	a	96.64	b	65.93	d	101.54	b	104.27	ab
	2.74		3.43		4.42		0.53		4.75		3.64	
∑ Coumaroylated anthocyanins	29.77	cd	43.69	a	37.98	ab	22.26	d	35.05	bc	42.46	ab
	2.32		2.93		3.01		1.04		2.49		2.60	
Polymeric pigments	21.07	c	34.78	a	38.14	a	10.11	d	25.11	b	20.72	c
	0.89		0.73		1.16		0.32		1.38		1.63	

4.3.6. Impact of vintage and ripeness on the cell wall structure and phenolic extractability

As previously discussed (section 4.3.4 and 4.3.5), differences found in the grape phenolics during ripening were often reflected in young wines. Additionally, large differences were found in the monosaccharide composition of the grape skin. From the previous results (section 4.3.2 and 4.3.3), the vintage had an important effect on the grape berry cell wall structure and composition. Likewise, differences in GalA and Ara were considerable. These differences may be explained by the climatic differences between the two vintages (2015 and 2016). The 2016 harvest season was characterised as being a particularly dry one, preceded by a warm summer with lower amount of rain compared to the previous season (VinPro, 2016).

It is thus clear that 2015 grape berry skins were more intact compared to 2016. Since the grape maturity (in ºBrix) was the same between the two vintages, changes that occurred during ripening and the subsequent extractability was influenced by the initial structure of the grape pomace. Climatic differences, such as the impact of the higher temperatures in the early maturation stages, may have affected the intactness of the grape berry. From there, a mild de-pectination process occurred during grape maturation for both vintages. However, the impact of ripeness was more clearly shown after the completion of the alcoholic fermentation. The results from cell wall analysis in the fermented grape pomace will be described later on (Chapter 5). Therefore, the pectin layer in 2015 seemed to be not only more intact, but it could also have had a lower porosity structure compared to 2016, with a lower degree of procyanidin retention.

The largest modifications during grape ripening for both vintages seemed to occur mainly in the cell wall's pectin layer. Overall the monosaccharide and polysaccharide results suggest a more intact berry structure in 2015 compared to 2016 (more de-pectinated from an early ripeness stages). The "initial" grape cell wall structure, can potentially be relevant for the winemaking, as it will influence the cell wall de-pectination, the phenolic biosynthesis and therefore the subsequent phenolic extractability during wine maceration. Studies in apple tissue showed how the interactions between pectin and proanthocyanidins were stronger with a higher degree of methyl-esterification of the HG (Le Bourvellec, *et al.*, 2009; Watrelot, *et al.*, 2013). From our results, a greater amount of highly methyl-esterified HG (JIM7) was exposed in 2016. In less intact and more de-pectinated pomace, the increase in highly-methyl esterified HG extractable with the CDTA could be related to greater exposure of this epitope due to an increase in the skin porosity. From our results we cannot obtain specific information about the grape porosity. Additionally, a higher amount of RG-I (mAbs INRA-RU1, INRA-RU2) or "hairy region" was also found in 2016. These polysaccharides have also been reported to be associated with a lower proanthocyanidin extractability (Quijada-Morín, *et al.*, 2015). These results confirmed the better exposure of the RG-I in the hemicellulose layer of 2016 grapes, due to a more degraded skin layer.

The lower phenolic content found in the 2016 wines, especially in polymeric phenols concentrations, could possibly explained by a lower phenolic synthesis in 2016 grapes. Climatic conditions can influence the plant photosynthesis and physiology, therefore affecting the biosynthesis and accumulation patterns of certain phenolic compounds (Teixeira *et al.*, 2013; Sun *et al.*, 2017). These differences may be influenced by differences in the plant physiology and berry metabolism between the two vintages (such as the higher temperatures in 2016). Temperature has been shown to influence the concentration of flavonols and anthocyanins concentrations, however, this effect on flavon-3-ol or proanthocyanidin concentration are not that clear (Cohen, *et al.*, 2012). Skin proanthocyanidin accumulation and their mean degree of polymerisation (mDP) seem to increase at véraison with higher temperature, but it seems to have little impact post-véraison (Cohen, *et al.*, 2008). However, higher temperatures have been shown to alter the cell wall polysaccharide structure in other plants, especially due to a decrease in pectin polysaccharides and a greater arabinose and galactose content (Lima, *et al.*, 2013). A second hypothesis could explain the lower phenolic content in 2016 wines due to a greater diffusion of certain cell wall components (as a consequence of more degraded cell walls) into the wines, interacting with the wine phenolics. Partial Least Square (PLS) analysis were used to assess the relationship between the HG and RG epitopes with the levels of catechin, B1-dimer and polymeric phenols in the grapes for all the grape treatments. The loading plot in Figure 4.5 shows a negative correlation between the polymeric phenols and the HG epitope JIM7 and with the RG-I backbones (INRA-RU1, INRA-RU2).

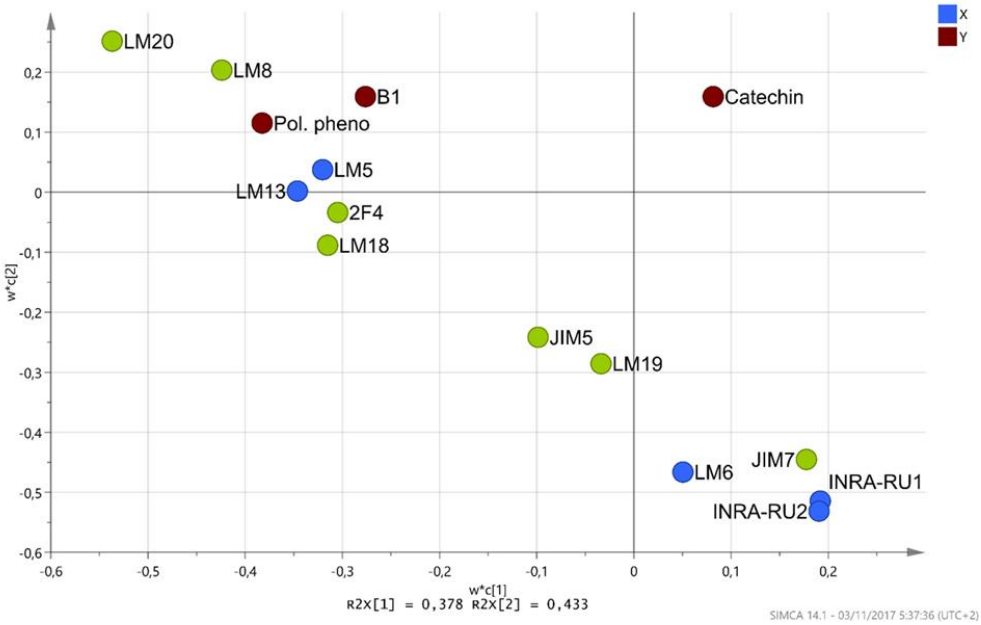

Figure 4.5. PLS loading plot. Relationship between the different HG (JIM5, JIM7, LM18, LM19, LM20, 2F4, LM8) and RG (INRA-RU1, INRA-RU2, LM5, LM6, LM13) epitopes composition and the catechin, B1 and polymeric phenol concentration in fresh grapes for all three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) and vintages (2015 and 2016).

Further cell wall modifications occurred during the grape maceration. Results from the monosaccharide composition in fermented pomace supports the idea of a more intact grape berry in 2015. Both, Ara and GalA showed lower mol % in the fermented pomace (Figure 4.6), compared to the fresh grapes, reducing their differences between the two vintages. This decreasing trend in GalA and Ara % was already reported by Zietsman *et al.* (2015). A greater de-pectination was found in 2015, shown by the % of Gal. The more depectinated or degraded the fresh grapes are, the smaller the changes in mol% GalA that will take place during fermentation (as in 2016). A relative increase in Man % and Glu % was also observed, associated with the co-precipitation of polysaccharides from the yeast cell walls was also observed (Figure 4.6).

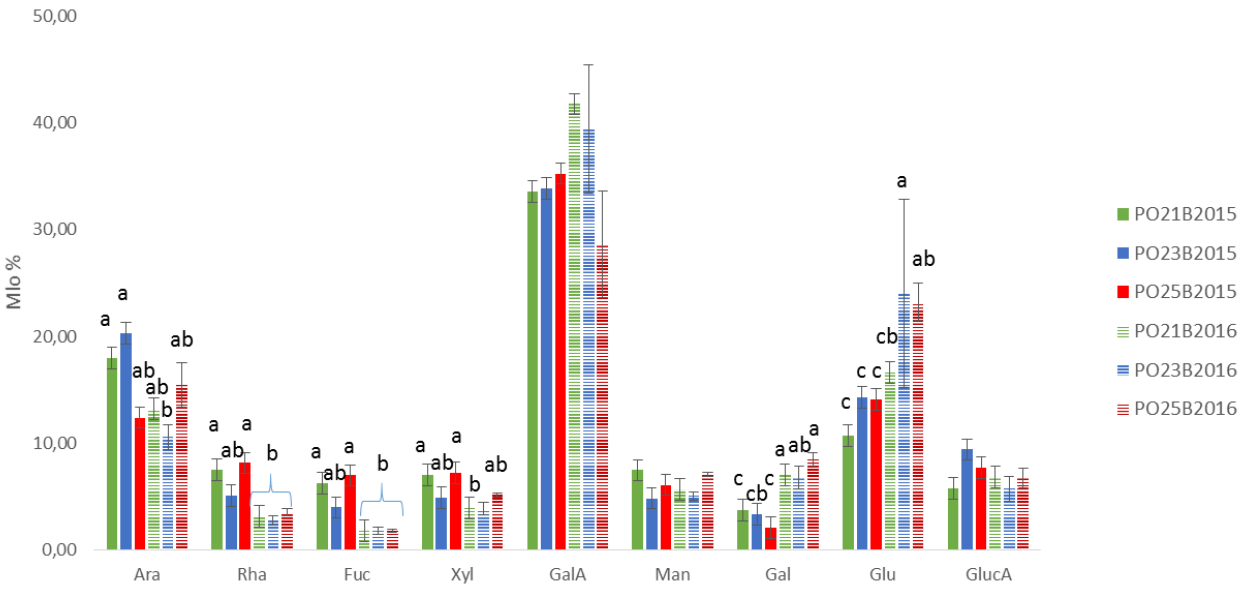

Figure 4.6. Monosaccharide composition (mol %) in the fermented pomace (PO) in three different ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) during two consecutive vintages (2015 and 2016). Different letters illustrate the significant differences (ANOVA, LSD test) between the three ripeness and vintage within the individual monosaccharides. The nine major monosaccharides are displayed: Ara (arabinose), Rha (rhamnose), Fuc (fucose), Xyl (xylose) GalA (galacturonic acid), Man (mannose), Gal (galactose), Glu (glucose), GlucA (glucuronic acid).

The polysaccharides results (Figure 4.7) in fermented grape pomace confirmed the idea of a more intact grape berry in 2015. The impact of ripeness in the cell wall polysaccharide and protein composition was more clearly shown after the completion of alcoholic fermentation. Firstly, an increase in the extractability of some of the HG epitopes (LM18, 2F4) with the CDTA was more obvious in 2016 than in 2015. Also, the increase during ripening in AGPs (JIM13). These differences between vintages were more obvious in the NaOH extract. Similar as in fresh grapes, the xyloglucan (LM15 and LM25) and cellulose (CBM3a) layer was more exposed in 2016 than in that of 2015. Furthermore, in 2016, an increased was observed with the course of grape ripeness in the RG-I

epitopes (mAbs INRA-RU1 and INRA-RU2) and arabinan side chains (mAb LM6). Also, a greater extractability in xyloglucan (mAbs LM15), extensins (LM1) and AGPs (JIM13) during ripening. A more depectinated berry structure, such as in 2016, enhanced the extractability of cell wall polysaccharides and proteins in the NaOH extract after the alcoholic fermentation. The increase in BS-400-2 signals was related to the formation of callose during the wine fermentation (Zietsman *et al*., 2015).

	Year	Ripening	HG partially/de-esterified (mAb JIM5)	HG partially esterified (mAb JIM7)	HG partially/de-esterified (mAb LM18)	HG partially/de-esterified (mAb LM19)	HG partially esterified (mAbLM20)	HG Ca2+ crosslinked (mAb 2F4)	Xylogalacturonan (mAb LM8)	Backbone of rhamnogalacturonan I (mAb INRA-RU1)	Backbone of rhamnogalacturonan I (mAb INRA-RU2)	(1→4)-β-D-galactan (mAb LM5)	(1→5)-α-L-arabinan (mAb LM6)	Linearised (1→5)-α-L-arabinan (mAb LM13)	(1→4)-β-D-(galacto)(gluco)mannan (mAb LM21)	(1→4)-β-D-(gluco)mannan (mAb LM22)	(1→3)-β-D-glucan (mAb BS-400-2)	Xyloglucan (XXXG motif) (mAb LM15)	Xyloglucan/unsubstituted β-D-glucan (mAb LM25)	(1→4)-β-D-xylan (mAb LM10)	(1→4)-β-D-xylan/arabinoxylan (mAb LM11)	Cellulose (crystalline) (mAb CBM3a)	Extensin (mAb LM1)	Extensin (mAb JIM11)	Extensin (mAb JIM20)	AGP (mAb JIM8)	AGP (mAb JIM13)	AGP (mAb LM14)	AGP, β-linked GlcA (mAb LM2)
CDTA POMACE	2015	21B	39	61	29	27	0	19	0	33	29	9	31	12	0	0	0	0	8	0	0	0	0	6	0	6	20	0	9
	2015	23B	39	61	28	28	5	21	0	36	32	10	33	14	0	0	0	0	9	0	0	0	0	8	0	5	21	0	10
	2015	25B	44	63	32	32	7	24	0	36	32	8	31	14	0	0	0	0	8	0	0	0	0	7	0	6	19	0	11
	2016	21B	40	62	21	31	0	17	0	39	38	6	30	7	0	0	0	0	6	0	0	6	0	0	0	0	14	0	5
	2016	23B	46	71	28	29	0	18	0	35	33	8	28	9	0	0	0	0	0	0	0	0	0	0	0	0	16	0	5
	2016	25B	41	68	33	35	0	30	0	46	45	10	39	13	0	0	0	0	9	0	0	5	0	6	9	7	25	5	11
NaOH POMACE	2015	21B	0	0	0	5	0	0	0	37	37	25	36	19	33	11	36	55	53	0	0	32	23	11	16	9	26	0	7
	2015	23B	0	0	0	6	0	0	0	42	46	27	43	25	33	8	35	52	51	0	0	30	23	10	15	9	25	0	8
	2015	25B	0	0	0	6	0	0	0	40	41	23	38	22	37	8	31	52	48	0	0	34	25	10	16	8	24	0	7
	2016	21B	0	0	0	13	0	0	0	42	46	24	43	21	27	6	32	63	75	7	0	56	21	6	18	0	22	9	8
	2016	23B	0	0	0	9	0	0	0	32	50	22	38	24	19	6	17	82	68	8	8	57	26	10	22	9	30	10	8
	2016	25B	6	0	8	21	0	0	0	45	59	27	48	25	29	16	17	73	71	11	12	59	31	8	21	9	28	9	8
										0		10		20		30		40		50		60		70		80		90		100

Figure 4.7. Heat map of the epitope abundance (0-100) in the CDTA and NaOH of fermented pomace for the three ripening levels (21ºBrix, 23ºBrix and 25ºBrix) during two consecutive seasons (2015 and 2016). Values lower than 5 are indicated as 0.

4.4. CONCLUSION

Climatic conditions affect vine growth, berry metabolism and physiology and therefore the grape evolution during harvest. A clear vintage effect was observed, possibly influenced by the drought and differences in temperature between the two harvest seasons. The study aimed to establish a link between changes in the berry cell wall composition, influenced by the effect of ripeness (Zietsman *et al.*, 2015) and the diverse climatic conditions per vintage, and the extractability of the phenolic compounds from grapes to wines (Gao *et al.*, 2015; Gao, *et al.*, 2016). Furthermore, it also brings out the possibility of a relationship between the berry intactness and the phenolic extractability, with a higher proanthocyanidin retention in more degraded grape pomaces. Additionally a possible relationship between heat stress and de-pectination in grape berries might exist. To date, contradictory trends have been reported on tannin extractability during ripening. These differences may not only depend on the phenolic synthesis/accumulation, but also on

differences in the cell wall composition and structure, influenced by the climatic conditions and the vine growth and berry physiology. In short, this is the first study performed in Shiraz using the combination of these methods allowing us to reach a better understanding of the relationship between the cell wall components and the phenolic compounds. This can potentially contribute to a better understanding of this field, which is necessary for wine producers to have better control of phenolic extractability and therefore the subsequent wine phenolic composition. Changes in the phenolic profiles and cell wall composition during fermentation will be presented in the following chapter.

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Chapter 5

Research results

Investigating the relationship between the cell wall polysaccharide structure and grape phenolic compounds in Shiraz.

Part II: extractability during fermentation in wines made from different grape ripeness levels

Chapter 5: Investigating the relationship between the cell wall polysaccharide structure and grape phenolic compounds in Shiraz.

Part II: extractability during fermentation in wines made from different grape ripeness levels

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ABSTRACT

The extent of phenolic compounds released during alcoholic fermentation is dependent on several structural and conformational changes occurring in the grape skins during ripening and during alcoholic fermentation. The present study evaluated the relationship between the cell wall breakdown and the colour and phenolic extractability during alcoholic fermentation in Shiraz wines made from grapes harvested at different ripeness levels (21ºBrix, 23ºBrix and 25ºBrix). The phenolic differences between the ripeness treatments were minimal after ¼ of the fermentation was completed. However, colour and phenolic content were significantly higher in completed wine made from 25ºBrix grapes compared to those made from harvested at 23ºBrix and especially 21ºBrix. In brief, the level of grape cell wall polysaccharide deconstruction during fermentation was a determining factor in the phenolic extractability. In this context, the de-pectination during ripening was found to enhance this deconstruction or “opening-up” of the grape pomace produced during the alcoholic fermentation, thus increasing the differential extraction of specific polyphenols into the wines. Additionally, this cell wall deconstruction also played a role in the possible retention and extraction of specific proanthocyanidins, depending on their nature and polymer length.

5.1. INTRODUCTION

The composition of the grapes is the main variable influencing the initial amount and nature of wine phenolics; however, due to their reactivity, phenolic compounds continuously evolve over time (Pérez-Magariño & González-SanJosé, 2004; Monagas, *et al.*, 2005; Garrido & Borges, 2013). These molecules are gradually extracted during the alcoholic fermentation due to grape tissue breakdown and the degradation of the grape berry cell wall structure during maceration. Different winemaking techniques will thus also influence the final phenolic concentration in the wines (Marais, 2003; Canals, *et al.*, 2005; Sacchi, *et al.*, 2005; Yacco, *et al.*, 2016). However, there is a lack of information on the relationship between the different groups of grape phenolics extracted into the wine and the berry cell wall polysaccharides, in fresh grapes and during fermentation.

Grape phenolics are represented by a large range of chemical structures, essentially divided in non-flavonoid and flavonoid compounds. Hydroxycinnamic acids (such as caftaric acid and coutaric acid) are normally the most abundant non-flavonoid compounds in wines. The hydroxycinnamic acids are extracted from the cellular vacuoles not only from the grape skin, but also from the cells in the pulp (Monagas, *et al.*, 2005; Adams, 2006; Garrido & Borges, 2013). On the other hand, flavonoids are subdivided into several groups and classes according to the unsaturation of on the pyran ring and the substitutions in the B ring. Anthocyanins are extracted from the hypodermal layers of grape skins (Ribéreau-Gayon, *et al.*, 2006). These compounds generally accumulate during ripening, becoming more extractable at commercial harvest (Hernández-Hierro, *et al.*, 2012, 2014). Flavonols are a group of compounds also found in the grape skin whose biosynthesis is highly influenced by sunlight exposure of the grapes (Makris, *et al.*, 2006). On the other hand, flavan-3-ols and proanthocyanidins (or condensed tannins) can be extracted not only from the grape skins, where they can specifically be found as free compounds in the tissue cell vacuoles or bound to cell wall components, notably proteins or polysaccharides (Amrani Joutei, *et al.*, 1994); but also from the grape seeds (Geny, *et al.*, 2003; Ribéreau-Gayon, *et al.*, 2006). Differences in the proanthocyanidins polymer length and molecular structure can affect its reactivity with other phenolic compounds or with cell wall material, affecting the extractability of these compounds from grapes into wine (Hanlin, *et al.*, 2010; Bindon & Kennedy, 2011; Bindon, *et al.*, 2014a; Quijada-Morín, *et al.*, 2015). Additionally, skin proanthocyanidins can be divided into procyanidins and prodelphinidins. Seed proanthocyanidins consist of procyanidins. Recent studies have shown that skin-derived proanthocyanidin concentrations in wine increase during grape ripening, partly as a consequence of a more extractable grape pomace (Ribéreau-Gayon, *et al.*, 2006; Bindon, *et al.*, 2013), whereas the seed tannins extractability seems to decrease during ripening (Kennedy, *et al.*, 2000; Peyrot Des Gachons & Kennedy, 2003; Bautista-Ortín, *et al.*, 2012). However, changes in tannin extractability remains a controversial topic as some other authors have showed a decrease in the tannin extractability during ripening (Adams, 2006; Hanlin & Downey, 2009).

The release of these grape phenolic compounds is accompanied by the diffusion of certain cell wall components as a consequence of the grape pomace cell wall breakdown occurring during the alcoholic fermentation (Guadalupe & Ayestarán, 2007; Gil Cortiella & Peña-Neira, 2017). Grape berry cell walls thus experience a progressive degradation of their pectin layer (i.e. de-pectination). The addition of commercial enzymes enhanced the de-pectination process and consequently the extraction of phenolic compounds in Cabernet Sauvignon (Gao, *et al.*, 2016). However, this cell wall de-pectination begins during the grape ripening process in the vineyard. A recent study in Pinotage showed the lower impact of commercial enzymes in the de-pectination of riper berries, which are naturally more degraded than those of than those harvested at a lower sugar level (Zietsman, *et al.*, 2015). However, the different ripeness levels were obtained from two different harvesting seasons. These changes entail the softening of the fruit, which is linked to pectin degradation, induced by pectolytic enzymes, and the solubilisation of the cell wall polysaccharides together with an increase in its protein content (Nunan, *et al.*, 1998; Huang, *et al.*, 2005; Ortega-Regules, *et al.*, 2008; Goulao, *et al.*, 2012).

As a consequence of the cell wall breakdown occurring during the alcoholic fermentation, a fraction of these cell wall components is also extracted into the wine. The concentration of these cell wall derived proteins and polysaccharides can thus influence the wine's sensory properties and phenolic stability (Revilla & González-SanJosé, 2003; Vidal, *et al.*, 2003, 2004; Guadalupe & Ayestarán, 2007). Wine polysaccharides, mainly extracted from grapes and yeasts, can enhance or inhibit tannin aggregation (Riou, *et al.*, 2002; Poncet-Legrand, *et al.*, 2007) affecting the colloidal stability. Several studies have analysed the impact of different winemaking techniques, such as the addition of commercial enzymes, on the enhanced extraction of grape phenolics (Ducasse *et al.*, 2010; Apolinar-Valiente *et al.*, 2015; Río-Segade *et al.*, 2015; Zietsman *et al*., 2015; Castro-López *et al.*, 2016; Gao *et al.*, 2016).

Different studies on fruits have demonstrated how all these changes can influence the fruit cell wall composition as well as the skin's porosity consequently increasing the amount of active binding sites (Bindon, *et al.*, 2010, 2012; Castro-López, *et al.*, 2016) and thereby increasing the phenolic retention by the fruit matrix (Renard, *et al.*, 2001; Le Bourvellec, *et al.*, 2004; Bindon, *et al.*, 2012; Watrelot, *et al.*, 2013, 2017). The high affinity between certain phenolic compounds and cell wall material (CWM), especially homogalacturonans (HG) and rhamnogalacturonan-I (RG-I), leads to a retention phenomenon known as adsorption.

The main aim of this study was to investigate the link between the cell wall disassembly, on a polysaccharide level, and the release of the grape phenolic compounds during wine fermentation. Most studies have approached both variables individually (Gao, *et al.*, 2015; Zietsman, *et al.*, 2015; Yacco *et al.*, 2016), but there is little information available about the relationship between cell wall material, especially polysaccharides, and the extraction of phenolic compounds during fermentation (Guadalupe & Ayestarán, 2007; Springer, *et al.*, 2016). The use of grapes harvested at three different

ripeness levels from the same vineyard, could help to better understand the influence of the natural de-pectination of the grape tissues occurs during ripening on the subsequent phenolic extractability during fermentation. The information obtained from this work could shed more light on the role of specific cell wall polysaccharides in the phenolic extraction during alcoholic fermentation.

5.2. MATERIALS AND METHODS

5.2.1. Grapes and winemaking procedures

How the grapes and wines were made can be found in Chapter 4, but a brief summary will be presented again. Grapes were harvested from the same Shiraz vines as in Chapter 3, Smart-Dyson training system on a 101-14Mgt rootstock, at three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) in 2016 from the Welgevallen experimental farm (GPS coordinates: 33°56'25.0"S 18°51'56.4"E) of the Department of Viticulture and Oenology of Stellenbosch University. A variable number of vines (up to approximately 120 kg) from the same rows were alternatively harvested for the three ripeness stages. Once in the cellar, grapes from the same ripeness treatment were pooled and divided into three fermentation buckets (40 kg), to reduced the intra-vineyard variability. The winemaking is described in Chapter 3. Fresh grapes from the three ripeness levels were sampled. Fermenting pomace and must samples were taken at several stages of the alcoholic fermentation (¼F, ½F, ¾F), while maintaining the skin to liquid ratio during fermentation. The fermentation sampling stages were assessed by the proportional decrease in ºBrix which was monitored with the use of a Ballingmeter. Grape skins were pressed after alcoholic fermentation (AF) in a basket press and the pressed wine added to the free run wine.

5.2.2. Skin cell wall preparation

Fresh berries (GR) and fermenting grape pomace from the three ripeness levels were collected during the same fermentation stages (¼F, ½F, ¾F) and in pressed pomace (PO). The alcohol-insoluble residue (AIR) was prepared from grape skins as described in Chapter 4. Between four to five AIR extractions were performed for each biological ferment of each ripeness level.

5.2.3. Monosaccharide analysis of cell wall samples

The de-pectinaction occurring in the cell wall during ripening and fermentation was analysed with gas chromatography coupled with mass spectrometry (GC–MS) by measuring the nine major cell wall monosaccharides: arabinose (Ara), fucose (Fuc), rhamnose (Rha), xylose (Xyl), mannose

(Man), galacturonic acid (GalA), galactose (Gal), glucose (Glu) and glucuronic acid (GlcA). The chromatography conditions of the GC-MS are described in Chapter 4. The hydrolysis of AIR samples was carried out as described in Chapter 4.

5.2.4. Comprehensive Microarray Polymer Profiling (CoMPP) analysis of cell wall fractions

The polysaccharide composition in the pectin- and hemicellulose-rich fractions of the grape pomace was profiled with the use of CoMPP. Approximately 10 mg of AIR samples were analysed as described in Chapter 4.

5.2.5. Colour and phenolic measurements

5.2.5.1. Spectrophotometric analysis

To assess the phenolic extraction during fermentation, colour and phenolic analysis were performed for all three grape ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) at different stages during alcoholic fermentation (¼F, ½F, ¾F) in the fermenting must and in the pressed fermented samples (AF). Different colour and phenolic parameters were performed as described in Chapter 3. All measurements were performed in triplicate.

The wine colour of all samples was also assessed with the CIELab colour space. This colorimetric approach was initially proposed by the Commission International de l'eclairage (CIE) (CIE, 1978). The CIELab method, recognised by the OIV (OIV, 2006), is used in wine as a more realistic approach to evaluate the wine's colour in a multidimensional space (Ayala, *et al.*, 1997). The wine colour was measured in a three dimensional colour space, defined by the following chromatic characteristics: L* as the clarity of the sample (L*=0 is black whereas L*=100 is colourless), a* as the red/green colour component (a* > 0 red, a* < 0 green), and b* as the blue/yellow component (b* > 0 yellow, b* < 0 blue) and its derivatives magnitudes C_{ab}* (chroma, i.e. saturation) and H_{ab}* (hue angle). The minimal colour differences that can be detected by the human eye have been established as ΔE* = 2.7 (Pérez-Caballero, *et al.*, 2003). All wine samples were centrifuged at 8000 rpm during 5 minutes prior to analyses. The spectra between 380 and 770 nm was recorded with the use of a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

5.2.5.2. HPLC analysis

Monomeric and polymeric phenolics were individually quantified in the different sampling stages of the wines at three ripeness levels (21ºBrix, 23ºBrix, 25ºBrix). The HPLC method and conditions are described in Chapter 3. Data processing was performed with Agilent ChemStation software (Agilent

Technologies – Santa Clara, California, USA) using the following phenolic standards: gallic acid, (+)-catechin, caffeic acid, *p*-coumaric acid, quercetin-3-glucoside, quercetin from Sigma-Aldrich Chemie (Steinheim, Germany), and malvidin-3-glucoside from Extrasynthese (Lyon, France).

5.2.5.3. Phloroglucinolysis

The proanthocyanidin fraction from the grape pomace was analysed by acid-cleavage in the presence of phloroglucinol using the conditions from Oberholster *et al.*, (2013). The grape skins were peeled from fresh berries and the grape pomace at different stages during the alcoholic fermentation (¼F, ½F, ¾F and end of fermentation - AF) and then blended in an extraction solvent (70% acetone) using an IKA Ultra-Turrax T 18b (IKA Labortechnik, Staufen, Germany) homogeniser. All extractions were carried stirring at 4ºC during 24 hours. The extracts were centrifuged (5 min at 800 rpm), evaporated and lyophilised. The final extract was then dissolved in methanol. Cleavage reactions were performed by the addition of 100 µL of phloroglucinol solution (100 g/L phloroglucinol and 2g/L ascorbic acid in a methanol solution containing 0.2N HCl) to 100 µL of grape sample. Samples were heated up to 50ºC during 20 min. The cleavage reaction was then stopped by the addition of 1 mL of 40 mM sodium acetate. The samples were centrifuged before the injection. Each of the three biological replicates were analysed in duplicate. Phloroglucinol, ascorbic acid and sodium acetate reagents were obtained from Sigma-Aldrich (Johannesburg, South Africa).

The chromatographic separation was carried out by RP-HPLC, using two Chromolith Performance RP-18e columns in series (100 mm x 4.6 mm, 3µm) provided with a pre-column (Merck (Pty) Ltd, Johannesburg, South Africa) on a Agilent 1260 operated with Chemstation software. Mobile phases were 1% (v/v) aqueous acetic acid (A) and acetonitrile containing 1% (v/v) acetic acid (B). The column temperature was 30°C and the flow rate was 2 mL/min. The method conditions were as follows: 0 min to 6 min (B: 3%), followed by a linear gradient in 15 min (from 3 to 18% of B) and 80% B for 3 min. The column was washed with 3% B. The instrument conditions were re-equilibrated for 3 min prior to the next injection. The proanthocyanidin cleavage products were determined by means of their response factor relative to (+)-catechin, which was used as the quantitative standard. The molar absorptivity determined by Kennedy & Jones (2001a) was used. The mean degree of polymerisation (mDP) was calculated as the sum (in moles) of all the terminal and extension subunits divided by the terminal units.

These extracts were also used to assess the remaining individual phenolics in the skins during fermentation with HPLC as described in section 5.2.5.2 in the 21ºBrix fermentations.

5.2.6. Statistical analysis

All analyses were carried out using Statistica 13.2 (TIBCO Statistica software, Palo Alto, CA, USA). Mixed model repeated measures ANOVAs were used and Fisher's least significant difference (LSD) corrections were used for post-hoc analyses. Significant differences were judged on a 5% significance level ($p \leq 0.05$). The distribution of certain chemical and sensory datasets were analysed with Principal Component Analysis (PCA) using SIMCA 14.1 software (Sartorium Stedim Biotech - Malmö, Sweden).

5.3. RESULTS AND DISCUSSION

All wines completed fermentation to dryness. The changes in pH and the increase in the ethanol content during the alcoholic fermentation were also monitored during the same sampling stages. The pH in the wines made from 21ºBrix grapes was significantly lower than the wines from 23ºBrix and 25ºBrix grapes (Table 5.1). No significant differences were found between 23ºBrix and 25ºBrix in terms of pH. The differences in pH, together with the alcohol levels can influence the phenolic extractability. The alcohol levels did not differ between the different wines at ¼ F and ½ F. Nonetheless, as the fermentation progressed, from ¾ F to AF, these differences became significant (Table 5.1). In AF wines, the alcohol level was significantly higher in the wines made from 25ºBrix grapes compared to wines made from 21ºBrix and 23ºBrix (Table 5.1).

Table 5.1. Changes during fermentation in the wine alcohol content and pH of wines made from Shiraz grapes from three different ripeness levels. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

		21ºBrix	23ºBrix	25ºBrix
Alcohol (%v/v)	**¼ F**	2.74 ± 0.52 ^{g}	3.06 ± 0.15 ^{g}	3.03 ± 0.04 ^{g}
	½ F	5.86 ± 0.86 ^{f}	6.02 ± 0.43 ^{f}	5.74 ± 0.14 ^{f}
	¾ F	7.35 ± 0.25 ^{e}	8.75 ± 0.30^{d}	8.80 ± 0.27 ^{d}
	AF	12.02 ± 0.14 ^{c}	13.70 ± 0.32 ^{b}	15.08 ± 0.19^{a}
pH	**¼ F**	3.29 ± 0.04^{cd}	3.37 ± 0.04 ^{b}	3.37 ± 0.04 ^{b}
	½ F	3.30 ± 0.04^{d}	3.37 ± 0.03 ^{b}	3.38 ± 0.02 ^{b}
	¾ F	3.26 ± 0.02^{d}	3.37 ± 0.04 ^{b}	3.37 ± 0.02 ^{b}
	AF	3.36 ± 0.02^{b}	3.47 ± 0.05 ^{a}	3.52 ± 0.02 ^{a}

5.3.1. Cell wall deconstruction during alcoholic fermentation

5.3.1.1. Changes in the grape skin monosaccharides during fermentation

Apart from the grape berry cell wall modifications occurring during ripening, further structural changes take place during the alcoholic fermentation. A progressive disruption of the grape cell wall network leads to the diffusion of aroma and phenolics compounds into the wine. This process is partly due to natural grape enzymatic activity, the pH and also the ethanol formed. Therefore, some of these cell wall components are probably also extracted into the wines as a consequence of this pomace breakdown (Vidal, *et al.*, 2003; Guadalupe & Ayestarán, 2007). We therefore analysed the evolution in the relative monosaccharide composition (mol %) in the fresh berries (GR), the fermenting skins (¼ F, ½ F and ¾ F) and the fermented pomace (PO). These monosaccharides are the constituents of the grape cell wall polysaccharides. Thus, fluctuations in monosaccharides can suggest changes occurring in the grape cell wall polysaccharides. When all the samples were compared, the alcoholic fermentation caused the main changes in the monosaccharide composition (Appendix Table 5.1) whereas the grape ripeness did not show a significant impact (Figure 5.1). Firstly, the increase in the alcohol content of the wines led to a possible solubilisation of the soluble polysaccharides into the fermenting must. From the monosaccharide data, no clear evidence of de-pectination were found from the evolution of GalA (Figure 5.1). However, a loss of arabinose (Ara) levels was found as the fermentation progressed in the 21 ºBrix and 23ºBrix treatments (Figure 5.1). Likewise a similar tendency was observed for rhamnose (Rha). Both parameters represent the loss in arabinogalactan-proteins (AGPs) and rhamnogalacturonans-I (RG-I), a consequence which is due to the de-pectination process. This decrease, in Ara and Rha, was not significant in the 25ºBrix treatments, which were probably naturally more depectinated. Other studies have also reported this loss in Ara, as a consequence of the release of AGPs and RG-I into the wine (Pellerin *et al.*, 1995; Vidal *et al.*, 2003; Guadalupe & Ayestarán, 2007; Zietsman, *et al.*, 2015).

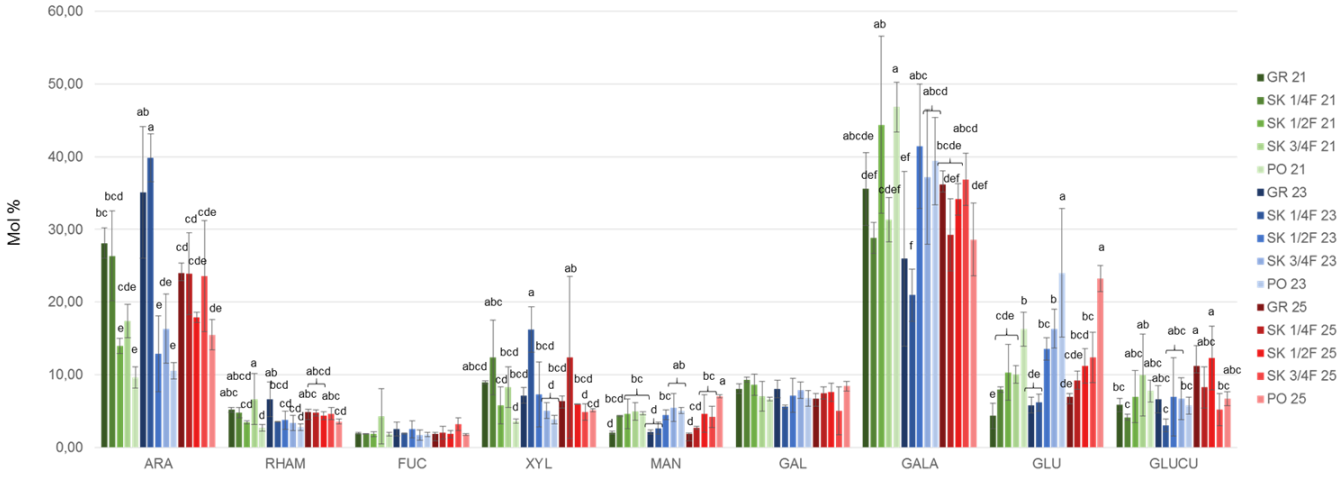

Figure 5.1. Monosaccharide composition (mol %) of AIR source from different stages of the fermenting pomace of Shiraz grape from three different grape ripeness levels. The different letters illustrate significant differences (ANOVA, LSD test $p < 0.05$) within the individual monosaccharides. Samples were coloured according to the grape ripeness, becoming lighter with the progression of the alcoholic fermentation. The sampling stages are represented by GR (fresh grapes), SK (fermenting skin) during different stages of the F (fermentation) and PO (fermented pomace). The nine major monosaccharides are displayed: Ara (arabinose), Rha (rhamnose), Fuc (fucose), Xyl (xylose) GalA (galacturonic acid), Man (mannose), Gal (galactose), Glu (glucose), GlucA (glucuronic acid).

With regards to the rest of the monosaccharides, xylose (Xyl) also showed a decreasing trend, but the variation in levels of some of these neutral sugars, such as fucose (Fuc) and galactose (Gal) was not significant. Nevertheless, an increase in Man levels, and especially Glu was observed. Since the results are expressed in relative mol %, this increase is associated with the progressive disruption and loss of the pectin polymers (e.g. Ara and Rha), but could also due to a possible co-precipitation and adsorption of certain yeast cell wall polymers, rich in mannoproteins, onto the grape pomace. Although similar trends were observed in all three treatments, a greater % of Glu was found in 23ºBrix and 25ºBrix fermented pomace compared to 21ºBrix (Figure 5.1).

5.3.1.2. Changes in the skin polysaccharide and protein fraction

CoMPP results shows the relative abundance of cell wall polymers and proteins that could be extracted with CDTA (primarily extracting pectins) (Figure 5.2 and 5.3) and NaOH (primarily extracting hemicelluloses) (Figure 5.4 and 5.5) from the fresh grapes and the pomace at the different stages throughout the fermentation (¼ F, ½ F, ¾ F and AF). Cell wall breakdown was observed in all three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) with the course of fermentation. The CoMPP results show a mild de-pectination and de-esterification, expected in the absence of macerating enzymes. Nonetheless, the grape ripeness also influenced the changes in the cell wall polysaccharides, enhancing the impact of the fermentative effect (Appendix Table 5.2 and Appendix Table 5.3). Firstly, a higher extraction of total homogalacturonan (HG) epitopes (JIM5, JIM7, LM18,

LM19, LM20 and 2F4) proved the greater de-pectination in 25ºBrix fresh grapes (Appendix Figure 5.1). The CoMPP data in fresh grapes suggest a greater de-pectination in riper berries (25ºBrix) due to a greater signal of the highly methyl-esterified HG labelled as JIM7 (Appendix Figure 5.1).

		HG partially/de-esterified (mAb JIM5)	HG partially esterified (mAb JIM7)	HG partially/de-esterified (mAb LM18)	HG partially/de-esterified (mAb LM19)	HG partially esterified (mAbLM20)	HG Ca2+ crosslinked (mAb 2F4)	Xylogalacturonan (mAb LM8)	Backbone of rhamnogalacturonan I (mAb INRA-RU1)	Backbone of rhamnogalacturonan I (mAb INRA-RU2)	(1→4)-β-D-galactan (mAb LM5)	(1→5)-α-L-arabinan (mAb LM6)	Linearised (1→5)-α-L-arabinan (mAb LM13)	(1→4)-β-D-(galacto)(gluco)mannan (mAb LM21)	(1→4)-β-D-(gluco)mannan (mAb LM22)	(1→3)-β-D-glucan (mAb BS-400-2)	Xyloglucan (XXXG motif) (mAb LM15)	Xyloglucan/unsubstituted β-D-glucan (mAb LM25)	(1→4)-β-D-xylan (mAb LM10)	(1→4)-β-D-xylan/arabinoxylan (mAb LM11)	Cellulose (crystalline) (mAb CBM3a)	Extensin (mAb LM1)	Extensin (mAb JIM11)	Extensin (mAb JIM20)	AGP (mAb JIM8)	AGP (mAb JIM13)	AGP (mAb LM14)	AGP, β-linked GlcA (mAb LM2)
21ºB	GRAPES	46	82	21	28	0	11	0	37	26	7	26	0	9	0	0	0	9	0	0	14	5	0	7	0	18	5	8
	1/4F	51	76	26	37	0	19	0	39	33	6	27	5	6	0	0	0	7	0	0	9	0	0	5	0	16	0	8
	1/2F	44	66	24	33	0	17	0	34	29	5	24	0	0	0	0	0	6	0	0	7	0	0	0	0	13	0	6
	3/4F	44	64	24	33	0	16	0	34	29	5	24	5	0	0	0	0	5	0	0	6	0	0	0	0	12	0	5
	POMACE	40	62	21	31	0	17	0	39	38	6	30	7	0	0	0	0	6	0	0	6	0	0	0	0	14	0	5
23ºB	GRAPES	47	86	23	25	0	13	0	32	20	5	24	0	8	0	0	0	0	0	0	9	0	0	0	0	20	0	8
	1/4F	45	71	24	26	0	14	0	30	22	7	21	0	13	0	0	0	0	0	0	5	0	0	0	0	16	0	7
	1/2F	53	77	30	32	0	18	0	35	28	10	25	5	6	0	0	0	0	0	0	6	0	0	0	0	17	0	7
	3/4F	49	71	29	30	0	18	0	36	34	10	29	10	0	0	0	0	0	0	0	5	0	0	0	0	16	0	6
	POMACE	45	70	27	29	0	18	0	34	32	8	27	9	0	0	0	0	0	0	0	0	0	0	0	0	16	0	5
25ºB	GRAPES	45	90	29	29	0	22	0	39	30	5	32	8	0	0	0	0	9	0	0	10	5	0	8	10	31	7	16
	1/4F	60	85	44	43	0	35	0	43	38	10	33	8	8	0	0	0	9	0	0	5	0	0	7	6	24	5	10
	1/2F	56	81	42	42	0	37	0	44	40	10	35	10	0	0	0	0	9	0	0	5	0	0	7	6	23	5	11
	3/4F	52	77	39	39	0	33	0	48	45	11	40	14	0	0	0	0	12	0	0	5	0	6	10	7	27	6	12
	POMACE	41	68	33	35	0	30	0	46	45	10	39	13	0	0	0	0	9	0	0	5	0	6	9	7	25	5	11
									0		10		20		30		40		50		60		70		80		90	100

Figure 5.2. Heatmap of the epitope abundance (0-100) in the CDTA extract (CoMPP results) of Shiraz fresh grapes and fermenting pomace at different stages of the fermentation made from grapes at three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix).The values are average of three biological repeats. The sampling stages are represented by GRAPES (fresh grapes), different stages of the alcoholic fermentation (¼ F, ½ F and ¾ F) and POMACE (fermented pomace). Values lower than 5 are indicated as 0.

Afterwards, a decrease in the JIM7 signal was observed during the alcoholic fermentation (Appendix Figure 5.2) which might indirectly indicate a release of these HG polymers into the fermenting must (Gao, *et al.*, 2015). In short, the more depectinated the greater the exposure of the hemicellulose polymers, as found in riper berries. Thus, as illustrated in Figure 5.3, the separation between fresh grapes and skin pomace ¼ F, along the corresponding PC1 axis for the CDTA extract, was larger than in PCA-23ºBrix (Appendix Figure 5.3) and PCA-25ºBrix (Appendix Figure 5.4). These results suggest a more intact pectin layer in 21ºBrix, more compact and less accessible. There was an increase in the amount of Ca^{2+} crosslinked HG (2F4) extracted in fresh grapes during ripening that was significantly higher when 25ºBrix grapes were compared to 21ºBrix and 23ºBrix grapes. The significantly higher response of the 2F4 epitope represents a greater extraction of the Ca^{2+} cross-linked HG gel. 3 As a consequence, more degraded cell wall tissue layers may thereby favour the extraction of the specific phenolic compounds in the wines made from 25ºBrix grapes.

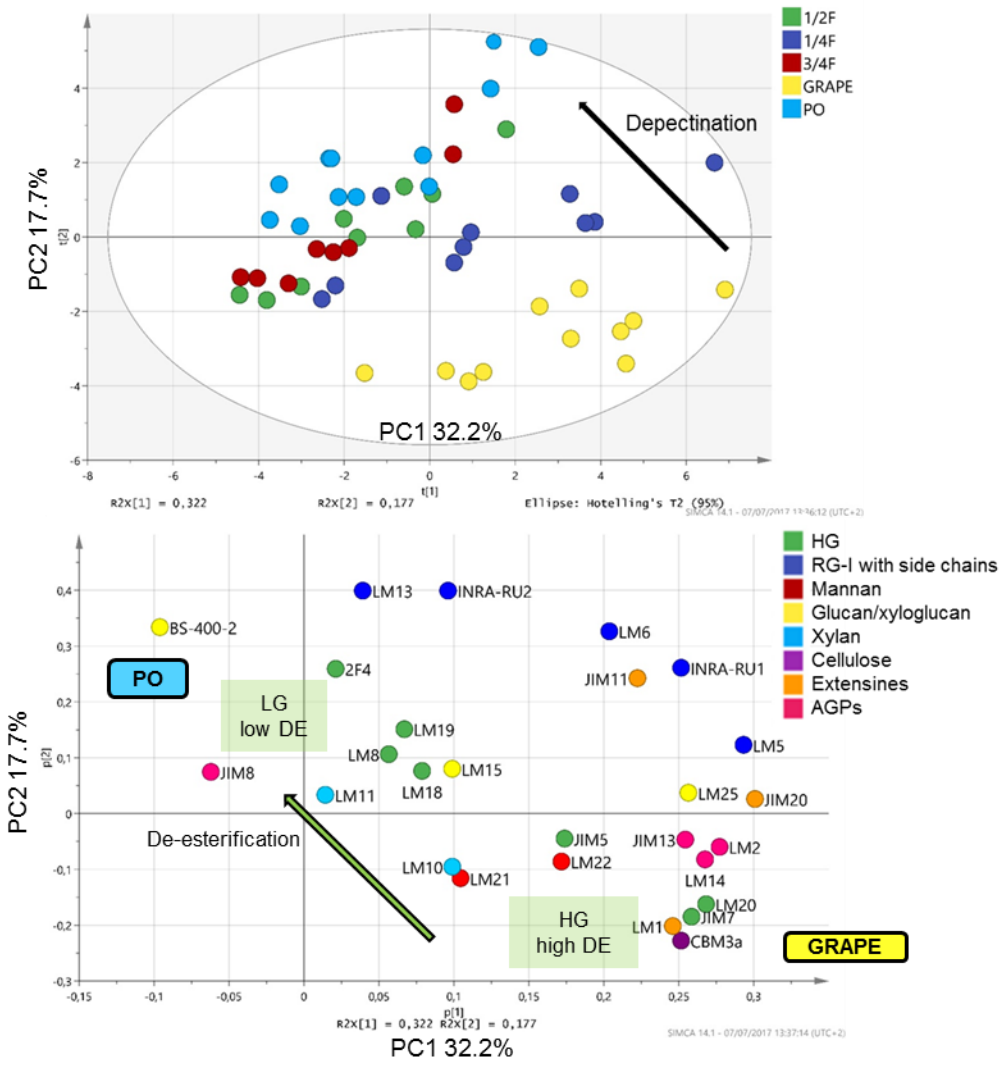

Figure 5.3. PCA score and loading plot for the CDTA extract (pectin-rich fraction) of Shiraz 21ºBrix treatment. The samples are coloured according to the alcoholic fermentation stage (GRAPE, ¼ F, ½ F, ¾ F and PO – fermented pomace) and the epitope category (loading plot). Degree of Esterification (DE).

From the NaOH extract (Figure 5.4), the exposure of the xyloglucans and cellulose is another factor driving the separation during fermentation of 21ºBrix along PC1 (37.2%) (Figure 5.5). The distribution of the samples according to the mAbs extracted with NaOH suggest a more intact hemicellulose rich fraction in 21ºBrix when compared to 23ºBrix (Appendix Figure 5.5) and 25ºBrix (Appendix Figure 5.6). The degradation of the hemicellulose rich fraction during fermentation is thereby enhanced by the grape ripeness as suggested Figure 5.5 when results from the 21 ºBrix treatment are compared with that of 23ºBrix (Appendix 5.5) and 25ºBrix (Appendix Figure 5.6).

		HG partially/de-esterified (mAb JIM5)	HG partially esterified (mAb JIM7)	HG partially/de-esterified (mAb LM18)	HG partially/de-esterified (mAb LM19)	HG partially esterified (mAbLM20)	HG Ca2+ crosslinked (mAb 2F4)	Xylogalacturonan (mAb LM8)	Backbone of rhamnogalacturonan I (mAb INRA-RU1)	Backbone of rhamnogalacturonan I (mAb INRA-RU2)	(1→4)-β-D-galactan (mAb LM5)	(1→5)-α-L-arabinan (mAb LM6)	Linearised (1→5)-α-L-arabinan (mAb LM13)	(1→4)-β-D-(galacto)(gluco)mannan (mAb LM21)	(1→4)-β-D-(gluco)mannan (mAb LM22)	(1→3)-β-D-glucan (mAb BS-400-2)	Xyloglucan (XXXG motif) (mAb LM15)	Xyloglucan/unsubstituted β-D-glucan (mAb LM25)	(1→4)-β-D-xylan (mAb LM10)	(1→4)-β-D-xylan/arabinoxylan (mAb LM11)	Cellulose (crystalline) (mAb CBM3a)	Extensin (mAb LM1)	Extensin (mAb JIM11)	Extensin (mAb JIM20)	AGP (mAb JIM8)	AGP (mAb JIM13)	AGP (mAb LM14)	AGP, β-linked GlcA (mAb LM2)
21ºB	GRAPES	0	0	0	12	0	0	0	20	14	18	19	6	22	0	8	62	72	6	0	53	16	5	15	0	17	6	0
	1/4F	5	0	0	12	0	0	0	24	19	19	22	8	24	0	17	64	74	7	0	54	18	6	17	0	20	7	6
	1/2F	0	0	0	0	0	0	0	27	26	19	25	11	13	0	19	60	72	7	0	52	15	5	14	0	18	6	5
	3/4F	0	0	0	11	0	0	0	29	28	20	29	13	19	0	21	62	74	7	5	54	15	5	14	0	18	6	6
	POMACE	0	0	0	13	0	0	0	42	46	24	43	21	27	6	32	63	75	7	0	56	21	6	18	0	22	9	8
23ºB	GRAPES	6	0	14	14	0	0	0	13	14	14	17	7	18	0	0	81	64	0	0	50	22	9	18	6	20	5	0
	1/4F	9	0	0	19	0	0	0	17	21	16	19	10	23	0	12	78	65	7	7	51	15	7	13	5	21	5	0
	1/2F	5	0	0	15	0	0	0	23	31	19	26	16	20	0	14	82	67	8	8	53	21	10	19	8	28	8	6
	3/4F	0	0	0	10	0	0	0	29	43	21	32	21	17	5	15	80	66	8	8	53	24	10	20	9	30	9	7
	POMACE	0	0	0	9	0	0	0	32	50	22	38	24	19	6	17	82	68	8	8	57	26	10	22	9	30	10	8
25ºB	GRAPES	0	0	0	0	0	0	0	17	20	12	18	7	14	17	5	76	66	7	6	55	29	8	21	6	17	5	0
	1/4F	7	0	6	15	0	0	0	24	30	18	27	12	21	17	13	79	70	12	11	58	26	8	19	7	22	7	6
	1/2F	0	0	6	13	0	0	0	32	41	20	34	16	21	18	15	78	70	12	11	57	31	9	23	9	27	9	7
	3/4F	0	0	5	10	0	0	0	36	46	22	38	18	21	18	15	78	71	12	11	58	29	9	21	9	26	9	7
	POMACE	6	0	8	21	0	0	0	45	59	27	48	25	29	16	17	73	71	11	12	59	31	8	21	9	28	9	8
									0	10	20	30	40	50	60	70	80	90	100								

Figure 5.4. Heatmap of the epitope abundance (0-100) in the NaOH extract (CoMPP results) of Shiraz fresh grapes and fermenting pomace at different stages of the fermentation made from grapes at three ripeness levels (21ºBrix, 23ºBrix and 25ºBrix).The values are average of three biological repeats. The sampling stages are represented by GRAPES (fresh grapes), different stages of the alcoholic fermentation (¼ F, ½ F and ¾ F) and POMACE (fermented pomace). Values lower than 5 are indicated as 0.

Therefore, the ripeness effect thus firstly enhanced the pectin layer's disruption during fermentation. The "opening-up" of the grape pomace was confirmed by a greater extraction of the cellulose (CBM3a) and xyloglucans epitopes (LM15 and LM25) in the NaOH extract (Figure 5.4) during the progression of fermentation, as these are found in the inner layer of the pomace (hemicellulose fraction) (Zietsman, *et al.*, 2017). The BS-400-2 epitope which recognises glucan polymers was also found to increase, especially in 21ºBrix. In this case, it is related to the formation of callose during the fermentative process (Zietsman, *et al.*, 2015).

The above-mentioned decrease in Ara (section 4.5.1.1) was not explained by the relative abundance of the RG-I epitopes (INRA-RU1, INRA-RU2). The results in fresh grapes showed a greater abundance of RG-I in the pectin layer. However, as a consequence of the "opening-up" of the grape pomace during the fermentation, a higher abundance in mAbs INRA-RU1, INRA-RU2, LM5, LM13 and especially in LM6 was found not only in the pectin layer, but also in the hemicellulose fraction (Figure 5.4) A higher exposure of the RG-I coating layer was generally found in riper berries (25ºBrix), supporting the idea of a greater "opening-up" of the grape pomace enhanced by the grape ripeness. From these results, a higher presence of RG-I with D-galactan side chains (mAb LM5) was also determined in the hemicellulose layers of the grape berry cell walls.

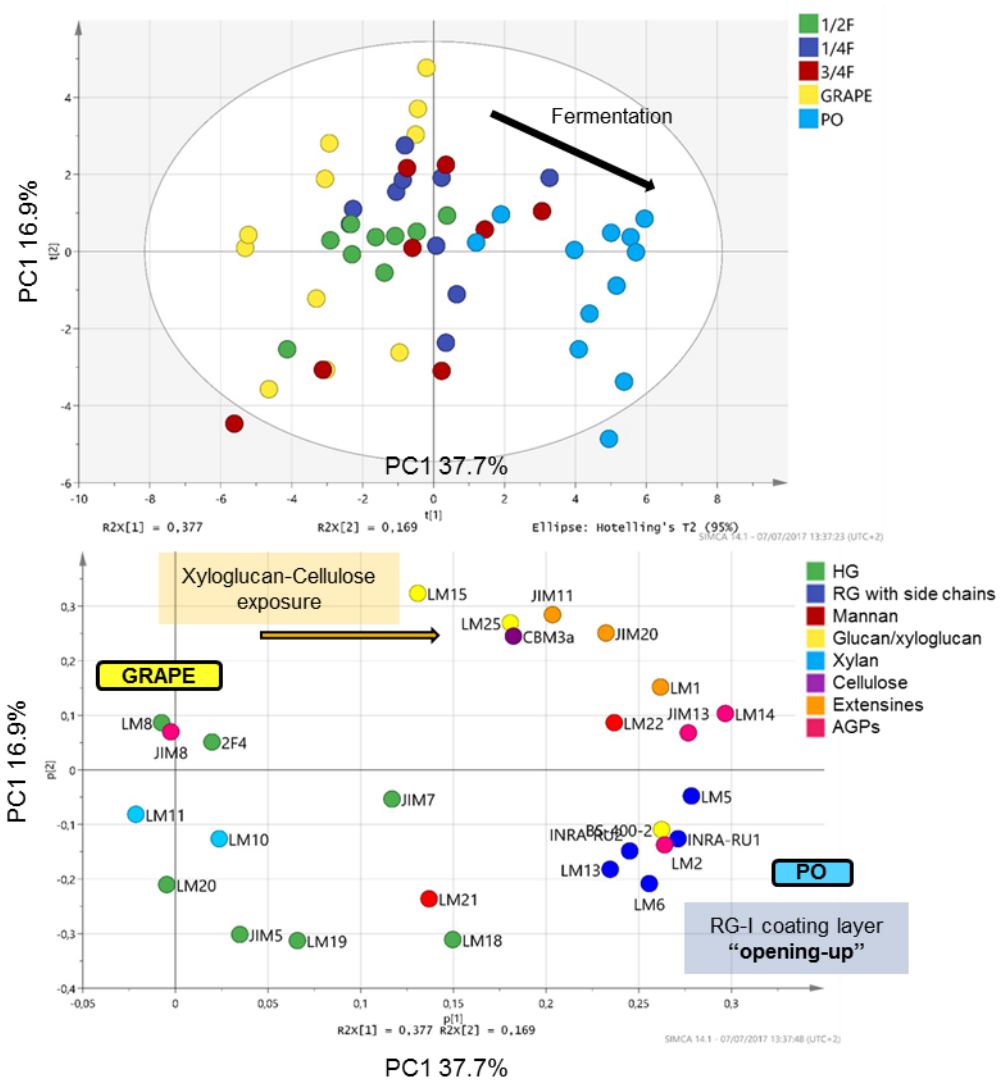

Figure 5.5. PCA score and loading plot for the NaOH extract (hemicellulose-rich fraction) of Shiraz 21ºBrix treatment. The samples are coloured according to the alcoholic fermentation stage (GRAPE, ¼ F, ½ F, ¾ F and PO – fermented pomace) and the epitope category (loading plot).

Although the AGPs epitopes were present in both the CDTA and NaOH extracts, the decrease in the relative abundance of Ara (Figure 5.4) might partly be explained by a decrease in the AGPs of the pectin fraction. On the other hand, clear signals for AGPs (mAbs JIM8, JIM13, LM14 and LM2) was found during fermentation in the NaOH extract. This increase in mAbs JIM8 and JIM13 was higher in 23ºBrix and 25ºBrix than in 21ºBrix.

5.3.2. Evolution of proanthocyanidins during fermentation determined by phloroglucinolysis in the grape skin

The phloroglucinolysis method allowed for the evaluation of the nature and complexity of the non-extractable tannins in the grape skins and pomace during alcoholic fermentation. As expected, from

the results in Table 5.2, the greatest changes in the tannin composition of the skins were mainly brought about by the alcoholic fermentation (Appendix Table 5.4). However, when the fresh grapes data was analysed separately, a few parameters, such as the amount of terminal units or the % of gallo units (prodelphinidins), were already significantly different at different ripeness levels (21ºBrix, 23ºBrix and 25ºBrix). In brief, a higher % of prodelphinidins was found in 23ºBrix and 25ºBrix grapes (Appendix Figure 5.7). In contrast, no significant differences were found for the mean degree of polymerisation (mDP) or average molecular weight (avMW) in fresh grapes (Table 5.2). Results reported in literature are not in agreement on the evolution of the proanthocyanidin polymer length during ripening, as some authors have reported an increase in the mDP (Kennedy, *et al.*, 2001; Bindon, *et al.*, 2013) whereas the tannin polymer length remained constant (Llaudy, *et al.*, 2008) or decreased, especially from véraison (Downey, *et al.*, 2003; Bordiga, *et al.*, 2011). Nevertheless, these trends could also differ between different cultivars as described by Obreque-Slier *et al.*, (2010,2013). The range of mDP (11,3 – 14,7) found in our results were somewhat higher than those of some authors (Obreque-Slier, *et al.*, 2010, 2013; Bindon, *et al.*, 2013), but notably lower compared to the results of skin tannins reported by Bordiga *et al.*,(2011). Values found in other studies with Shiraz grapes (Downey *et al*. 2003) at similar ripeness levels were higher (mDP 24-28; ºBrix: 23-25) than those found in our work.

To our knowledge, the only study investigating the influence of the maceration time on the mDP of the proanthocyanidins from the grapes at different ripeness levels, focused on the release of these compounds into a model solution (Llaudy, *et al.*, 2008). However, according to our knowledge, no information is available on the polymer length of proanthocyanidins found in fermenting skins. Our results showed a release of the larger phenolics as the mDP decreased in the grape pomace during the fermentative process (Table 5.2). From our results, the extraction trends during fermentation were similar between the three ripeness levels, however, the grape ripeness may have enhanced the extraction kinetics of the different proanthocyanidins. As shown in Table 5.2, the largest phenolics (i.e. higher mDP) were more rapidly released from 25ºBrix berries, as reflected by the earlier decrease in the mDP of the compounds remaining in the fermenting skins. This decrease in the mDP was already significant mDP after ¼ of the fermentation was completed in the wines made from 25ºBrix grapes, whereas in the wines made from 21ºBrix grapes it was only after ¾ F (Table 5.2). This earlier release in the 25ºBrix treatment could be related with an easier extraction from grape cells into the fermenting must due to the cell wall de-pectination that occurred during ripening.

Table 5.2. Phloroglucinolysis analysis in the fermenting skin pomace of three different ripeness levels (21ºBrix, 23ºBrix and 25ºBrix) during different stages of the alcoholic fermentation. The different letters indicate significant differences (ANOVA p<0.05) between the treatments (ripeness and fermentation stage).

		21ºBrix	23ºBrix	25ºBrix
mDP	**GR**	12.86 ± 1.24 ^{ab}	11.34 ± 0.15 ^{abcd}	14.72 ± 2.52 ^{a}
	¼ F	11.87 ± 0.13 ^{abc}	8.90 ± 0.40 ^{efc}	10.83 ± 1.06 ^{eb}
	½ F	12.05 ± 5.06 ^{abc}	7.67 ± 0.24 ^{efd}	10.10 ± 0.76 ^{efb}
	¾ F	6.87 ± 0.29 ^{f}	9.20 ± 1.20 ^{efc}	7.25 ± 0.55 ^{ef}
	AF	7.84 ± 0.51 ^{ef}	9.15 ± 1.15 ^{efc}	8.05 ± 1.02 ^{efc}
%Gallo units	**GR**	49.59 ± 0.47 ^{ab}	53.59 ± 1.28 ^{a}	53.53 ± 1.01 ^{a}
	¼ F	50.29 ± 1.52 ^{ab}	47.11 ± 0.25 ^{cbd}	48.27 ± 0.27 ^{cb}
	½ F	42.72 ± 4.94 ^{hdg}	42.67 ± 0.95 ^{fdg}	44.74 ± 0.81 ^{cde}
	¾ F	38.27 ± 3.64 ^{hg}	42.31 ± 1.33 ^{feg}	40.10 ± 1.33 ^{fh}
	AF	42.60 ± 0.98 ^{fe}	38.58 ± 1.82 ^{fh}	35.84 ± 0.95 ^{h}
% Galloyl units	**GR**	5.95 ± 0.41 ^{cd}	6.68 ± 0.17 ^{cb}	5.23 ± 0.33 ^{d}
	¼ F	6.47 ± 0.08 ^{cdb}	7.40 ± 0.42 ^{ab}	6.42 ± 0.50 ^{cdb}
	½ F	5.71 ± 0.71 ^{cd}	7.25 ± 0.15 ^{ab}	6.78 ± 0.49 ^{cb}
	¾ F	6.67 ± 0.53 ^{cb}	5.84 ± 0.71 ^{cd}	7.32 ± 0.29 ^{ab}
	AF	6.30 ± 0.37 ^{cdb}	6.43 ± 0.02 ^{cb}	8.28 ± 0.14 ^{a}
av MW	**GR**	3929.3 ± 376.0 ^{ab}	3471.0 ± 46.8 ^{abcde}	4523.6 ± 796.5 ^{a}
	¼ F	3696.9 ± 58.1 ^{abc}	2951.9 ± 232.0 ^{fb}	3460.9 ± 352.0 ^{abcde}
	½ F	3652.1 ± 1535.8 ^{abcd}	2369.3 ± 86.0 ^{fe}	3133.4 ± 255.9 ^{fb}
	¾ F	2103.1 ± 86.6 ^{f}	2785.0 ± 355.3 ^{fc}	2279.0 ± 159.1 ^{f}
	AF	2443.0 ± 158.4 ^{fd}	2922.8 ± 315.4 ^{fb}	2468.2 ± 314.5 ^{fc}

mDP: mean degree of polymerisation
av MW: average molecular weight

Additionally, comparing all the fermentation stages for the three ripeness levels, the % of prodelphinidins and the average avMW showed a significant decreased during the fermentation for all the ripeness levels (except for MW in 23ºBrix). The decrease in % prodelphinidins was higher in 23ºBrix and 25ºBrix when compared to 21ºBrix (Table 5.2). Interestingly, for 25ºBrix, the % of galloyl units showed an increase in the grape pomace as the fermentation progressed, whereas in 21 ºBrix and 23ºBrix it remained relatively constant. Seed phenolics are especially rich in these galloylated compounds. A high affinity of the cell wall material for galloylated units have been described in literature (Renard, *et al.*, 2001; Hanlin, *et al.*, 2010). Therefore, as a consequence of the higher levels of alcohol produced from the riper berries (25ºBrix), the ripeness level seemed to have influenced the seeds' extractability (Canals, *et al.*, 2005; Hernández-Jiménez, *et al.*, 2012), thereby releasing a higher amount of galloylated units into the wines.

From the results in Appendix Table 5.5, in fresh grapes, catechin (C) was the most representative compound in the terminal units of the skin proanthocyanidins (for all ripeness levels) whereas epigallocatechin (EGC), followed by the epicatechin (EC) were the major monomers part of the extension units (measured as EGC-P and EC-P respectively). EC was found to be the most abundant extension unit in the majority of grape cultivars investigated in a study conducted by Mattivi

et al., (2009), however, a higher proportion of EGC was also found for some of the grape cultivars (Mattivi, *et al.*, 2009). A similar evolution was found, between the three ripeness levels, in the % of the skin proanthocyanidin extension units during fermentation. Whilst the EGC-P % decreased, the % of EC-P increased. This may indicate a greater release of prodelphinidins (rich in epigallocatechin subunits) compared to procyanidins. Higher levels of prodelphinidins were found in riper berries (Appendix Table 5.5).

5.3.3. Colour and phenolic extraction during fermentation from three different grape ripeness levels

The extractability of certain phenolic compounds can be associated to the conformational and structural changes of the cell wall occurring during grape ripeness (Bindon, *et al.*, 2014b). In this study, the release of these phenolic compounds into the wines was followed by spectrophotometric (Table 5.3) and HPLC (Table 5.4) analysis at several stages of the alcoholic fermentation (¼F, ½F, ¾F and AF) for the three ripeness stages. From the results, a strong ripeness impact was observed, especially between 21ºBrix and 23º-25ºBrix, as the extraction kinetics differed between them. Firstly, as described in Table 5.3, the colour and phenolics parameters (CD, MCD, TRP, TP, SO_2 resistant pigments and tannins) measured in wines after pressing (AF), were significantly lower in the wines made from the least ripe berries (21ºBrix) compared to wines made from 23ºBrix and 25ºBrix grapes. The concentration of copigments was similar in wines made from 21ºBrix and 25ºBrix grapes. A higher extraction was expected in the wines made from the 25ºBrix harvest, however, only the copigments and MCP tannins levels (Appendix Figure 5.8) were significantly different in the wines made from 23 ºBrix and 25ºBrix grapes compared with those of 21ºBrix.

As expected, the overall results showed a general increase in phenolics with the progression of fermentation. Nevertheless, the extraction trends of specific classes of phenolic compounds during fermentation differed between the different grape ripeness levels. The impact of the ethanol content on grapes from different ripeness levels had previously been reported (Canals, *et al.*, 2005). In agreement with Canals *et al*. (2005), the ripeness seemed to enhance the extractability during fermentation in our case, too. Regarding colour extraction, the CD only reached its maximum peak at ¾F for musts from 21ºBrix grapes, followed by a decrease towards the end of the fermentation. For wines made from 21ºBrix grapes, copigments and TRP content also reached their peak at ¾F, but then remained stable. The highest amount of MCP tannins extracted were found half way through the alcoholic fermentation. In brief, from these findings it seemed that, in the 21ºBrix treatment, the phenolic compounds reached a plateau during the alcoholic fermentation. On the other hand, the 23ºBrix and 25ºBrix wines showed a gradual increase in most of these parameters, except for the concentration of copigments in the wines made from 25ºBrix grapes (they reached their peak concentration halfway through the fermentation), reaching maximum values at the end of the

fermentation (Table 5.3). The concentration of SO_2 resistant pigments significantly increased during the fermentation for all three grape ripeness levels. The amount of SO_2 resistant pigments was significantly higher in wines made from 23º and 25ºBrix grapes, compared to those made from 21ºBrix grapes (Table 5.3).

Table 5.3. Colour and phenolic analysis during fermentation from grapes harvested at three different grape ripeness levels (21ºBrix, 23ºBrix and 25ºBrix). The different letters indicate significant differences (ANOVA $p<0.05$) between the treatments (ripeness and fermentation stage).

		21ºBrix	23ºBrix	25ºBrix
CD (AU)	¼ F	8.90 ± 0.67 ^{g}	9.35 ± 1.34 ^{gf}	10.32 ± 1.10 ^{eg}
	½ F	12.32 ± 0.96 ^{ed}	14.03 ± 1.34 ^{cd}	15.38 ± 1.78 ^{cb}
	¾ F	13.68 ± 0.60 ^{cd}	16.91 ± 0.24 ^{ab}	14.30 ± 2.01 ^{cd}
	AF	11.35 ± 1.34 ^{f}	17.84 ± 1.32 ^{a}	17.64 ± 2.84 ^{a}
Hue	¼ F	0.38 ± 0.01 ^{eg}	0.44 ± 0.01 ^{bc}	0.41 ± 0.02 ^{ab}
	½ F	0.37 ± 0.01 ^{gf}	0.40 ± 0.02 ^{ecd}	0.40 ± 0.02 ^{ecd}
	¾ F	0.36 ± 0.01 ^{g}	0.39 ± 0.02 ^{egc}	0.39 ± 0.02 ^{egc}
	AF	0.41 ± 0.01 ^{abc}	0.44 ± 0.22 ^{a}	0.44 ± 0.02 ^{a}
MCD (AU)	¼ F	6.86 ± 0.87 ^{f}	9.61 ± 0.78 ^{de}	10.61 ± 0.92 ^{d}
	½ F	8.97 ± 0.64 ^{de}	12.76 ± 1.05 ^{c}	14.79 ± 1.49 ^{b}
	¾ F	9.17 ± 0.23 ^{de}	14.11 ± 1.56 ^{b}	14.53 ± 0.64 ^{b}
	AF	8.28 ± 0.87 ^{e}	15.12 ± 1.00 ^{ab}	16.73 ± 1.44 ^{a}
TRP (AU)	¼ F	16.39 ± 1.04 ^{e}	20.02 ± 1.76 ^{d}	19.45 ± 0.92 ^{d}
	½ F	19.93 ± 1.17 ^{d}	25.31 ± 2.48 ^{c}	28.92 ± 3.12 ^{b}
	¾ F	23.35 ± 0.51 ^{c}	30.79 ± 2.63 ^{b}	29.86 ± 0.49
	AF	24.17 ± 0.60 ^{c}	37.50 ± 1.70 ^{a}	37.82 ± 0.30 ^{a}
TP (AU)	¼ F	27.08 ± 1.17 ^{e}	29.35 ± 1.86 ^{ed}	28.54 ± 0.64 ^{e}
	½ F	31.97 ± 1.53 ^{cd}	32.01 ± 1.70 ^{cd}	39.21 ± 3.18 ^{b}
	¾ F	34.29 ± 2.39 ^{c}	38.81 ± 2.48 ^{b}	40.29 ± 1.14 ^{b}
	AF	39.40 ± 2.30 ^{b}	51.62 ± 2.21 ^{a}	53.37 ± 0.11 ^{a}
Copigments (AU)	¼ F	9.69 ± 0.73 ^{f}	11.68 ± 0.55 ^{efd}	11.33 ± 0.30 ^{ef}
	½ F	11.85 ± 0.90 ^{ed}	15.89 ± 0.38 ^{cb}	16.31 ± 3.98 ^{ab}
	¾ F	13.18 ± 0.59 ^{ce}	16.91 ± 0.86 ^{ab}	15.18 ± 2.14 ^{cb}
	AF	11.38 ± 0.42 ^{efd}	18.13 ± 0.89 ^{a}	13.99 ± 2.55 ^{cd}
SO_2 resistant pigments (AU)	¼ F	0.63 ± 0.08 ^{j}	1.18 ± 0.12 ^{gf}	1.31 ± 0.38 ^{eg}
	½ F	0.72 ± 0.06 ^{jh}	1.67 ± 0.11 ^{ed}	1.71 ± 0.20 ^{cd}
	¾ F	1.00 ± 0.35 ^{gh}	2.06 ± 0.21 ^{cb}	1.93 ± 0.23 ^{cd}
	AF	1.52 ± 0.35 ^{edf}	2.45 ± 0.29 ^{a}	2.33 ± 0.25 ^{ab}
Tannins (mg/L)	¼ F	244.66 ± 14.73 ^{e}	282.97 ± 59.87 ^{de}	380.00 ± 27.28 ^{d}
	½ F	399.57 ± 84.01 ^{d}	376.14 ± 83.91 ^{d}	676.85 ± 88.04 ^{cb}
	¾ F	344.25 ± 77.83 ^{de}	382.49 ± 91.62 ^{d}	729.49 ± 66.96 ^{ab}
	AF	311.57 ± 68.82 ^{de}	584.99 ± 20.54 ^{c}	842.03 ± 96.17 ^{a}

CD: colour density (AU). MCD: modified colour density (AU). TRP: total red pigments (AU). TP: total phenolics (AU).

Interestingly, although the wines made from 21ºBrix grapes had in general a lower phenolic profile at AF, differences between the 21ºBrix, 23ºBrix and 25ºBrix treatments at ¼F were smaller for some of these colour and phenolic parameters compared to AF. For example, the CD or TP were similar after ¼ F (Table 5.3). At the same fermentation stage (¼F), the tannin concentration was already higher in the must from 25ºBrix compared to those from 21ºBrix grapes (Appendix Figure 5.8), but

with the progress of fermentation, these differences in between the different treatment became larger.

These differences were confirmed by the phenolic results analysed by HPLC (Table 5.4). A very low concentration of these individual phenolics was obtained from the HPLC analysis in 23ºBrix treatments after ¼ of the alcoholic fermentation (23B ¼F). At the same fermentation stage (¼ F), no significant differences were found between 23º and 25ºBrix treatments from the spectrophotometric results. We can therefore consider the possibility of a sampling or instrumental error. Nevertheless, from the overall HPLC results, a significantly lower anthocyanin (total glucosylated anthocyanins, total acetylated anthocyanins and total coumaroylated anthocyanins) and polymeric pigment content was found in 21ºBrix at the end of fermentation (AF) compared to wines made from 23ºBrix and 25ºBrix grapes (Table 5.4). However, ANOVA analysis did not show significant differences for the concentration of catechins, the total amount of hydroxycinnamic acids or total amount of flavonols between the final wines (AF).

A greater tannin content was found in wines made from 23ºBrix and 25ºBrix grapes. The concentration of B1-dimer and polymeric phenols was also significantly higher in 23ºBrix (not for B1) and 25ºBrix finished wines compared to wines of the 21º Brix treatment (Table 5.4). In all wines, B-1 reached its maximum at ¾ of the alcoholic fermentation, decreasing towards the end in the 21ºBrix wine treatment. The amount of polymeric phenolics gradually increased, reaching its peak at AF. These polymeric phenols were positively correlated with the increase of the wine alcohol content (R^2=0.74). However, the differences in concentration for the final wines made from 23ºBrix and 25ºBrix grapes were not significant. Thus, differences found in wine MCP tannin concentrations, especially between 23 and 25ºBrix treatments, were not reflected by the HPLC results (R^2=0.47 between MCP tannins and polymeric phenols). These could be explained by a higher amount of tannins possibly binding to cell wall polysaccharides (possibly as the result of a greater cell wall deconstruction).

In contrast, a lower CD observed in 21ºBrix wines towards the end of alcoholic fermentation (Table 5.3) can be linked to the decrease in the total free anthocyanins (not significant for glucoside and acetylated anthocyanins, but significant for coumaroylated anthocyanins) levels from ¾F onwards. This loss in CD and anthocyanin concentration was not observed in the wines of the 23ºBrix and 25ºBrix treatments, as they remained constant during the latter stages of the fermentation, except for the coumaroylated anthocyanins in the 23ºBrix treatment. Additionally, as in the case of the concentration of SO_2 resistant pigments, the concentration of polymeric pigments in the wines generally increased during fermentation (R^2=0.65 between SO_2 resistant pigments and polymeric pigments), explained by the formation of these anthocyanin-derivatives after crushing. However, in wines made from 21ºBrix grapes, the amount of polymeric pigments reached its peak half way through the fermentation (½F) whereas in wines from made from 23ºBrix and 25ºBrix grapes it increased until ¾F and AF respectively. These differences can be explained by a greater

concentration of polymeric pigments in riper grapes or a greater extraction. Also, this increase could be due to a greater extraction of phenolic compounds susceptible to form these polymeric pigments during fermentation.

Table 5.4. Groups of individual phenolic compounds (mg/L) during fermentation from grapes harvested at three different grape ripeness levels (21ºBrix, 23ºBrix and 25ºBrix). The different letters indicate significant differences (ANOVA p<0.05) between the treatments (ripeness and fermentation stage).

		21ºBrix	23ºBrix	25ºBrix
Gallic acid	¼ F	2.63 ± 0.06 ^{i}	0.63 ± 0.26 ^{j}	3.91 ± 0.25 ^{fgh}
	½ F	3.07 ± 0.05 ^{hi}	4.19 ± 0.14 ^{efg}	4.51 ± 0.25 ^{def}
	¾ F	3.56 ± 0.03 ^{gh}	4.87 ± 0.45 ^{de}	5.10 ± 0.11 ^{d}
	AF	6.13 ± 0.17 ^{c}	7.42 ± 0.69 ^{b}	8.61 ± 0.37 ^{a}
Catechin	¼ F	6.00 ± 1.14 ^{cd}	0.72 ± 0.22 ^{d}	3.92 ± 0.39 ^{cd}
	½ F	6.17 ± 1.78 ^{cd}	7.74 ± 0.51 ^{ab}	5.05 ± 0.31 ^{cd}
	¾ F	7.80 ± 0.77 ^{bcd}	15.49 ± 9.16 ^{a}	5.80 ± 0.50 ^{cd}
	AF	10.14 ± 0.22 ^{abc}	10.07 ± 0.46 ^{abc}	9.70 ± 0.18 ^{abc}
B1	¼ F	51.22 ± 4.36 ^{cd}	4.86 ± 3.46 ^{e}	52.74 ± 3.98 ^{bcd}
	½ F	61.52 ± 3.58 ^{abcd}	67.09 ± 3.75 ^{ab}	63.92 ± 4.58 ^{abc}
	¾ F	66.47 ± 3.10 ^{ab}	70.49 ± 11.92 ^{a}	65.88 ± 4.41 ^{abc}
	AF	47.51 ± 2.78 ^{d}	62.19 ± 8.73 ^{abcd}	64.47 ± 5.14 ^{abc}
Polymeric phenols	¼ F	90.07 ± 4.86 ^{fg}	31.49 ± 12.52 ^{g}	125.59 ± 6.07 ^{ef}
	½ F	134.87 ± 8.12 ^{e}	204.69 ± 13.90 ^{cd}	190.11 ± 14.18 ^{d}
	¾ F	136.48 ± 9.16 ^{e}	245.75 ± 28.71 ^{b}	228.90 ± 16.32 ^{bc}
	AF	182.76 ± 17.11 ^{cd}	329.84 ± 34.48 ^{a}	372.98 ± 35.41 ^{a}
∑ Hydroxycinnamic acids	¼ F	35.83 ± 0.72 ^{a}	12..00 ± 7.87 ^{g}	33.10 ± 0.71 ^{abc}
	½ F	35.16 ± 0.91 ^{ab}	28.15 ± 1.12 ^{bcd}	30.92 ± 0.72 ^{abcd}
	¾ F	30.58 ± 0.55 ^{abcde}	24.69 ± 1.12 ^{def}	26.75 ± 0.31 ^{cde}
	AF	17.95 ± 0.30 ^{fg}	17.95 ± 1.26 ^{fg}	23.64 ± 0.56 ^{ef}
∑ Flavonol	¼ F	61.83 ± 3.09 ^{d}	19.68 ± 12.85 ^{f}	41.81 ± 2.40 ^{e}
	½ F	78.64 ± 2.91 ^{bc}	73.93 ± 3.29 ^{bcd}	59.15 ± 3.58 ^{d}
	¾ F	87.16 ± 1.03 ^{ab}	86.56 ± 5.32 ^{ab}	69.31 ± 2.61 ^{cd}
	AF	83.49 ± 1.70 ^{abc}	93.82 ± 7.23 ^{a}	82.57 ± 3.62 ^{abc}
∑ Glucosylated anthocyanins	¼ F	124.01 ± 4..73 ^{e}	54.34 ± 36..81 ^{f}	156.59 ± 5.63 ^{de}
	½ F	152.21 ± 6..04 ^{de}	199.30 ± 4..12 ^{bc}	200.60 ± 8.71 ^{bc}
	¾ F	173.03 ± 2..09 ^{cd}	233.49 ± 10..50 ^{ab}	211.48 ± 7.41 ^{ab}
	AF	153.18 ± 0.79 ^{de}	238.14 ± 12.09 ^{a}	237.87 ± 7.83 ^{a}
∑ Acetylated anthocyanins	¼ F	51.04 ± 2.12 ^{f}	22.88 ± 15.49 ^{g}	61.25 ± 2.09 ^{ef}
	½ F	64.49 ± 3.03 ^{ef}	82.56 ± 0.99 ^{cd}	86.48 ± 3.69 ^{bcd}
	¾ F	73.39 ± 1.08 ^{de}	98.90 ± 5.20 ^{ab}	92.02 ± 3.09 ^{abc}
	AF	65.93 ± 0.53 ^{ef}	101.54 ± 4.75 ^{ab}	104.27 ± 3.64 ^{a}
∑ Coumaroylated anthocyanins	¼ F	26.00 ± 1.20 ^{ef}	11.34 ± 6.92 ^{g}	32.12 ± 1.69 ^{de}
	½ F	33.74 ± 2.36 ^{de}	38.90 ± 1.14 ^{bcd}	47.15 ± 3.19 ^{ab}
	¾ F	35.33 ± 1.15 ^{cd}	44.06 ± 2.61 ^{ab}	48.20 ± 3.71 ^{a}
	AF	22.26 ± 1.04 ^{f}	35.05 ± 2.49 ^{cd}	42.46 ± 2.60 ^{abc}
Polymeric pigment	¼ F	5.06 ± 1.83 ^{hi}	2.19 ± 0.92 ^{j}	7.24 ± 2.14 ^{ghi}
	½ F	9.25 ± 2.48 ^{fg}	18.26 ± 1.17 ^{cd}	12.18 ± 0.16 ^{ef}
	¾ F	8.99 ± 0.55 ^{fgh}	23.17 ± 3.89 ^{ab}	14.51 ± 3.03 ^{de}
	AF	10.11 ± 0.56 ^{fg}	25.11 ± 2.39 ^{a}	20.72 ± 2.82 ^{bc}

5.3.4. CIELab

The colour extraction kinetics was also analysed using the CIELab parameters. A negative correlation between monomeric anthocyanins and L*, b* or H_{ab}* has been found, whereas a positive correlation with C_{ab}*, has previously been reported in young red wines (Han, *et al.*, 2008). Also, a significant correlation was found between the concentration of gallic acid in wines and the C_{ab}* and b* values (Caivano & del Pilar, 2012).

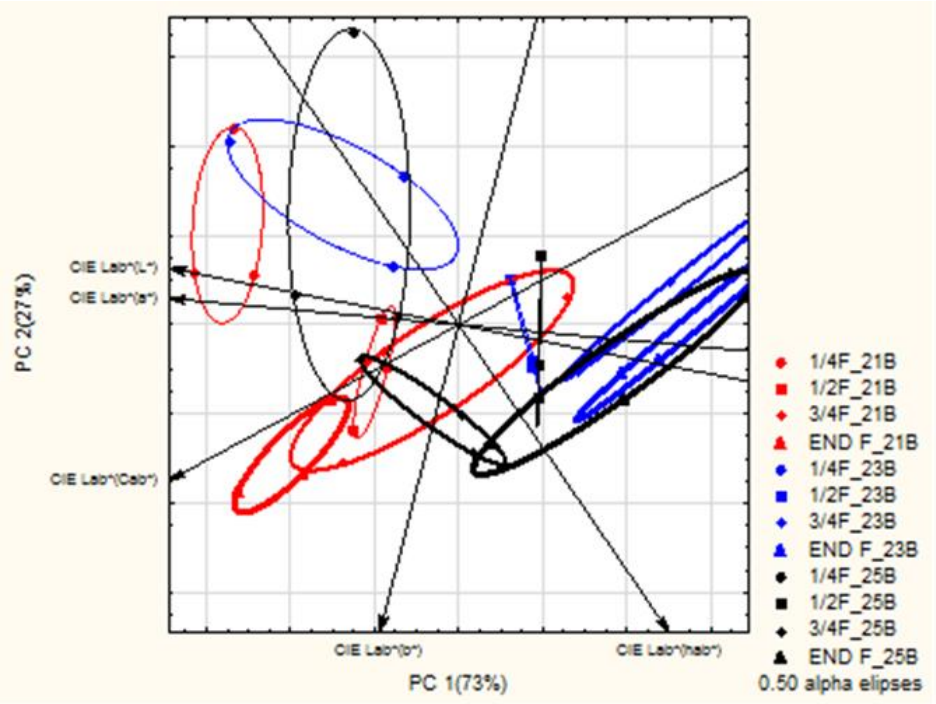

Figure 5.6. Wine sample distribution according to the CIELab parameters in the wine. The ellipses are coloured according to the grape ripeness levels (21ºBrix, 23ºBrix and 25ºBrix), becoming thicker as the fermentation progresses (¼ F, ½ F, ¾ F and AF).

As illustrated in Figure 5.6, the samples were mainly separated along PC1 (73%) by ripeness and fermentation stage in terms of the CIELab results. The biplot shows the sample distribution according to the different CIELab colour parameters. The ellipses, which become thicker with over the course of the fermentative process, represent the variability in the measurements. Thus, on the left axis on PC1, characterised by higher values of L*, a*, and C_{ab}*, are the wines made from 21ºBrix grapes (Appendix Table 5.6). These parameters described 21ºBrix as clearer wines with a higher red component, possibly explained by a lower content of polymeric pigments. Ethanol content and pH also influence these chromatic differences (Hermosín-Gutiérrez, 2003; Canals, *et al.*, 2005; Torskangerpoll & Andersen, 2005). The lower pH of 21ºB wines compared with 23-25ºBrix wines (Table 5.1) could help to explain the higher a* values, as the red components decrease with a higher pH (Sarni-Manchado, *et al.*, 1996). Additionally, 23ºBrix and 25ºBrix wines were also found in the

left axis of PC1 for their first sampling stage of the alcoholic fermentation (¼ F). Therefore, the grape pigments extracted at the early stages of the alcoholic fermentation were probably more water soluble and therefore easily extractable into the wines, independent of ripeness or alcohol potential of the wines. As the wines fermented, the 23ºBrix and 25ºBrix treatments showed a greater colour, with a lower red but a higher yellow component, associated with the contribution of polymeric pigments to the wine colour (Appendix Table 5.6). Colour differences between the different treatments wines were more marked as the fermentation progressed.

5.3.5. Unravelling the relationship between extractable and non-extractable phenolic compounds and the cell wall deconstruction

Several polymerisation reactions between phenolics, as well as possible interactions between CWM and phenolic compounds, are involved during the fermentation process. Therefore, it is difficult to establish a specific relationship between the changes in specific grape cell wall polysaccharides and the release of grape phenolics into the wines. In brief, a progressive phenolic extraction was found during fermentation (Table 5.3 and 5.4), enhanced by the prior disassembly induced in the skin cell walls by the grape ripeness. The grape berry cell wall model proposed by Gao et al (2015) describe two main layers in the grape pomace of Cabernet Sauvignon: a pectin-rich fraction, with a large amount of highly-esterified HG and Ca^{2+} crosslinked HG, and an internal hemicellulose-rich fraction, associated with RG-I (RG-I coating layer). The authors describe the possible start of the de-pectination process (occurring during the fermentation) from the pulp cells, characterised by a thinner cell walls. Thus, based on the cell wall model proposed by Gao *et al*., (2015) and the CoMPP results, Figure 5.7 illustrates the disassembly and phenolic release occurring during fermentation and enhanced by grape ripeness. A more de-pectinated berry, partly explained by a higher signal of Ca^{2+} crossed-linked HG to the mAbs 2F4, was found in grapes harvested at 25ºBrix, compared to 21ºBrix and 23ºBrix. This signal increased during the fermentation (Appendix Figure 5.2), especially in 25ºBrix, due to the de-pectination process. The greater de-esterification in the pectin layer of 25ºBrix fresh grapes probably led to better access to the inner layers of the pomace and subsequent easier release of phenolic compounds. The mDP in the grape skins for 21ºBrix was constant up to ¾F, whereas it significantly decreased after ¼F in the 23ºBrix and 25ºBrix treatments. The proanthocyanidin extraction may have been influenced by their polymer size and their retention, which are influenced by the degree of cell wall de-pectination. The greater loss in the % of gallo units found in the fermenting grape pomace of the riper berries (23º and 25ºBrix) may also be explained by a different retention on the cell wall polymers or a lower accessibility due to more rigid cell wall layers in the 21ºBrix grapes.

Additionally, the individual phenolic compounds remaining in the fermenting grape pomace were also quantified with HPLC, but only for 21ºBrix grapes (Table 5.5). The analysis were performed on

the same acetone grape homogenate used for the phloroglucinol reaction. Surprisingly, not all the compounds decreased in concentration as the fermentation progressed. Notably the polymeric forms (polymeric phenols and polymeric pigments) remained relative stable during the fermentation. A retention by the CWM or low accessibility to a part of the phenolic polymeric fraction, due to their molecular size, probably explains this occurrence. The formation of cavities or pores can be formed via grape loosening, as a consequence of cell wall de-pectination. This increase in the grape skin porosity can lead to the encapsulation of grape phenolics, creating a more difficult extraction into the must or wine and therefore affecting the extractability of grape tannins (Bindon, *et al.*, 2012; Bindon, *et al.*, 2014a). The affinity and the strong interactions formed between the highly methylated HG and the procyanidins is well known in apples (Watrelot, *et al.*, 2013). Furthermore, with the fermentative process, certain cell wall components, such as the HG labelled with mAb JIM7 (Appendix Figure 5.2), can also be extracted into the wines as demonstrated by (Gao, *et al.*, 2015). These HG molecules released into the wines might be either be free or bound to wine phenolics, influencing the tannin self-aggregation and therefore potentially influencing the wine's phenolic stability. In this matter, as mentioned before, the MCP tannin concentration in finished wines (AF) was significantly higher in the wines made from 25ºBrix grapes compared to those of 23ºBrix, whereas no significant differences were found for the polymeric phenols.

When the individual phenolics that remained in the skins of the 21 ºBrix treatments were assessed during fermentation a decreasing trend was observed for anthocyanins and flavonols according to the concentration of these compounds remained in the fermenting skins (Table 5.5). The release of free-anthocyanins (all glucosylated, acetylated and coumaroylated anthocyanins forms) was evident as their concentration in the grape skins decreased significantly during fermentation. Anthocyanins are water soluble compounds and consequently more easily extractable (Castañeda-Ovando, *et al.*, 2009). In a similar way, most of the flavonols were also extracted into the wines (Table 5.5). In both cases, the greatest decrease occurred from crushing until the midway through the alcoholic fermentation.

Table 5.5. Group of non-extracted individual phenolic compounds (mg/L) in the fermenting grape skins and pomace of 21ºBrix grapes at different stages during the alcoholic fermentation (GR: fresh grapes, F as the fermentation progress and PO: fermented pomace). The different letters indicate significant differences (ANOVA $p<0.05$) between the fermentation stages.

	GR	¼ F	½ F	¾ F	PO
Gallic acid	1.69 ± 0.07 ^{c}	4.21 ± 1.16 ^{bc}	6.20 ± 1.18 ^{ab}	8.24 ± 0.94 ^{a}	6.09 ± 0.21 ^{ab}
Catechin	38.2 ± 7.1 ^{b}	70.5 ± 17.3 ^{ab}	73.3 ± 13.1 ^{a}	80.8 ± 5.9 ^{a}	86.7 ± 4.1 ^{a}
B1	156.3 ± 38.4 ^{a}	115.4 ± 22.2 ^{ab}	101.9 ± 27.3 ^{ab}	77.5 ± 2.9 ^{b}	106.5 ± 1.2 ^{ab}
Polymeric phenols	4762.9 ± 411.7 ^{abc}	5522.7 ± 260.7 ^{ab}	4345.8 ± 350.6 ^{c}	4555.7 ± 387.5 ^{bc}	5827.8 ± 326.5 ^{a}
∑ hydroxycinnamic	2.61 ± 0.49 ^{b}	3.85 ± 1.38 ^{ab}	5.35 ± 1.58 ^{ab}	7.04 ± 0.64 ^{a}	6.96 ± 0.41 ^{a}
∑ flavonols	370.88 ± 26.95 ^{a}	294.32 ± 4.82 ^{ab}	253.12 ± 49.52 ^{b}	243.72 ± 16.94 ^{b}	268.10 ± 30.08 ^{b}
∑ Glucosylated anthocyanins	391.6 ± 141.2 ^{a}	174.9 ± 56.9 ^{ab}	117.4 ± 104.5 ^{b}	36.4 ± 12.0 ^{b}	26.0 ± 1.8 ^{b}
∑ Acetylated anthocyanins	185.0 ± 64.8 ^{a}	81.1 ± 22.7 ^{ab}	49.6 ± 40.4 ^{b}	18.3 ± 7.2 ^{b}	17.4 ± 1.0 ^{b}
∑ Coumaroylated anthocyanins	319.1 ± 104.0 ^{a}	164.5 ± 52.1 ^{ab}	79.5 ± 60.8 ^{b}	33.5 ± 9.8 ^{b}	28.8 ± 1.5 ^{b}
Polymeric pigments	47.4 ± 6.4 ^{ab}	63.2 ± 1.0 ^{a}	42.6 ± 7.9 ^{b}	47.0 ± 5.0 ^{ab}	42.4 ± 3.1 ^{b}

An interesting increase was observed for gallic acid content and total hydroxycinnamic acids. Firstly, the increase in gallic acid in 21ºBrix grape pomace during fermentation was corroborated by the increase in the % galloylated units with phloroglucinolysis analysis (Table 5.2), although it was not significant when compared to the other ripeness levels. Additionally, the wine's gallic acid concentrations also increased (Table 5.5). Grape seed phenolics may have a role in the phenolic extractability explained by the possible adsorption of gallic acid, hydroxycinnamic acids and their derivatives onto the skin cell walls.

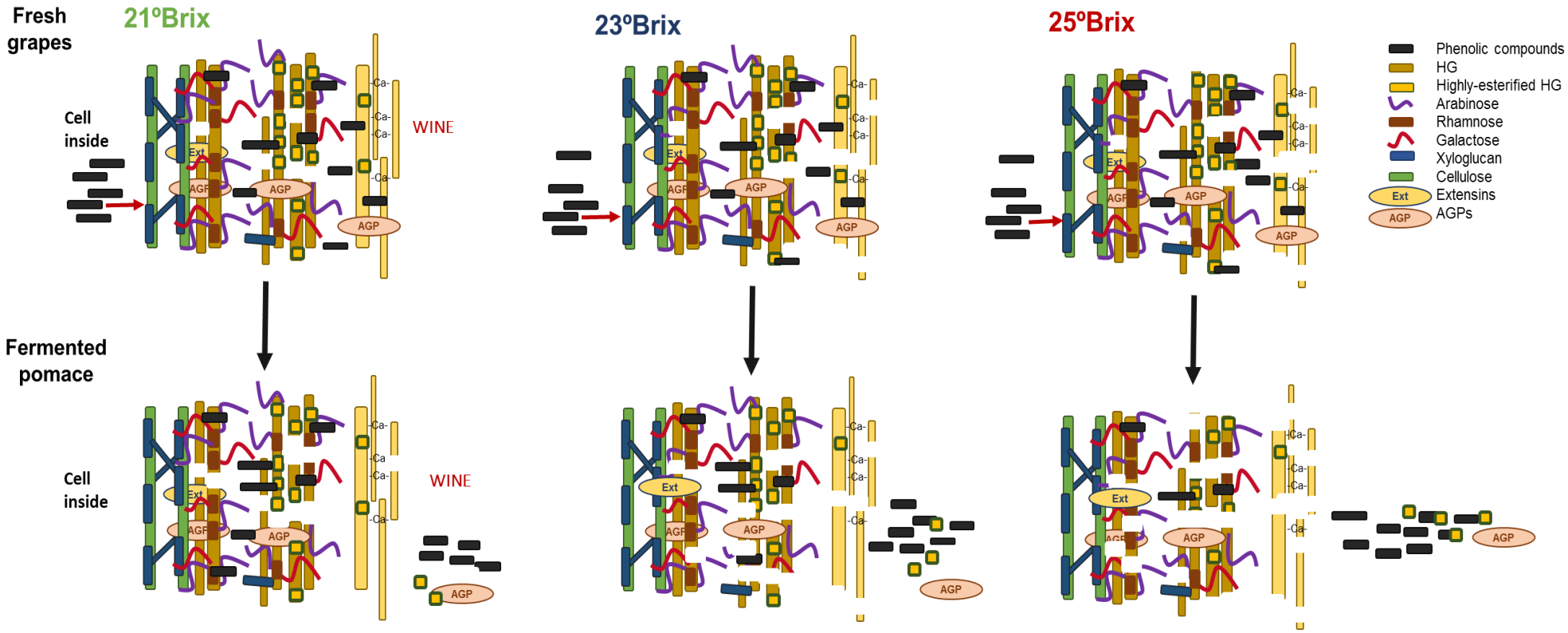

Figure 5.7. Proposed model of the cell wall disassembly and the subsequent phenolic extractability occurring during fermentation of grape from three different grape ripeness levels (21ºBrix, 23ºBrix and 25ºBrix).

5.4. CONCLUSION

In conclusion, cell wall modification occurring during ripening was instrumental in understanding the enhanced de-pectination observed in the grape berry cell walls and thereby in the release of the grape phenolic compounds during alcoholic fermentation. The greater extraction observed in riper berries was clearly associated with a greater de-pectination of the grape berry cell walls. During the fermentative process, grape phenolics were gradually extracted into the wines, probably with cell wall derived polysaccharides, enhanced by the degradation that occurred during grape ripening, which influenced the wine composition. These results has improved the understanding of the relationship between grape polysaccharide deconstruction and differential extraction of grape phenolics during fermentation. Additionally, this research, although preliminary, indicates a possible retention of skin tannins as a function of their nature and polymer complexity. In general, this work is a first step to obtain a better understanding of the relationship between the changes in the occurring in the grape cell wall, especially in the pectin-rich fraction, and phenolics compounds extracted during alcoholic fermentation.

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Chapter 6

Research results

Impact of oxygen and anthocyanin/tannin ratios on the colour, phenolic and precipitate composition in a model wine solution

Chapter 6: Impact of oxygen and anthocyanin/tannin ratios on the colour, phenolic and precipitate composition in a model wine solution

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ABSTRACT

Wine colour and phenolic stability over time is influenced by the amount of phenolics in young wines. The ratio between different phenolic compounds can also be determinant in the colour and phenolic development of red wines. Three anthocyanin/tannin ratios (A/T) extracted in a wine-like system were saturated with oxygen several times during sample storage. The impact of a forced oxidation and different A/T on the colour and phenolic composition was evaluated over time. The precipitate formed over time was also studied. From our results, a greater amount of seed tannins can enhance phenolic polymer formation, especially in the presence of oxygen. In this matter, the oxygen seemed to favour certain polymerisation reactions between tannins, leading to higher concentration of monomeric anthocyanins in solution. This excess of phenolics also led to a greater precipitation formed over time.

6.1. INTRODUCTION

The production of red wines with good ageing potential is important to the global wine industry. A large number of biological interactions and chemical reactions during the winemaking process and storage and can lead to changes in the composition and stability of red wines (Pérez-Magariño & González-SanJosé, 2004; Arapitsas, *et al.*, 2016). Red wine's colour and phenolic stability are essential parameters which are directly related to consumer acceptance of the product. The wine's matrix composition is also playing a role in influencing the wine's stability. As an important part of the wine matrix, phenolic compounds are involved not only in affecting a red wine's colour, but also certain sensory properties. Wine phenolics are highly reactive molecules, partaking in different reactions from crushing throughout the entire winemaking process, which may lead to the formation of more complex and stable molecules over time (Pérez-Magariño & González-SanJosé, 2004;

Monagas, *et al.*, 2006; Arapitsas, *et al.*, 2014; Bimpilas, *et al.*, 2015). Different winemaking techniques can thus have an indirect impact on a wine's stability as these may also influence phenolic extractability and evolution (Sacchi, *et al.*, 2005; Smith, *et al.*, 2015).

Anthocyanins, mainly responsible for the colour in young red wines (Ribéreau-Gayon, *et al.*, 2006; He, *et al.*, 2012), are extracted from the grape skins, whereas grape tannins are extracted from both skins and seeds. Skin and seed tannins differ in nature, structure and extractability (Mattivi, *et al.*, 2009). Whilst anthocyanin concentrations normally reach a maximum during the early stages of the alcoholic fermentation, tannins are progressively extracted as the ethanol concentration increases (Peyrot Des Gachons & Kennedy, 2003; González-Manzano, *et al.*, 2004; Ribéreau-Gayon, *et al.*, 2006; Garrido & Borges, 2013). Moreover, as is the case with anthocyanins, skin tannin levels reach a plateau during fermentation, whereas seed tannins follow a linear extraction partly due to the hydration of the seed coat (Cerpa-Calderón & Kennedy, 2008; Hernández-Jiménez, *et al.*, 2012; Yacco, *et al.*, 2016). The initial A/T ratio will affect the subsequent wine polymerisation reactions (Singleton & Trousdale, 1992; Sparrow, *et al.*, 2015) and the interaction of phenolic compounds with other wine components (Bindon, *et al.*, 2010; Springer, *et al.*, 2016). The ratio of anthocyanins to tannins may therefore not only affect the wine's stability, but also its sensory properties (Canals, *et al.*, 2008). Techniques such as early seed removal or the addition of mannoproteins (Poncet-Legrand, *et al.*, 2007) are used as winemaking practices to alter the skin/seed tannin ratio, thereby trying to reduce the bitterness (associated with seed tannins) in the final red wines (Meyer & Hernandez, 1970; Lee, *et al.*, 2008; Guaita, *et al.*, 2017).

Apart from the initial phenolic content, several other factors like the presence of oxygen, will influence the evolution of red wine over time. Indeed, oxygen is thought to play a crucial role in red wine's colour and phenolic stabilization during the entire winemaking process (Du Toit, *et al.*, 2006). It is involved in several reactions, such as phenolic polymerization, which leads to the formation of new and more stable phenolic compounds over time (Fulcrand, *et al.*, 1996; Atanasova, *et al.*, 2002). Oxidation of ethanol produces acetaldehyde, which enhances the formation of ethyl-bridges between phenolics (Timberlake & Bridle, 1977; Dallas, *et al.*, 1996; Saucier, *et al.*, 1997; Es-Safi, *et al.*, 1999). Additionally, the oxidative degradation rate differs between the different groups of phenolics. For example, as shown in a study by Jorgensen *et al.*, (2004), skin procyanidins degrade faster than those extracted from the seeds in the presence of oxygen. The impact of oxygen on altered A/T ratios in wine has been shown to improve the colour density (CD) and the polymeric pigment concentration as the tannin content increases (Picariello, *et al.*, 2017). Thus, the impact of oxygen will probably vary as a function of the nature and relative ratios of the wine phenolics.

There are still lot of unknowns regarding the complexity and the different structures of phenolic compounds, especially tannins, in grapes and wine. In this regard, the continuous development of more powerful analytical methods and techniques is leading to the generation of larger datasets with valuable information for wine science. The impact of oxygen on wine phenolics has been examined

extensively (Castellari, *et al.*, 2000; Atanasova, *et al.*, 2002; Wirth, *et al.*, 2010; Arapitsas, *et al.*, 2012; McRae, *et al.*, 2015; Quaglieri, *et al.*, 2017). However, only two recent publications have evaluated the effect of oxygen on different anthocyanin/tannin ratios (Picariello, *et al.*, 2017; Carrascón, *et al.*, 2018). Nonetheless, in the study from Picariello *et al.* (2017) the commercial tannins used in this study may contain some other compounds, thereby not only altering the tannin concentration (Versari, *et al.*, 2013).

The present study was divided into two parts. Firstly, a descriptive study was performed that aimed to provide more valuable information on how oxygen affects the polymerisation reactions of phenolic compounds obtained through different anthocyanin/tannin ratios (A/T) in a wine-like system (WL). Different amounts of grape seeds were used to alter the tannin levels of in the A/T ratios. The second part of the study, based on an untargeted approach, evaluated and tentatively identified the composition of the precipitate that was formed over time as a consequence of the different phenolic ratios. To our knowledge, only two previous study analysed the composition of the phenolic derived precipitate formed in real wines (Waters, *et al.*, 1994; Prakash, *et al.*, 2016), but this is the first time that the precipitation phenomena as a consequence of different phenolic ratios is studied. In short, the aim of the present work was to assess, with the help of targeted and untargeted analyses, the evolution over time of an initially different phenolic composition in a WL (obtained with three A/T ratios) in the presence or absence of oxygen.

6.2. MATERIAL AND METHODS

6.2.1. Wine-like A/T ratios

Shiraz grapes (27ºBrix) were obtained in 2015 from the Welgevallen experimental vineyard of the Department of Viticulture and Oenology at Stellenbosch University. The amount of grape seeds, chosen from preliminary trials, was the main variable in the treatments as indicated in Table 6.1. Grape skins (240 g) were peeled off and manually separated from the flesh and seeds, rinsed with deionised water and extracted with or without seeds according to the seed to skin ratios (no seeds, SK normal seed to skin ratio, SKSD and four times the normal seed to skin ratio, SK4SD) as indicated in Table 6.1 for 9 days in 1L hydroalcoholic solution (15% ethanol), at pH 3.4 and containing 6.0 g/L tartaric acid. A single extraction was performed per A/T ratio. The extractions were carried at 25ºC and manually shaken three times per day. To avoid the possibility of any spontaneous fermentations, 20 mg/L NaN_3 were added to the extracts. After nine days, the skins and seeds were and separated from their WL extracts and softly pressed by hand (in the presence of CO_2). The three final WL ratios (SK, SKSD, and SK4SD) were then centrifuged at 8000 rpm (5 min), to remove any residual grape skin. The iron and copper concentrations were then adjusted to 5 mg/L and 0.3 mg/L respectively with the use of $FeSO_4.7H_2O$ and $CuSO_4.5H_2O$ (Sigma-Aldrich, St. Louis, MO, USA) according to Danilewicz (2007). Finally, the wine extracts were divided into Control (C) and Oxygen treatments

(Ox) and transferred to vials (20 mL vials for C and 100 mL vials for Ox) and sealed hermetically. C vials were previously sparged with nitrogen. Then, the C treatments were transferred into the vials using CO_2 gas. Before being transferred to the vials, Ox samples were vigorously shaken by hand in a 500 mL volumetric flask for 2 minutes allowing air to enter every 10 seconds to reach oxygen saturation. The oxygen consumption was monitored, in C and Ox samples. All vials were stored in the dark at 15ºC until the required analysis after 3 (3M), 6 (6M) and 9 months (9M) of storage. Once opened and analysed, C treatments vials were discarded whereas in the case of Ox samples, 20 mL were drawn from the vials and the remainder again saturated with oxygen before further storage. Glass beads were used to fill the headspace in the Ox vials at each of the sampling stages. In total, Ox samples were saturated with oxygen three times (at time 0 - 0M, after 3M and 6M) during the study. After 6 months, Ox samples were transferred to new vials, with new oxygen spots (see below), allowing for the recovery of the precipitate formed in the WL at this specific sample stage.

Table 6.1. Treatments induced to obtain different Anthocyanin/tannin (A/T) ratios.

Treatments	
SK	Skins with no seeds
SKSD	Skin: seeds = 1:1 (natural seed to skin ratio)
SK4SD	Skins: seeds = 1:4 (four times the natural seed to skin ratio)

6.2.2. Oxygen measurement

Oxygen spots (Pst3, PreSens, Regensburg, Germany) were placed in several vials (control and oxygen vials) to avoid invasive measurements and used to monitor the oxygen uptake rate (Coetzee, *et al.*, 2016) for the first 3 days after oxygen addition. Vials were stored in the dark to avoid possible damage to the spots.

6.2.3. Colour and phenolic measurements

6.2.3.1. Spectrophotometric analysis

Colour and phenolic parameters (CD, TRP, TP, copigments and tannins) for all treatments were determined at 0M and after 3M, 6M and 9M in the same manner as described in Chapter 3. At each time point three vials of each treatment were opened and analysed. Additionally, the colour and phenolic composition was also analysed at time 0M in each of the three extracts. The CIELab parameters were determined for all the treatments only after 3M, 6M and 9M (not at 0M).

6.2.3.2. HPLC analysis

The analysis of individual phenolics were performed for all ratios and C/Ox samples at 0M and after 3M, 6M and 9M of storage, using the HPLC method used to analyse for individual phenolics previously described in Chapter 3.

6.2.3.3. Hydrophilic Interaction Liquid Chromatography (HILIC)-FLD.

To assess the changes in the flavan-3-ols and proanthocyanidins oligomers, the WL at 0M and after 3M, 6M and 9M of storage, were also analysed using a novel HILIC method (Terblanche, 2017). Sample preparation was performed by solid phase extraction (SPE) of the WL systems using 6 mL/500 mg Oasis HLB cartridges (Waters - Milford, MA, USA). The HLB cartridges were conditioned with 2 mL methanol followed by 2 mL deionised water. Then, 12 mL of wine extract were diluted with 38 mL deionised water prior to loading the sample onto the cartridge. The cartridge was then washed with 2 mL distilled water, followed by elution with 10 mL methanol. The final eluent was evaporated to dryness with the use of a Rotavapor R-134 (Büchi, Flawil, Switzerland) and reconstituted in 2 mL methanol. The sample was then diluted (1:1) with acetonitrile for HILIC analyses.

HILIC separations were performed following the procedure described by Terblanche (2017). A volume of 15 µL was injected in an XBridge Amide column (150 mm × 4.6 mm, 2.5 µm particles, Waters), with a Phenomenex KrudKatcher pre-column filter. The mobile phases used consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in de-ionised water (B). The oven temperature was set at 40ºC. The separations were performed with the use of the following gradient: 5 – 40% B (0 – 60 min), 40 – 70% B (60 – 62 min), 70% for 2 min, followed by a re-equilibration period of 8 min. The flavan-3-ol and oligomer detection was carried out with a fluorescence detector (FLD). The chemical standards used were: (-)-Epicatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate and (-)-epicatechin gallate (Sigma Aldrich, Steinheim, Germany). Proanthocyanidin oligomeric standards were obtained from semi-preparative HPLC separations from cocoa beans.

6.2.4. Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS)

For the second part of the study, the precipitates formed over time in all the treatments were recovered after 6M and 9M. This was done by removing the liquid part from the vial and placing it in a fume hood. Once the precipitate were dry it was dissolved in 5 mL methanol. A sample volume of 2 µL was injected into a Synapt G2 quadrupole-time-of-flight (Q-TOF-MS) mass spectrometer (Waters Corporation). The separation was performed on an Acquity UPLC HSS T3 column (1.8 µm internal diameter, 2.1 mm x 100 mm, Waters Corporation) using 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) and a scouting gradient, with a flow rate of 0.3 mL/min. The column temperature was 55 ºC. The instrument was operated with an electrospray ionization probe in both positive and negative mode. Data acquisition was performed in MS^e mode which consisted of a low

collision scan (from m/z 150 to 600) and a high collision energy scan (from m/z 40 to 600), using a ramp of 30-60 V. The MS parameters were optimized for best sensitivity as follows: cone voltage 15 V, nitrogen desolvation gas at 650 L/hr and desolvation temperature 275ºC. The data was obtained as RT m/z, intensity, using MassLynx (v. 4.1) software (Waters, Milford, MA, USA). The WL ratios and their corresponding precipitate (P) samples were injected from the same vials (for C/Ox samples).

6.2.5. Statistical analysis

All analyses were carried out using Statistica 13.2 (TIBCO Statistica software, Palo Alto, CA, USA). Significant differences were judged on a 5% significance level ($p \leq 0.05$) with LSD Post Hoc tests. Additionally, Principal Component Analysis (PCA), Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) and their corresponding S-plots were performed with SIMCA 14.1 software (Sartorium Stedim Biotech - Malmö, Sweden).

6.3. RESULTS AND DISCUSSION

6.3.1. Colour and phenolic extraction in the different A/T ratios

The extraction kinetics for all three A/T ratios (SK, SKSD and SK4SD) were daily monitored by spectrophotometric and HPLC analyses (data not shown). From previous trials and in order to allow for a better extraction from the grape seeds, all extractions were performed for nine days. At 0M, (after nine days of extraction) the colour density (CD) and the amount of total red pigments (TRP) were similar between the three ratios (Table 6.2). However, a higher amount of tannins and total phenolics (TP) were found with an increase of grape seeds at this stage (Table 6.2). Considering the individual phenolic compounds, gallic acid and catechin levels were especially different between the different A/T ratios at 0M (Table 6.2). In addition, differences in the flavan-3-ols monomer, dimer and trimer composition were also observed between the A/T ratios. At 0M, a larger concentration of dimers and trimers was found in WL SK4SD (Table 6.2).

Table 6.2. Colour and phenolic composition of the A/T ratios at 0M.

	SK	SKSD	SK4SD
Spectrophotometric			
420 nm (AU)	3.04	3.45	3.30
520 nm (AU)	6.75	7.48	6.76
620 nm (AU)	1.18	1.39	1.34
CD (AU)	10.96	12.31	11.40
TRP (AU)	24.02	26.23	24.01
TP (AU)	32.37	39.86	48.99
Tannins (mg/L)	450.99	587.86	1092.77
HPLC (mg/L)			
Gallic acid	2.67	31.92	86.13
catechin	8.60	59.76	154.14
B1	16.35	47.50	88.28
Polymeric phenols	245.66	403.40	392.75
Total hydrox	4.82	7.07	7.78
Total flavonols	67.24	85.09	75.15
Total glucosylated	230.42	284.08	245.09
Total acetylated	116.79	144.60	121.15
Total coumaroylated	65.38	90.96	90.94
Polymeric pigments	14.24	25.50	32.02
Total anthocyanins	426.83	545.14	489.20
HILIC (mg/L)			
Monomers	0.85	53.38	85.68
Dimers	n.d.	1.72	8.94
Trimers	n.d.	n.d.	4.00
Total	0.85	55.10	98.62

6.3.2. Influence of a different A/T ratios on the oxygen consumption

The oxygen consumption (mg/L) was firstly monitored for the C and Ox samples at 0M, and only for Ox samples in the following oxygenations after 3M and 6M of storage. From the quick oxygen depletion observed at 0M, the oxygen consumption of the following oxidations was only monitored during the first three day (70-75 hours). As previously mentioned, the Pst3 oxygen spots were replaced after 6M, transferring the Ox samples to new vials for the last measurement and recovering the precipitate prior to the last oxygenation. The A/T composition, in concentration and probably nature, clearly played a role as the oxygen consumption rates differed between the storage times and treatments. These differences in the oxygen depletion rates could possibly be explained by changes occurring in the phenolic profile and concentration of the different A/T ratios over time. Firstly, as shown in Figure 6.1A, there was a quick depletion of the low amounts of oxygen present

in C samples (probably due to residual oxygen levels). From Figure 6.1B, which illustrates the oxygen consumption in Ox vials at 0M, minimal differences were found between the A/T ratios. The larger concentration and possibly different nature of phenolics at 0M did not seem to have an influence on the oxygen consumption. In both cases, the dissolved oxygen in the different A/T ratios was depleted after a few hours. Nevertheless, this oxygen consumption rate varied over time, as the phenolic profile of the WL ratios evolved. The oxygen consumption measured after 3M, was generally slower compared to 0M (Figure 6.2). Interestingly, the oxygen consumption took longer in the vials from SK4SD samples. The excess of seed phenolics may have had an influence on the formation rate of new polymeric forms, involving oxidative reactions, during the vial storage. This may have led to a WL media composition with a lower level of compounds susceptible to oxidation. Furthermore, as illustrated, the oxygen consumption observed in Figure 6.3, was even slower after 6M, probably also as a consequence of a lower substrate availability to react with oxygen in the WL media.

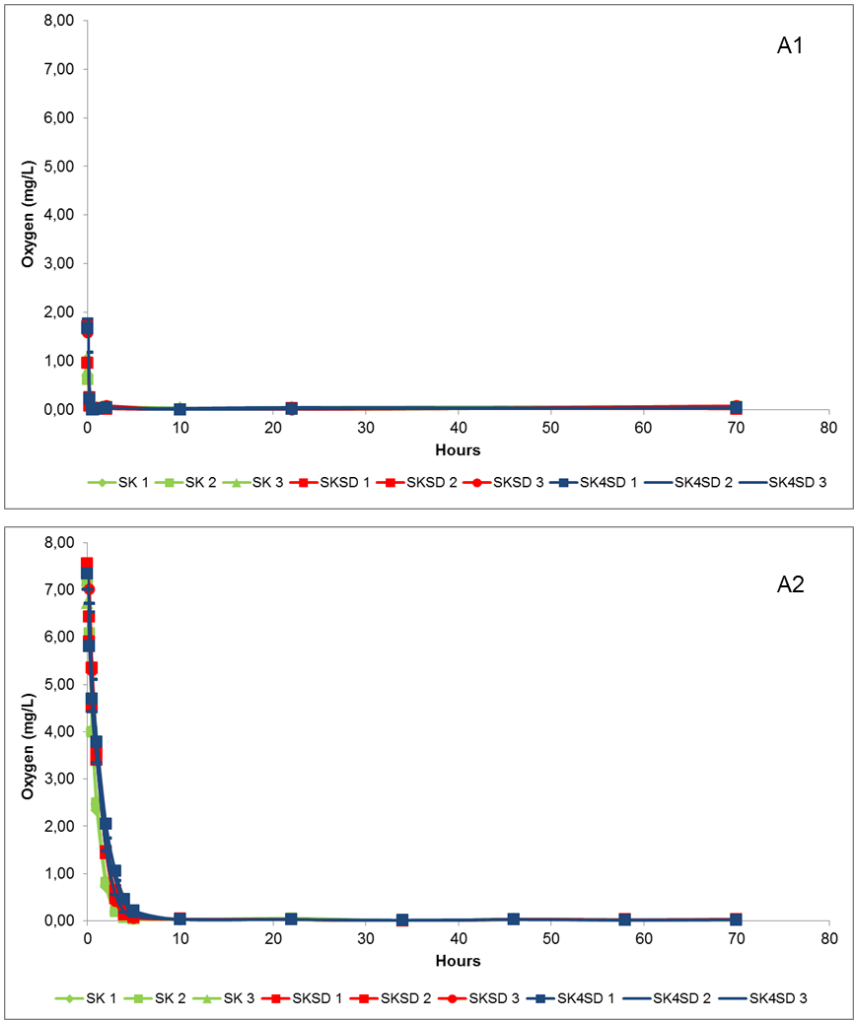

Figure 6.1. Oxygen consumption in the different A/T ratios (SK, SKSD and SK4SD) in C (A1) and Ox (A2) treatments at 0M. The oxygen consumption was monitored in triplicate.

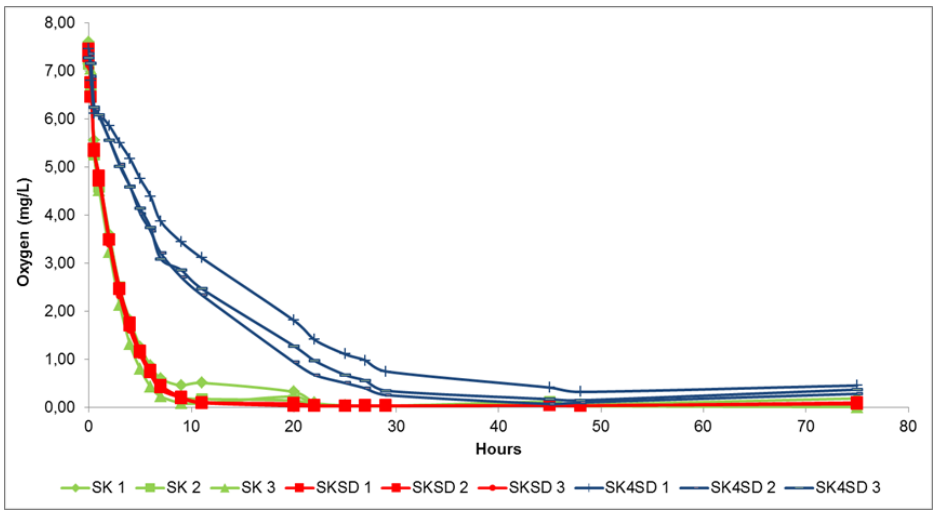

Figure 6.2. Oxygen consumption after 3M for the A/T Ox ratios (SK, SKSD and SK4SD). The oxygen consumption was monitored in triplicate.

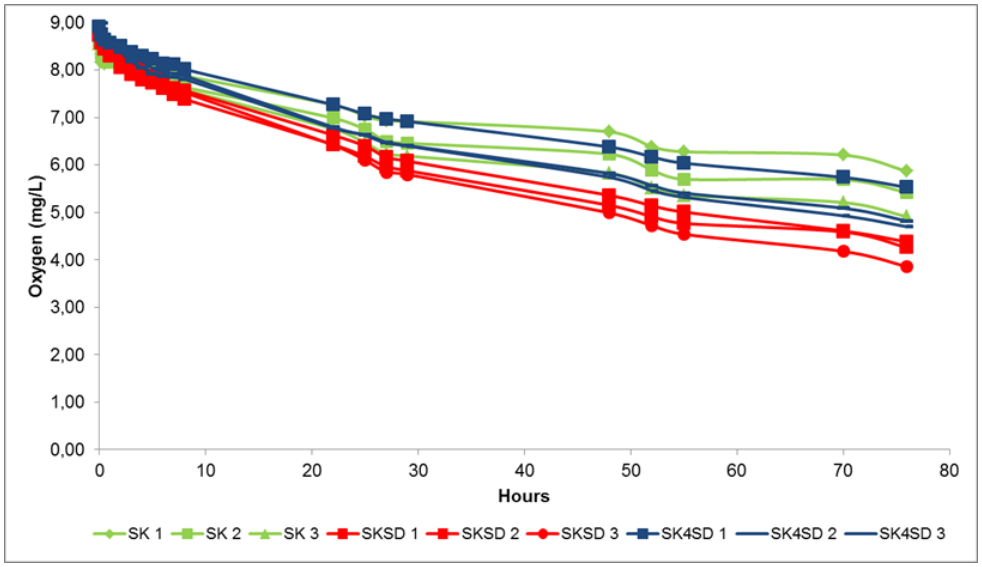

Figure 6.3. Oxygen consumption after 6M for the A/T Ox ratios (SK, SKSD and SK4SD). The oxygen consumption was monitored in triplicate.

6.3.3. Colour and phenolic evolution of the A/T ratios

Differences were observed over time in terms of the colour and phenolics as determined spectrophotometrically, of which some were already observed at 0M. After nine days of extraction, the three A/T ratios had similar CD and TRP values that represented the desired similar anthocyanin content between the A/T ratios (Table 6.2). As expected, higher TP and tannin levels were found in SKSD and especially SK4SD, with a greater amount of seeds (Table 6.2), when compared to SK (with only skin tannins). However, time played a significant role (Appendix Table 6.1 and 6.2) and after the subsequent storage, significant differences were also found in colour and the phenolic composition between the different A/T ratios over time (Appendix Table 6.3). Whilst the anthocyanin to tannin ratios was the most important factor for the TP levels and, obviously, the tannin

concentration, the oxygen had a large influence on the colour components, such as the TRP and CD, especially the absorbance at 420 nm (Appendix Table 6.3). Furthermore, time was also a determining factor, especially for the TRP and copigment concentrations.

The evolution of the different spectrophotometric parameters over time, for the three A/T ratios and C and Ox samples, are shown in Figure 6.4. The cumulative effect of all the parameters drives a clear separation between the samples. In Figure 6.4-A1, it can be seen that the different ratios were separated along the PC1 axis (54.4%). As previously mentioned, the TP and tannin levels were mainly influenced by the A/T ratio (Appendix Table 6.3). The loadings plot (Figure 6.4-B) showed a general larger phenolic content in SK4SD treatments, especially compared to SK treatments. Over the course of time, these differences between the A/T ratios became smaller, especially after 9M, probably as a consequence of phenolic degradation, but also as a result of over polymerisation reactions and subsequent precipitation of insoluble phenolic compounds. Figure 6.4-A2, the samples are coloured according to the sampling stages (0M, 3M, 6M and 9M) and after 9M, the A/T ratios samples were more closely distributed along the PC1 axis (54.4%). When the samples were coloured according to the C/Ox treatment, the samples distributed along the PC2 axis (21.3%), with Ox samples being characterised by a generally higher phenolic content and especially tannin concentration over time. Contrary to the results of Geldenhuys *et al.* (2012), oxygen may have also played an important role in the tannin concentration (Figure 6.4-A3). However, Geldenhuys *et al.* (2012) applied a progressive micro-oxygenation, whereas in this study a large amount of oxygen was added at a time.

A general loss of colour and reduction in phenolics levels was found over time, especially from 0M (Table 6.2) to 3M (Appendix Table 6.3), except in the tannin concentration (Figure 6.5). As an example, the amount of TRP decreased in all the samples during the first 3M, especially in most of the C treatments (Appendix Table 6.3). The oxygen seemed to have enhanced the polymerisation between certain compounds and limiting therefore the degradation of certain red pigments. The TRP content was significantly higher in the SKSD and SK4SD Ox treatments at 3M (Appendix Table 6.3). From then onwards, the differences between C and Ox treatments and between A/T ratios were reduced over time.

Conversely, the tannin content showed different patterns from 0 to 3M within the different treatments. As illustrated in Figure 6.5 (values at 0M are specified on the Y axes), clear differences were found between C and Ox samples. Whilst the MCP tannin levels were relatively constant from 0M to 3M in C samples (except for a slight increase in SKSD), an increase in the tannin levels was observed in Ox (SKSD and SK4SD) samples during the same period. However, after 3M, the tannin levels were only significantly higher in SK4SD-Ox when compared to the corresponding C samples (Figure 6.5). During the following three months, the C treatments showed a progressive decrease in tannin levels, except for the SK treatment (constant from 3M to 9M), while not changing significantly up to 9M. (Figure 6.5). On the other hand, the Ox treatments' tannin levels increased (SK and SKSD) or

remained stable (SK4SD) up to 6M of storage, which might also explain the different oxygen consumption rates of the second oxidation after 3M of storage. From then, all the Ox A/T ratios experienced a general decrease in tannin levels towards the last sampling stage (9M). This decrease can possibly be explained by the formation of larger and/or unstable polymers, no longer soluble in the hydroalcoholic solution. Thus, the oxygen had an impact on the tannin polymerisation and the reactivity of the polymerisation reaction products towards methylcellulose.

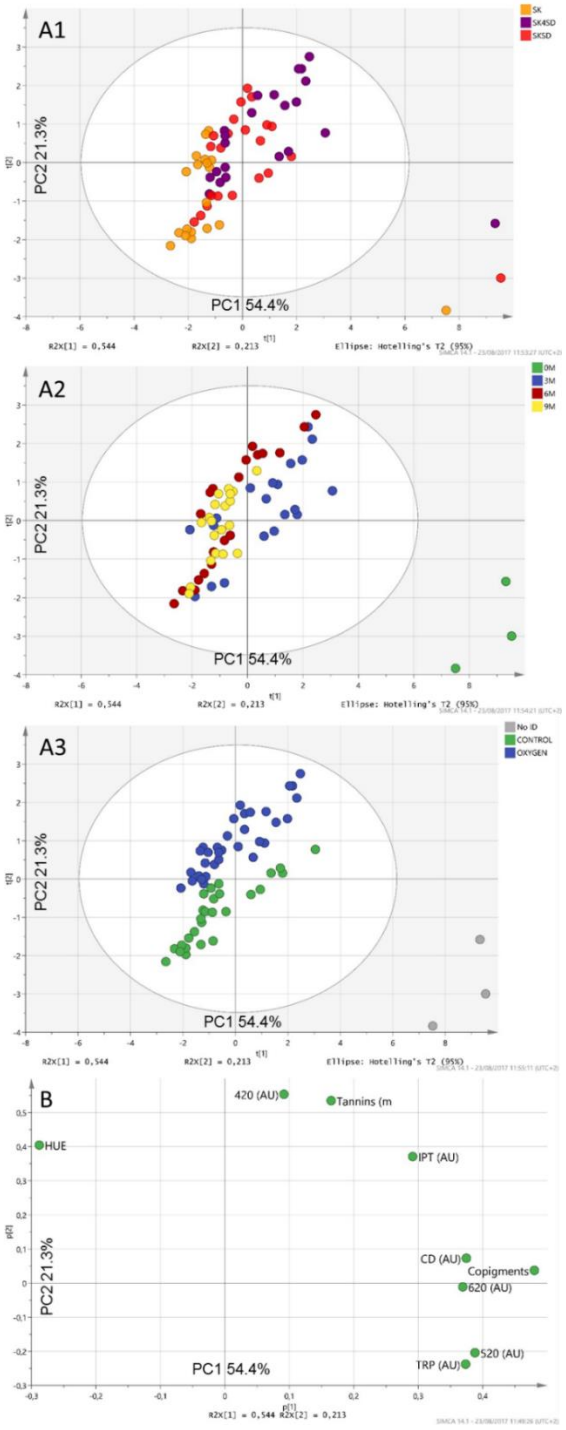

Figure 6.4. PCA and loading plot A/T samples distribution based on the spectrophotometric methods. A1 shows the samples according to the three different A/T ratios. A2 shows the samples according to the sampling stage. In A3 the samples were coloured according to the C/Ox treatments. B corresponds to the loading plot.

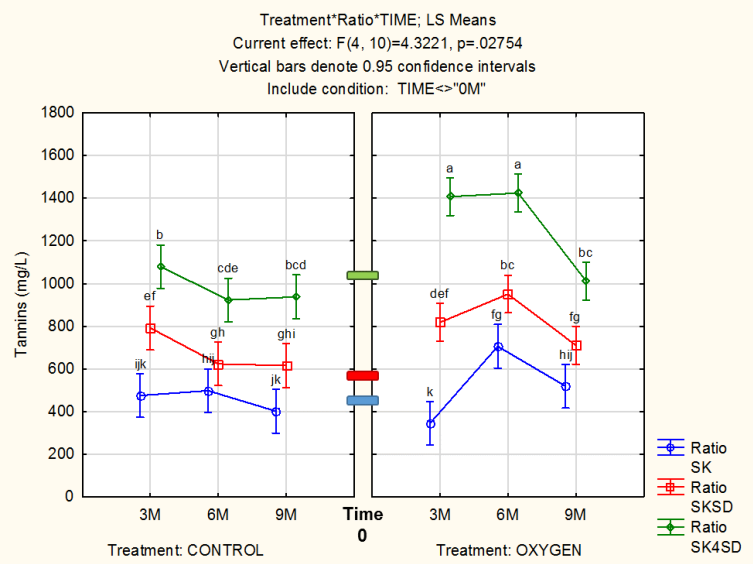

Figure 6.5. Evolution of the MCP tannin levels (mg/L) of all A/T treatments over time. Values at time 0 are placed on the Y axis. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

The impact of the different A/T ratios and their relevance on the A/T colour and phenolic composition as assessed spectrophotometrically is shown Figure 6.4. Furthermore, oxygen not only played a significant role in tannin polymerisation, as widely documented in literature (Singleton, 1987; Castellari, *et al.*, 2000; Atanasova, *et al.*, 2002; Waterhouse & Laurie, 2006; Gambuti, *et al.*, 2013; Quaglieri, *et al.*, 2017), but indirectly also influenced the protection of the total red pigments. In the presence of oxygen, a higher phenolics level might create a competitive effect for the reaction with oxygen, favouring specific polymerisation reactions. Thus, the higher pigment content can be explained by the depletion of oxygen as a consequence of the reaction of other phenolic compounds with oxygen, instead of the anthocyanins/pigments.

6.3.4. CIELab parameters

The storage time played an important role, together with the different A/T ratios in some cases, on the different CIELab parameters of the samples (Appendix 6.4). However, these differences between the A/T ratios seemed to be reduced by the impact of oxygen, especially notable in L*, b* and C_{ab}* (Appendix Table 6.5). In general, C treatments were clearer (higher L*) than Ox treatments (Figure 6.6). This clarity might be related to lower amounts of TRP in C samples.

Overall, the samples were characterised by an increase in the sample clarity (L*) accompanied by a loss in the a* component (red/green colour dimension: a* > 0 red, a* < 0 green) over time. At the same time, b* (yellow/blue component: b* > 0 yellow, b* < 0 blue) showed a general increase

(Appendix Table 8). The CIELab results in general agreed with the results 420 nm and 520 nm colour measurements and the TRP content previously reported (section 6.3.3).

Figure 6.6. Evolution of the clarity (L*) of the sample for all A/T treatments over time. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

6.3.5. HPLC data for individual phenolics

Results of individual phenolics are shown in (Table 6.2 and 6.3). The different A/T ratios, the presence/absence of oxygen and the time played a role in the phenolic composition of the treatments. A large difference between gallic acid concentrations were found between the three A/T ratios. A higher amount of seeds led to an obvious greater extraction of gallic acid (Table 6.2). At 0M, large differences were found in the gallic acid content between the treatments (Table 6.2). From time 0M to 3M, a decrease in the gallic acid concentration was observed and it was possibly linked to the formation of new polymeric forms (especially in SK4SD), precipitation or its degradation. The hypothetical interaction between gallic acid quinones and flavonol units have been recently reported (Mouls & Fulcrand, 2015). Thus, the concentration of polymeric phenols was also significantly higher in SKSD and SK4SD compared to SK (Table 6.3). These differences between the A/T ratios remained over time. Over the course of time (especially from 6 M), the polymeric phenols content was generally higher in Ox treatments. Therefore, the presence and reactivity of seed derived compounds may influence the polymerisation and the colour and phenolic stability.

Additionally, a higher total flavonol content was found in Ox samples; however, the total hydroxycinnamic acids concentration was higher in C samples. Unexpectedly, although in very small concentrations (not always significant), the total hydroxycinnamic acid content seemed to increase over time (Table 6.3). Literature had reported a general decrease of hydroxycinnamic acids

concentration during storage (García-Falcón, *et al.*, 2007). However, an increase of certain hydroxycinnamic acids has also been observed (García-Falcón, *et al.*, 2007; Arapitsas, *et al.*, 2014), possibly as a result of copigment degradation, expected to occur over time (Bimpilas, *et al.*, 2016).

Table 6.3. Levels of the individual and groups of phenolic compounds (mg/L) for all A/T treatments over time. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

Treatment		CONTROL			OXYGEN		
Ratio	Months	SK	SKSD	SK4SD	SK	SKSD	SK4SD
Gallic acid	3M	1.14 ± 0.04 ^{j}	8.73 ± 0.02 ^{gh}	28.78 ± 0.11 ^{c}	1.28 ± 0.01 ^{ij}	9.04 ± 0.09 ^{fg}	30.24 ± 0.13 ^{b}
	6M	1.51 ± 0.04 ^{ij}	8.55 ± 0.15 ^{h}	27.69 ± 0.54 ^{d}	1.17 ± 0.01 ^{ij}	9.21 ± 0.25 ^{f}	30.40 ± 0.04 ^{b}
	9M	1.53 ± 0.05 ^{i}	8.78 ± 0.03 ^{gh}	28.57 ± 0.32^{c}	1.27 ± 0.01 ^{ij}	9.58 ± 0.22 ^{e}	30.78 ± ± 0.41 ^{a}
Catechin	3M	8.28 ± 3.03 ^{de}	8.57 ± 1.30 ^{de}	20.47 ± 1.07 ^{b}	6.73 ± 0.19 ^{efg}	15.38 ± 3.43 ^{c}	35.70 ± 2.80 ^{a}
	6M	5.88 ± 2.41 ^{efg}	6.60 ± 1.61 ^{efg}	12.49 ± 0.50 ^{cd}	3.94 ± 0.85 ^{fg}	3.12 ± 0.18 ^{g}	13.37 ± 6.03 ^{c}
	9M	20.73 ± 3.01 ^{b}	6.91 ± 0.35 ^{efg}	8.93 ± 1.68 ^{de}	6.46 ± 1.50 ^{efg}	11.78 ± 1.66 ^{cd}	8.95 ± 2.52 ^{de}
B1	3M	14.02 ± 4.15 ^{ij}	19.97 ± 1.32 ^{gh}	66.58 ± 0.48 ^{a}	11.55 ± 2.00 ^{jk}	33.07 ± 4.73 ^{e}	69.49 ± 4.90 ^{a}
	6M	7.38 ± 0.63 ^{k}	22.68 ± 0.88 ^{fg}	44.62 ± 2.28 ^{d}	11.30 ± 1.99 ^{jk}	25.91 ± 0.39 ^{f}	46.59 ± 4.65 ^{d}
	9M	8.61 ± 0.33 ^{k}	25.88 ± 4.25 ^{f}	52.26 ± 3.70 ^{c}	16.38 ± 2.26 ^{hi}	35.17 ± 1.89 ^{e}	61.40 ± 2.14 ^{b}
Polymeric phenols	3M	513.75±1.55 ^{k}	707.86±5.50 ^{ef}	1002.65±20.15 ^{a}	525.20±0.54 ^{k}	731.42±20.87 ^{de}	1010.29±22.25 ^{a}
	6M	442.05±32.68 ^{l}	586.18±48.70 ^{i}	689.75±7.32 ^{fg}	529.33±23.93 ^{jk}	656.95±27.41^{gh}	832.36±31.70 ^{c}
	9M	490.99±16.63 ^{k}	645.19±3.77 ^{h}	758.85±28.89 ^{d}	568.59±6.63 ^{ij}	712.80±15.34 ^{ef}	903.30±16.05 ^{b}
∑ Hydroxycinnamic acids	3M	3.59 ± 0.04 ^{hij}	4.43 ± 0.06 ^{de}	3.92 ± 0.10 ^{gh}	2.97 ± 0.09 ^{k}	3.26 ± 0.42 ^{ijk}	3.61 ± 0.46 ^{hi}
	6M	4.06 ± 0.12 ^{efg}	4.52 ± 0.07 ^{cd}	4.86 ± 0.15 ^{bc}	3.19 ± 0.09 ^{jk}	3.36 ± 0.18 ^{ij}	3.58 ± 0.16 ^{hi}
	9M	4.37 ± 0.09 ^{def}	5.10 ± 0.10 ^{b}	5.54 ± 0.09 ^{a}	3.51 ± 0.14 ^{hij}	4.01 ± 0.41 ^{fg}	4.24 ± 0.07 ^{defg}
∑ Flavonols	3M	16.37 ± 0.61 ^{d}	18.12 ± 0.36 ^{c}	16.44 ± 0.48 ^{d}	20.15 ± 0.62 ^{b}	22.79 ± 0.76 ^{a}	20.78 ± 0.48 ^{b}
	6M	12.10 ± 0.65 ^{fg}	13.01 ± 1.01 ^{ef}	10.23 ± 0.56 ^{hi}	15.88 ± 0.44 ^{d}	16.08 ± 0.73 ^{d}	13.30 ± 0.64 ^{ef}
	9M	11.06 ± 0.52 ^{gh}	11.62 ± 0.20 ^{g}	9.58 ± 0.35 ^{i}	13.93 ± 0.77 ^{e}	14.07 ± 1.24 ^{e}	11.75 ± 0.60 ^{g}
∑ Glucosylated anthocyanins	3M	3.98 ± 0.37 ^{f}	4.46 ± 0.09 ^{e}	4.88 ± 0.12 ^{d}	7.53 ± 0.12 ^{c}	12.39 ± 0.42 ^{b}	18.81 ± 0.39 ^{a}
	6M	1.25 ± 0.19 ^{ij}	1.17 ± 0.04 ^{ijk}	1.07 ± 0.06 ^{ijk}	1.94 ± 0.20 ^{h}	2.46 ± 0.20 ^{g}	2.66 ± 0.10 ^{g}
	9M	0.87 ± 0.03 ^{k}	0.92 ± 0.02 ^{jk}	0.87 ± 0.02 ^{k}	1.05 ± 0.02 ^{ijk}	1.35 ± 0.15 ^{i}	1.33 ± 0.07 ^{i}
∑ Acetylated anthocyanins	3M	2.72 ± 0.88 ^{ef}	2.21 ± 0.11 ^{efg}	4.18 ± 1.53 ^{d}	6.24 ± 2.55 ^{c}	11.84 ± 0.54 ^{b}	16.88 ± 0.49 ^{a}
	6M	1.74 ± 0.06 ^{fg}	1.61 ± 0.06 ^{fg}	1.44 ± 0.03 ^{fg}	2.55 ± 0.13 ^{efg}	3.20 ± 0.27 ^{de}	3.39 ± 0.15 ^{de}
	9M	1.41 ± 0.11 ^{fg}	1.33 ± 0.02 ^{g}	1.28 ± 0.07 ^{g}	1.59 ± 0.04 ^{fg}	1.88 ± 0.19 ^{fg}	1.70 ± 0.10 ^{fg}
∑ Coumaroylated anthocyanins	3M	2.92 ± 0.41 ^{d}	2.95 ± 0.10 ^{d}	3.04 ± 0.08 ^{d}	4.95 ± 0.44 ^{c}	7.58 ± 0.45 ^{b}	9.46 ± 0.50 ^{a}
	6M	1.44 ± 0.07 ^{efg}	1.31 ± 0.06 ^{fg}	1.18 ± 0.03 ^{g}	1.74 ± 0.14 ^{e}	1.79 ± 0.03 ^{e}	1.63 ± 0.04 ^{ef}
	9M	1.24 ± 0.07 ^{g}	1.19 ± 0.06 ^{g}	1.14 ± 0.02 ^{g}	1.27 ± 0.06 ^{fg}	1.44 ± 0.08 ^{efg}	1.29 ± 0.05 ^{fg}
Polymeric pigments	3M	14.01± 0.35 ^{ijk}	16.75 ± 0.18 ^{def}	19.78 ± 0.86 ^{b}	14.83 ± 0.30 ^{ghij}	18.70 ± 0.34 ^{bc}	21.66 ± 0.41 ^{a}
	6M	12.57 ± 1.20 ^{l}	14.52 ± 1.51 ^{hijk}	13.50 ± 0.80 ^{kl}	14.56 ± 0.94 ^{hijk}	15.15 ± 1.01 ^{ghi}	15.51 ± 0.73 ^{fgh}
	9M	13.73 ± 0.51 ^{jkl}	16.08 ± 0.18 ^{efgj}	14.81 ± 0.49 ^{ghij}	15.66 ± 0.13 ^{efgh}	16.76 ± 0.52 ^{de}	17.62 ± 0.28 ^{cd}

Likewise, a large decrease was observed in the anthocyanin concentration of all treatments from 0M (Table 6.2) to 3M (Table 6.3). The larger decrease in anthocyanin levels observed in the C treatments was not associated with higher polymeric pigments formation (Table 6.3). Nevertheless, the HPLC results confirmed the idea of certain oxidative reactions between phenolics being favoured in the presence of oxygen. The oxidation of ethanol and tartaric acid could possibly have led to the formation of ethyl bridged structures between tannins moieties, thereby leading to a lower reactivity involving anthocyanins. This may explain the higher levels of monomeric anthocyanin, especially

during the first 3M (Figure 6.7), in the treatments where higher levels of seeds were present and oxygen added. Therefore, a higher amount of monomeric anthocyanins would remain as free forms in the solution. Supporting this, after 3M of storage, SK and SKSD samples showed a greater decrease in glucosylated, acetylated and coumaroylated anthocyanins in the absence of oxygen. On the other hand, SK4SD initially had a higher concentration of polymeric pigments, thereby influencing the polymerisation reactions. These differences between A/T ratios in the concentration of polymeric pigments, for both C and Ox, were also found at 3M, but disappeared after 6M of storage. Despite these results, we cannot discard the possibility that certain of these polymeric pigments are not detected by the current HPLC method. Nevertheless, after 6M, all treatments experienced anthocyanin degradation and differences between treatments became smaller. This anthocyanin degradation over time has been widely reported in red wines, partly as a consequence of the pigmented polymer formation (Somers, 1971; Somers & Evans, 1979; Pérez-Magariño & González-SanJosé, 2004; Arapitsas, *et al.*, 2014; Quaglieri, *et al.*, 2017).

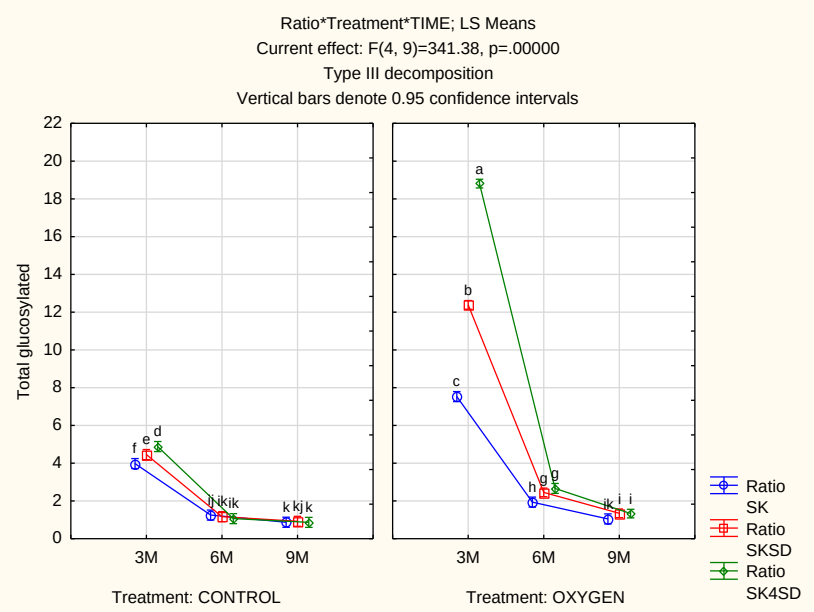

Figure 6.7. Evolution of the total glucosylated anthocyanin (mg/L) for all wine extract treatments over time. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

6.3.6. HILIC-FLD data for tannins

All samples were analysed with a HILIC method capable of separating proanthocyanidins of degree of polymerisation (DP) up to eight (octamers) (Terblanche, 2017). However, in our samples we only detected up to trimers. From the results, large differences were observed between the three A/T ratios. Firstly, as discussed in the spectrophotometric and HPLC analysis (section 6.4.4 and 6.4.5), the SK4SD treatments had a significantly higher phenolic concentration. These differences were further corroborated by the HILIC results that showed that at 0M the SK4SD treatment had the largest

concentration of monomers and dimers (not detected in SK) and was the only treatment where trimers were detected (Table 6.2). The WL SK4SD's treatments (C and Ox) also seemed to have more complex tannin based structures, as trimer molecules were exclusively found in them. After time 0M the oxygen and the time effect were also relevant to the WL composition. In short, the excess of phenolics, extracted from the grape seeds, had a major effect influencing the initial phenolic composition of the A/T, but also affecting the subsequent polymerisation reactions. However, the changes occurring over time (within the same compounds) were only significant in SK4SD.

Table 6.4. Evolution of flavan-3-ol monomers, dimers and trimers (mg/L) for all the A/T treatments over time. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

Treatment		CONTROL			OXYGEN		
Ratio	Months	SK	SKSD	SK4SD	SK	SKSD	SK4SD
Monomers	3M	0.11 ± 0.20 ^{d}	1.08 ± 0.10 ^{d}	28.17 ± 16.04 ^{b}	0.31 ± 0.03 ^{d}	7.78 ± 1.66^{cd}	53.65 ± 8.40 ^{a}
	6M	n.d. ^{d}	n.d. ^{d}	15.47 ± 1.61 ^{c}	n.d. ^{d}	4.76 ± 0.88 ^{cd}	30.28 ± 15.04 ^{b}
	9M	n.d. ^{d}	n.d. ^{d}	6.47 ± 0.87 ^{cd}	n.d. ^{d}	1.74 ± 0.96 ^{d}	3.85 ±0.34 ^{cd}
Dimers	3M	0.41 ± 0.38 ^{e}	0.55 ± 0.95 ^{e}	15.21 ± 2.55 ^{d}	0.54 ± 0.08 ^{e}	4.35 ± 2.02 ^{e}	37.19 ± 6.98 ^{b}
	6M	0.92 ± 1.30 ^{e}	3.01 ± 0.35^{e}	26.86 ± 4.16 ^{c}	n.d. ^{e}	5.42 ± 1.54 ^{e}	48.85 ± 1.97^{a}
	9M	n.d. ^{e}	1.52 ± .38 ^{e}	11.68 ± 6.50^{d}	n.d. ^{e}	3.07 ± 0.16 ^{e}	29.68± 0.96 ^{c}
Trimers	3M	n.d. ^{d}	n.d. ^{d}	0.42 ± 0.74 ^{d}	n.d. ^{d}	n.d. ^{d}	8.64 ± 2.11 ^{a}
	6M	n.d. ^{d}	n.d. ^{d}	7.04 ± 1.25 ^{b}	n.d. ^{d}	n.d. ^{d}	5.36 ± 0.18 ^{c}
	9M	n.d. ^{d}	n.d. ^{d}	n.d. ^{d}	n.d. ^{d}	n.d. ^{d}	n.d. ^{d}
Total	3M	0.52 ± 0.55 ^{e}	1.63 ± 0.97 ^{e}	43.81 ± 19.19 ^{b}	0.85 ± 0.07 ^{e}	12.13 ± 3.68 ^{de}	99.49 ± 16.74 ^{a}
	6M	0.92 ± 1.30 ^{e}	3.01 ± 0.35 ^{de}	49.37 ± 7.02 ^{b}	n.d. ^{e}	10.18 ± 2.42 ^{de}	84.48 ± 13.25 ^{a}
	9M	n.d. ^{e}	1.52 ± 0.38 ^{de}	18.16 ± 5.62 ^{cd}	n.d. ^{e}	4.81 ± 0.80 ^{de}	33.53 ± 1.30 ^{bc}

The analysis over time showed how polymerisation reactions took place regardless of the presence or absence of oxygen, as during the first 3M, the dimer concentration increased in most treatments (Table 6.4) from time 0 (0M) (Table 6.2). Furthermore, differences were also found between C and Ox and from our results, the oxygen had some influence, enhancing the polymerisation reactions at early stages in the SK4SD treatments. Dimers (Figure 6.8 - values at 0M are specified on axis Y) and trimers concentration in SK4SD samples kept being significantly higher in Ox treatments when compared to their respective C treatments over time. Apart from the concentration, the impact of oxygen was also linked to the speed in which these compounds were formed. The trimer concentration reached its peak after 3M in SK4SD Ox treatments, whereas in C samples the highest concentration in C samples was found only after 6M of storage (Table 6.4). This tannin polymerisation could possibly be explained by the formation of ethyl-bridges between tannin molecules, mediated by the acetaldehyde formed by the oxidation of the ethanol contained in the model solution (Timberlake & Bridle, 1976). The formation of ethyl-bridge dimers and trimers from grape seed phenolics was already reported (Rockenbach, *et al.*, 2012). The condensation mediated by acetaldehyde occurs at a higher rate than the direct condensation (Es-Safi, *et al.*, 1999). As

expected, monomer concentrations decreased over time as more complex molecules were formed. In addition, from 6 to 9M, a decrease in dimers and trimers was also observed (Table 6.4). This drop might be related to a possible breakdown or precipitation due instability in the WL media of certain phenolic derived polymers.

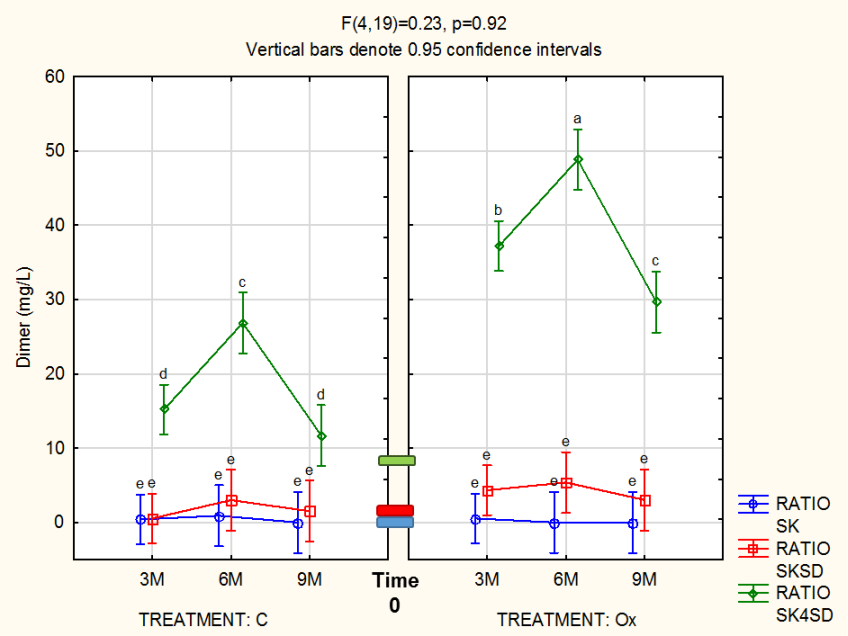

Figure 6.8. Evolution of the total dimers concentration (mg/L) for all A/T treatments over time. Values at time 0 are placed on the Y axis. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

The absence of condensed tannins in the WL SK (only grape skins) samples after 6M might be related to certain limitations of the sample preparation method for the HILIC method. MCP tannins were detected in all SK treatments (Figure 6.5). Thus, this discrepancy in the tannin values may be explained by a possible retention of certain pigmented tannins during the HILIC sample preparation which could react with the methylcellulose in the spectrophotometric tannin determination. However, Spearman coefficient correlations showed very good correlations between the monomer and dimer concentration measured with the HILIC-FLD and the catechin and B1 dimer concentrations determined by HPLC (R=0.83 and R=0.81 respectively). Moreover, the present method allowed us to evaluate the evolution of the seed derived flavan-3-ols over time.

6.3.7. Wine stability

Finally, for the second part of the study, untargeted MS analysis was used to evaluate the impact of oxygen on the colour and phenolic stability of the three A/T ratio samples. For this purpose, the WL media and its corresponding precipitates (P) were collected after 6M and 9M of storage, for all three ratios and C/Ox treatments. A large dataset was generated from the WL and P samples analysed with LC-HRMS. PCA was used to explore the sample distribution according to the MS dataset

generated. The PCA plot illustrated in Figure 6.9 shows a clear separation along PC2 (7.45%) between the two different matrices, WL and P, according to their chemical composition. The low explained variance found in the PCA plot is due to a consequence of the large number of ions detected in both matrices. Discriminant analyses were performed using this data to find which compounds were influenced by the various parameters (anthocyanin/tannin ratio, oxygen and time).

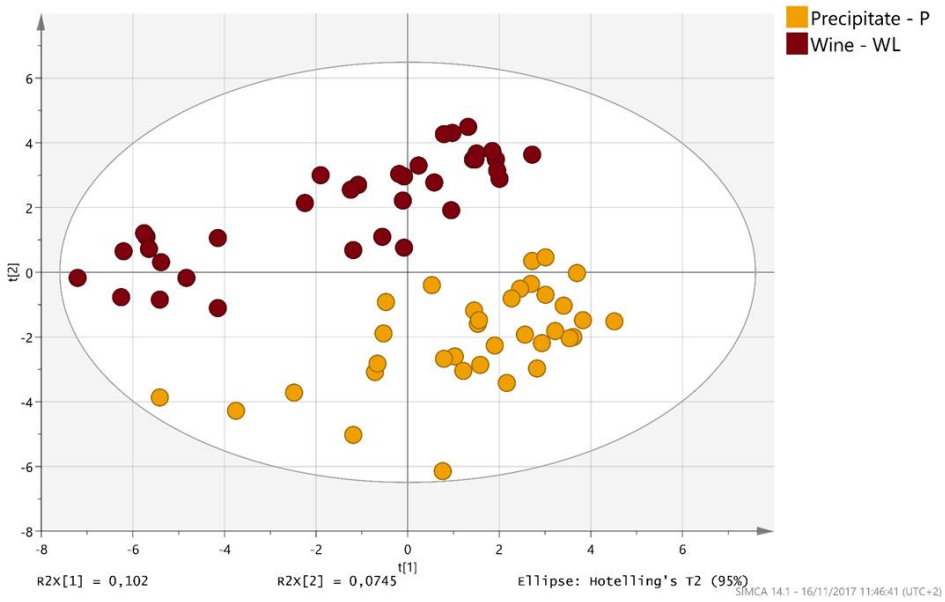

Figure 6.9. PCA plot showing the distribution of the two different matrix, WL and P, analysed by LC-HRMS.

6.3.8. Identification of the compounds

With the aim to further identify the compounds driving the differences between samples, discriminant analyses were performed on WL and P data independently. With this in mind, Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) is a more powerful technique as it concentrates the variance, and was therefore used for this purpose. To evaluate the impact of the three A/T ratios, the corresponding S-Plots (scaled to Pareto variance), were constructed based on pair-wise comparisons (i.e. WL SK vs. WL SKSD; WL SK vs. WL SK4SD or WL SKSD vs. WL SK4SD). The S-plots are a screening tool frequently used to target/identify the possible markers contributing specifically to each treatment. These potentially distinctive markers are generally distributed on the “S”-wings of the plot (upper-right and lower-left edge of the plot). From then, a list of possible markers which may influence the phenolic stability and precipitation was generated. All models were built and statistically validated using SIMCA 14.1 software. The CV-ANOVAs for the different pair-wise comparisons were performed to assess model reliability (Worley & Powers, 2016) (Table 6.5).

Table 6.5. CV-ANOVAs evaluating the model reliability for the different pair-wise comparison between treatments.

	F	p
Wine vs. precipitate	103.021	0.000
Wine		
SK / SK4SD	18.151	0.000
SK / SKSD	8.069	0.001
SKSD / SK4SD	2.724	0.045
C / Ox	2.125	0.082
6M vs. 9M	1.940	0.130
Precipitate		
SK / SK4SD	12.185	0.000
SK / SKSD	1.871	0.148
SKSD / SK4SD	1.427	0.264
C / Ox	2.270	0.120
6M / 9M	12.284	0.000

Some of the ions/compounds were found in both matrices, WL and P. Their presence in the precipitate may be explained by the possible saturation of the specific compound in solution, or as result of the degradation of a larger compound during the precipitation or analysis. Nevertheless, an OPLSA and the corresponding S-Plot was generated to visualise which ions might be driving the main differences between the two matrices (Figure 6.10). From the S-plot, the ions considered as the main contributors, but not the only ones, driving the differences between the matrices were m/z 477, 507, 637 and 655 m/z for the P (lower side of the S) and 177, 191 and 605 for the WL (upper side of the S). Tentative assignment of these influential ions to specific compounds was based on accurate mass data, low and high collision energy MS spectra and on-line UV spectra and relative RP-LC elution order in comparison with reported literature.

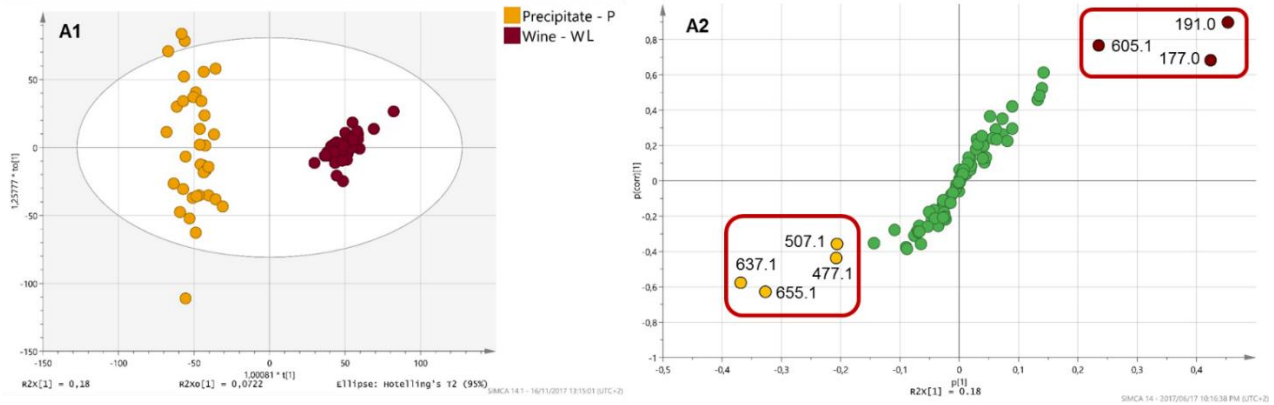

Figure 6.10. OPLS-DA (A1) and corresponding S-Plot (A2) comparing the two different matrices (WL and P) according to the different ions analysed by LC-HRMS.

As shown in Table 6.6, the ions found in the WL matrix and distinguishing WL from P, were identified as gluconolactone (m/z 177), citric acid (m/z 191) and a hemicellulose derivative (m/z 605). The presence of some cell wall polysaccharide derivatives in solution could be relevant to obtain a better understanding on how the polysaccharides influence wine stability. On the other hand, m/z 477 (petunidin glucoside), m/z 507 (syringetin-hexoside), m/z 637 (malvidin-coumaroyl-glucoside) and m/z 655 (its carbinol form) were identified in the precipitate, indicating that loss of colour over time is at least partially due to precipitation of anthocyanins (Table 6.6).

Table 6.6. Identification of the wine stability markers found with the untargeted analysis.

RT	Ions	Matrix	Identity	M-H	Formula	ppm	MSE fragments
2.61	605.1965	WL	Hemicellulose derivative	605.1929	$C_{22}H_{37}O_{19}$	5.9	n.a.
2.67	191.0199	WL	Citric acid	191.0192	$C_6H_8O_7$	0	155,136,111
5.53	169.0143	Both	Gallic acid	169.0138	$C_7H_5O_5$	0.6	125.0242 [M-CO2]
6.15	177.0403	Both	Gluconolactone	177.0393	$C_6H_{10}O_6$	-3.4	159,139.103
7.77	331.0668	Both	Gallic acid glucoside	331.0665	$C_{13}H_{15}O_{10}$	0.9	169,125
8.51	315.1082	P	Hydroxytyrosol glucoside	315.108	$C_{14}H_{19}O_8$	0.6	153,123
9.3	183.0295	WL	Methylgallate	183.0286	$C_8H_8O_5$	-3.8	-
10.48	341.0879	WL	Caffeic acid-3/4-O-glucoside	341.0873	$C_{15}H_{17}O_9$	1.8	289,281,179,161,135
11.08	443.1923	P	Unknown				
11.2	341.0868	P	Caffeic acid-3/4-O-glucoside	341.0873	$C_{15}H_{17}O_9$	-1.5	289,281,179,161,135
11.99	229.0979	Both	Unknown				
12.3	325.0927	WL	Coumaric acid glucoside	325.0927	$C_{15}H_{17}O_8$	-2.2	289,265,295,163,145 ,119
13.08	325.0926	Both	Coumaric acid glucoside	325.0923	$C_{15}H_{17}O_8$	1.2	289,265,295,163,145 ,119
13.17	289.067	P	Epicatechin	289.071	$C_{15}H_{13}O_6$	-0.7	245.0822, 125.0243
14.46	347.0763	P	Dihydrosyringetin	347.0767	$C_{17}H_{15}O_8$	-1.2	347,329,151
14.72	197.0449	Both	Ethylgallate	197.0458	$C_9H_{10}O_5$	4.1	169,125
16.36	369.1193	Both	Unknown	369.1186	$C_{17}H_{22}O_9$	1.9	161,133,125
17.56	477.067	WL	Quercetin-glucoronide	477.0669	$C_{21}H_{17}O_{13}$	0.2	301.0342. 271.0253, 151.0034, 179
17.63	463.0869	Both	Quercetin-hexoside	463.0877	$C_{21}H_{19}O_{12}$	-1.7	301.0342. 271.0253, 151.0034, 179
17.94	493.0976	Both	Laricitrin-hexoside	493.0982	$C_{22}H_{21}O_{13}$	-1.2	331,315, 301
18.59	353.1249	P	Unknown				
19.83	477.1038	Both	Petunidin-glucoside	477.1033	$C_{22}H_{21}O_{12}$	0.4	314,299,285
19.98	507.1144	Both	Syringetin-hexoside	507.1139	$C_{23}H_{23}O_{13}$	1	345,344, 329.316
21,42	637.1564	P	Malvidin-coum-gluc	637.1557	$C_{32}H_{29}O_{14}$	1.1	\

With regards to the specific differences between the different WL composition, the CV-ANOVA analysis showed that only the models built between the different A/T ratios in the WL were statistically significant ($p<0.05$) (Table 6.5). The models built for C/Ox and time were not statistically significant although these treatments played a role in the WL chemical composition, as previously described.

The graphs in Figure 6.11 show the cross-validated OPLS-DA plots and their corresponding S-plots for the different pair comparisons between the WL A/T ratios (R1 as SK, R2 as SKSD and R3 as SK4SD). The ions selected can be considered as the major contributors driving the differences between the A/T ratios. A maximum of five ions were selected when a large amount of ions were found on the edge of the S plot (like in Figure 6.11 C2). Firstly, from SK vs. SK4SD (Figure 6.11 A2) and SK vs. SKSD (Figure 6.11 B2), the m/z 177 and 341 were only present in WL from the SK

treatments. On the other side, the following ions: m/z 169, 197, 229 and 331 were found to appear on the upper-right edge of the S plot as the number of seeds increased during the extraction (Figure 6.11 A2 and B2). Furthermore, a few other ions were selected from the pair-wise comparisons performed between SKSD and SK4SD. As represented in Figure 6.11 C2, the ion 177 m/z was found on SKSD (lower-left side) whereas m/z 169, 229, 331 were found again in the SK4SD side, together with other ions such as m/z 347 and 443. Thus, the nature and the amount of the compounds extracted from the grape seeds played an important role in the phenolic stability. The formation of gluconolactone (m/z 177), naturally found in some food products and as an additive in cosmetics, had previously been reported in studies wine-like system (Bertrand & Barbe, 2002). Its formation is related to the presence of gluconic acid in solution, an oxidised byproduct of glucose. Additionally, caffeic acid-3/4-O-glucoside (m/z 341) was also found in solution when comparing SK vs. SKSD. On the other hand, some of the ions selected from the treatments with the presence of seeds were related to seed derived compounds such as gallic acid (m/z 169), ethyl gallate (m/z 197) and a gallic acid-glucoside (m/z 331). Additionally, when comparing SK4SD versus SKSD, a gallic acid derivative (m/z 331) was also identified. However, the ions m/z 229 and 443 remains unknown.

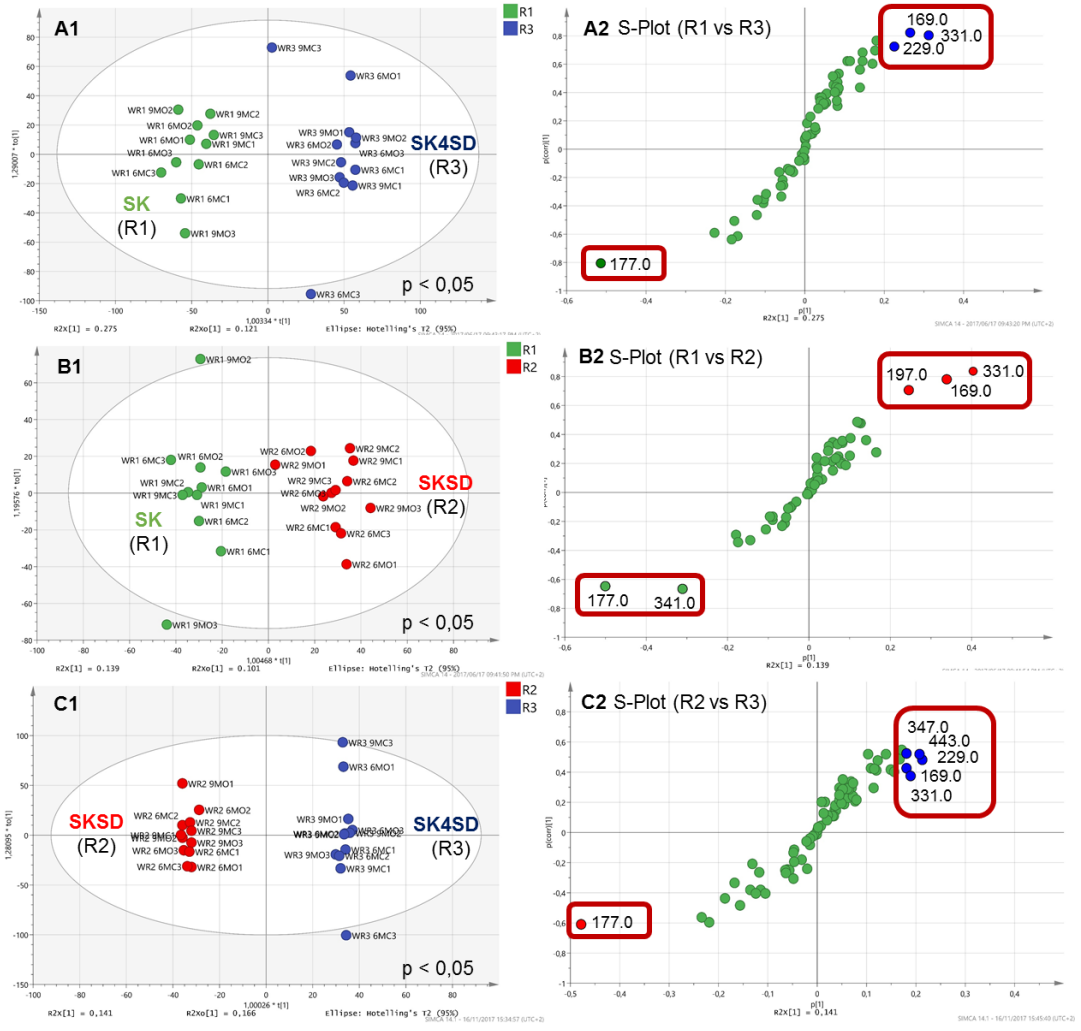

Figure 6.11. OPLS-DA and S-Plot pair-wise comparisons between the different A/T ratios on the WL. R1, R2 and R3 represent the SK, SKSD and SK4SD respectively. A1 and A2 represents the OPLS-DA plot and S-plot between SK and SK4SD. B1 and B2 compared SK vs. SKSD. C1 and C2 compared SKSD vs. SK4SD.

Although not significant, the cross-validated OPLS-DA models and the corresponding S-Plots evaluating the impact of oxygen and time were also performed in WL (data not shown). Interesting results were also obtained, such as the occurrence of methylgallate (m/z 183) in WL-C samples, compared to the presence of quercetin-glucoside (m/z 369) in the WL-Ox samples (Table 6.6).

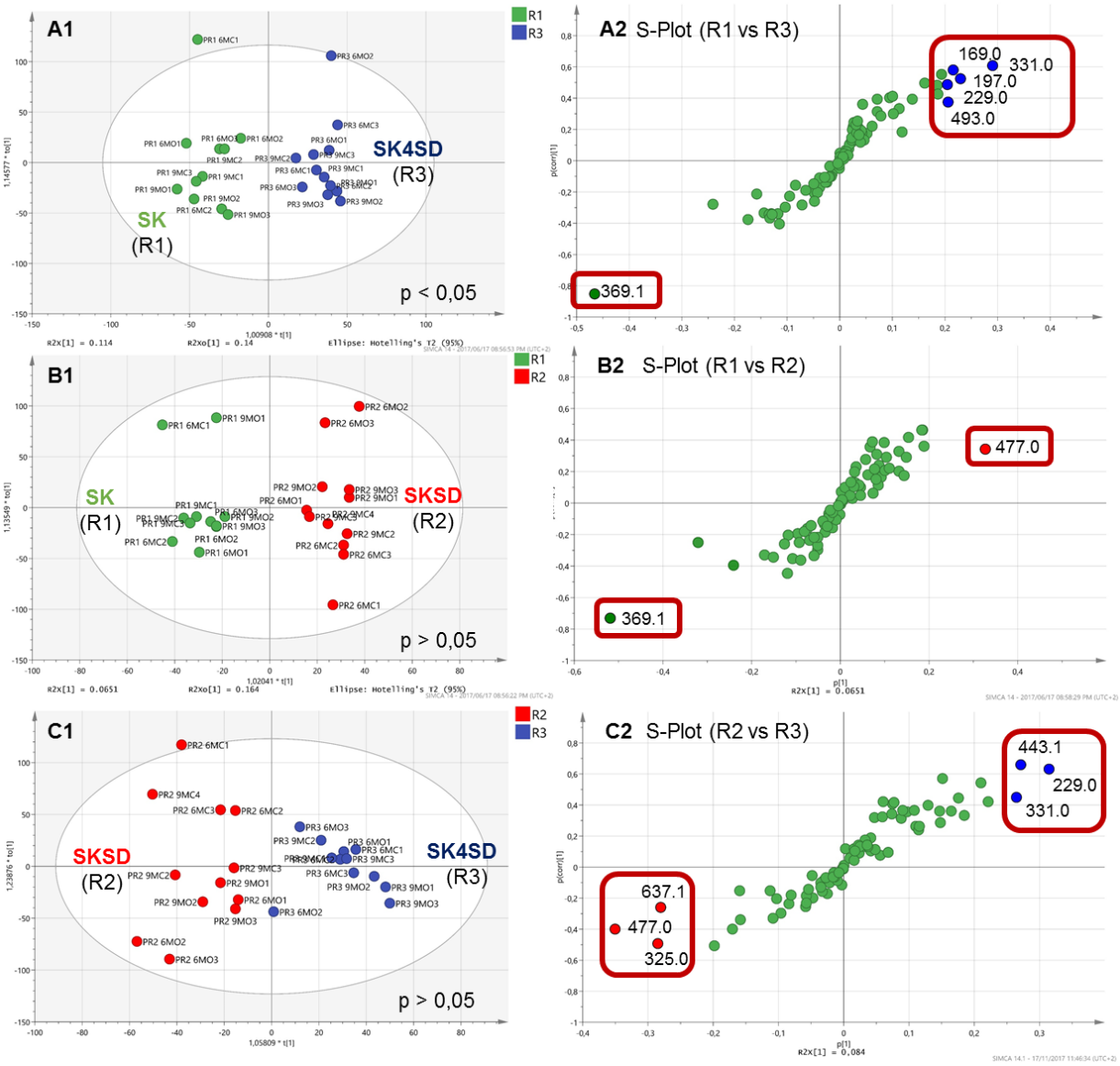

Figure 6.12. OPLS-DA and S-Plot pair-wise comparisons between the different A/T ratios on the P. R1, R2 and R3 represent the SK, SKSD and SK4SD respectively. A1 and A2 represents the OPLS-DA plot and S-plot between SK and SK4SD. B1 and B2 compared SK vs. SKSD. C1 and C2 compared SKSD vs. SK4SD.

On the other hand, interesting results were obtained from the discriminant analysis performed on the P data (Figure 6.12). To our knowledge, this is the first study that evaluated the composition of a wine precipitate influenced by specific A/T ratios. However, the impact of the different A/T ratios was found to be significant only for pair-wise comparisons between SK and SK4SD (Figure 6.12A2). These results indicated a role of the grape seed derived compounds not only in the concentration,

but also on the stability of certain compounds in a wine like system. Firstly, 369 m/z was found on the lower-left side of the S-plot (SK) from Figure 6.12 A2 and B2. The excess of seeds (SK4SD) led to the precipitation of the molecules with the following m/z: 169, 197, 229, 331 and 493. The ion m/z 369 remains unknown. On the other side of the S-plot, the excess of seed (SK4SD) led to the precipitation of specific compounds which were also identify in the WL such as gallic acid (m/z 169), ethyl gallate (m/z 197) and a gallic acid derivative (m/z 331). As mentioned, their presence in the P samples may be due to a possible saturation in the wine solution or as a result of the fragmentation of a larger molecule. The ion m/z 493 (Table 6.6) was identified as laricitrin-hexoside.

Moreover, contrary to the results in WL, the cross-validated OPLS-DA and S-Plot defined time as a significant factor in the compound precipitation. The oxygen as a factor was not significant. From the S-plot 6M vs. 9M (Figure 6.13), a few selected ions (m/z 315, 507, 637, and 655) originating from compounds that precipitated after 6M whilst 177, 325 and 341 m/z seemed to precipitate after 9M. Then, over the course of time, malvidin-coumaryl-glucoside and its carbinol form precipitated after 6M, as well as hydroxytyrosol-glucoside (m/z 315) (glucosylated of the bioactive compound hydroxytyrosol found in wine) and syringetin (m/z 507). Interestingly, some hydroxycinnamic acids derivatives only precipitated after 9M, such as the caffeic acid-3/4-O-glucoside (m/z 341) or coumaric acid glucoside (m/z 325).

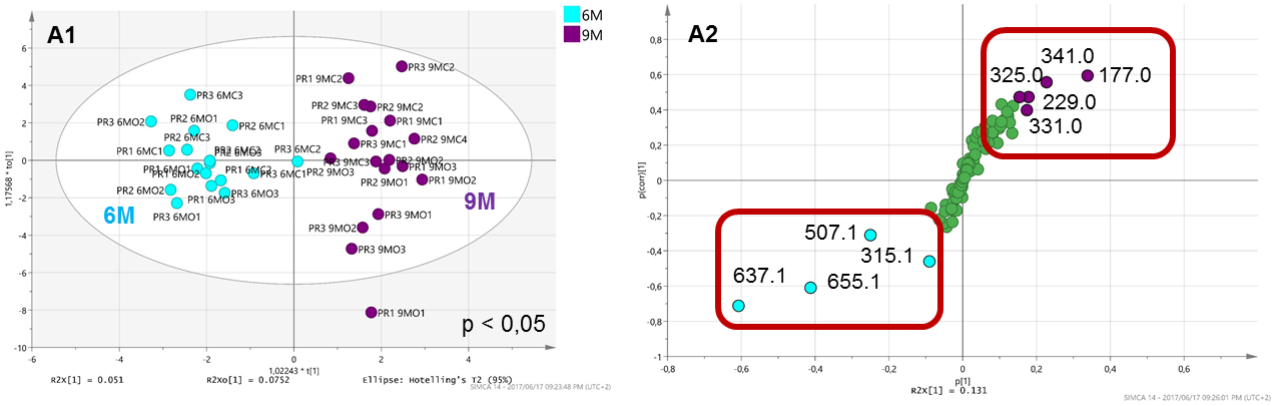

Figure 6.13. OPLS-DA (A1) and S-Plot (A2) pair-wise comparisons according to the precipitation time (6M vs.9M) on the P.

A few ions were also selected from the C/Ox S-plot (although the model was not significant) as they were clearly located on the S wings. As illustrated in Figure 6.14, malvidin-coumaryl-glucoside (637 m/z) was found in C samples. On the side the ions 463, 477 and 507 m/z were selected originating from compounds that were precipitated in the presence of oxygen. Quercetin-hexoside (m/z 463), petunidin glucoside (m/z 477, RT 19.83) and syringetin (m/z 507) were found in the Ox precipitate. The presence of m/z 637 in C samples confirms the results from Table 3 and the indirect protective

effect of oxygen towards certain monomeric anthocyanins during the first months of storage under our conditions.

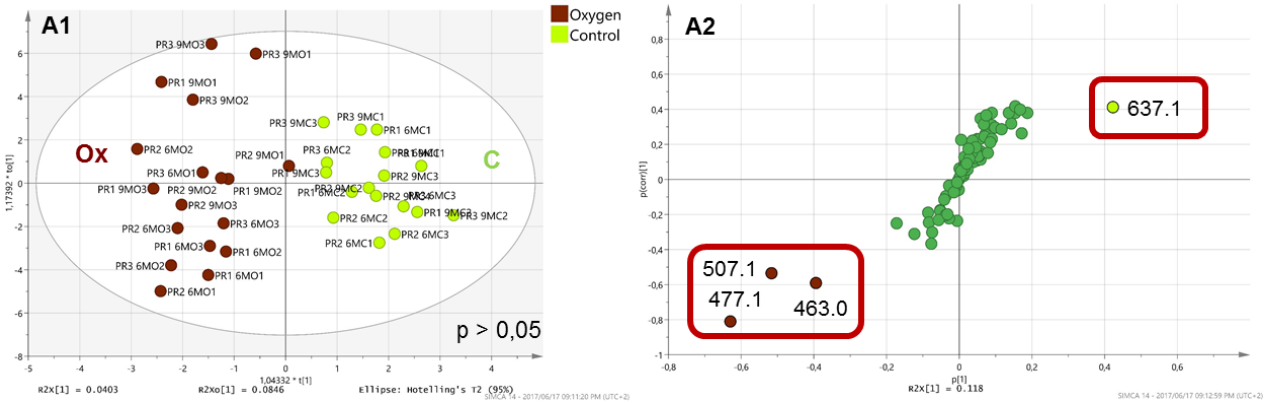

Figure 6.14. OPLS-DA (A1) and S-Plot (A2) pair-wise comparisons according to the C and Ox samples on the P.

6.4. CONCLUSION

The first goal was to assess the impact of the oxygen on three A/T ratios in a WL system. In this sense, the present study has proved the influence of these A/T ratios in phenolic polymerisation, and the colour and phenolic evolution over time. Firstly, our results showed a relevant role of the phenolics derived from grape seeds in the phenolic polymerisation, in absence or presence of oxygen, over time. Also, the study demonstrated how the polymerisation between certain compounds was favoured in the presence of oxygen. This might explain the larger TRP levels and the concentration of total glucosylated, acetylated and coumaroylated forms of the monomeric anthocyanins in WL-Ox samples at 3M (Table 6.3) found in Ox samples. Thus, the higher levels of seed derived compounds could have favoured these polymerisation reactions during the oxidative processes or reacted with the oxygen, leaving part of the monomeric anthocyanins free in the solution.

The impact of seed addition or removal on the colour of the wines had been previously reported (Canals, *et al.*, 2008). From our results, in agreement with Canals *et al.* (2008), a higher amount of seeds (SK4SD) showed a higher amount of TRP (especially oxidized samples at 3M), but it did not affect the final CD contrary to what Picarello *et al* (2017) described. The HILIC method permitted us to measure the specific concentration of oligomeric tannins. To date, a large number of studies had focused on the impact of seed addition or removal on the colour, phenolic profile and sensory properties of the wines (Meyer & Hernandez, 1970; Canals, *et al.*, 2008; Lee, *et al.*, 2008; Guaita, *et al.*, 2017), but there is a lack of information on their evolution and how oxygen influences this. This work has been carried in a model wine solutions. Nonetheless, in a red winemaking process, the presence of other grape derived compounds, such as skin proteins or polysaccharides, may alter

this self-aggregation, polymerisation and precipitation reactions (Poncet-Legrand, *et al.*, 2007; Watrelot, *et al.*, 2017).

Moreover, the present study also showed the influence of oxygen and a greater tannin content on the stability of certain compounds in a WL. The A/T ratios with an excess of seeds were characterised by a larger number of gallic acid derivatives in solution, but also these formed in the precipitate. Here again it was shown how the presence of seed derived compounds in the WL solution might have favoured the remaining of certain anthocyanins in solution. In absence of oxygen, malvidin-3-coumaroylglucoside was found to precipitate more than in the presence of oxygen. To our knowledge, this is the first time that the precipitation of phenolic compounds, as a consequence of altered A/T ratio and forced oxidation, was examined. Grape seeds are a very rich source of phenolics. Some of these compounds, such as dyhydroxytyrosol (from grape and olive seeds), are used as a bioactive compounds part of dietary supplements. A recent study has evaluated the impact of hydroxytyrosol addition in Syrah wines, as alternative to SO_2, with the result of an improvement in the wine colour at bottling (Raposo, *et al.*, 2016). Nevertheless, the negative impact of seed derived phenolic compounds on the sensory perception of certain wines is well known, especially related with the bitter taste (Arnold & Noble, 1978; Peleg, *et al.*, 1999; Pascual, *et al.*, 2016). Further studies need to be done that investigates not only the impact of the grape seeds on the wine stability, but also on their role on the phenolic extractability during wine red fermentation.

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Chapter 7

General discussions and conclusions

Chapter 7: General discussions and conclusions

7.1. GENERAL DISCUSSIONS

The quality of a red wine is undoubtedly associated with its colour and other organoleptic properties. Understanding wine chemistry, and more specifically grape and wine phenolic compounds, is very important to comprehend the evolution in colour, taste and mouth-feel of red wines. Extensive worldwide research has investigated the diversity of factors affecting phenolic synthesis and accumulation (Adams, 2006; Fournand, *et al.*, 2006; Cohen, *et al.*, 2008; Mattivi, *et al.*, 2009), phenolic extractability (Canals, *et al.*, 2005; Sacchi, *et al.*, 2005; Bindon, *et al.*, 2014; Hernández-Hierro, *et al.*, 2014; Smith, *et al.*, 2015), reactions involving phenolics over time (Gambuti, *et al.*, 2013; Arapitsas, *et al.*, 2014), as well as the final sensorial profiles of the wines (Wollmann & Hofmann, 2013; Ma, *et al.*, 2014; Sáenz-Navajas, *et al.*, 2017). All the work published in recent years has shown a large increase in interest in this topic as was shown by Aleixandre-Tudo *et al.* (2017). Fundamental research in this field is essential in order to obtain a better understanding on the wide range of chemical interactions involving phenolic compounds as well as to develop more advanced analytical methods which may be applicable to the wine industry.

This study has tried to highlight the importance of the phenolic composition of young Shiraz wines to better understand their colour and phenolic evolution during ageing. As part of this, we have also studied two very relevant fields such as the grape and wine phenolic composition and the berry cell wall associated polysaccharides and proteins and how these evolve during ripening and fermentation. For all three seasons studied, *Vitis vinifera* grapes were obtained from the same Shiraz vineyard from the Department of Viticulture and Oenology from Stellenbosch University. Therefore, the present results, although interconnect able, could obviously differ from findings with other red cultivars and different growing regions.

The first objective of this study (Chapter 3) was to monitor the colour and phenolic evolution, as well as the sensorial changes, occurring in Shiraz wines made from grapes from the same vineyard, but with different initial phenolic profiles. This took place over two different vintages (2014 and 2015) where differences in the young wines' phenolic concentrations/profiles came from grapes from different training systems, vigour zones and ripeness levels. However, for the second vintage (2015), the work focussed exclusively on the evolution of the wines made from different grape ripeness levels, as this was the only parameter which we could investigate in a more detailed manner. In the 2014 wines the colour and phenolic differences became smaller during wine ageing. However, in 2015 wines, differences in colour and phenolic composition between the wines made from the ripest berries (25ºBrix) and the rest (21ºBrix, 23ºBrix and 24ºBrix), became larger from the end of

fermentation to the bottling and remained over time. These results highlights the idea that only the grape sugar level is not always a good indicator to estimate the phenolic composition of the resulting wines. The greater concentration in polymeric phenols and polymeric pigments in certain wines could therefore be due to them being present at higher levels in the grapes or being more extractable. However, the formation from their monomeric moieties with a higher reactivity to form these polymers over time, could also be possible. Changes in phenolic concentrations and composition and the structural conformation of the grape skin cell walls during ripening can be a major factor determining the wine's final phenolic concentration.

From this idea, in the second part of this study we investigated the possible relationship between the cell wall polymer composition and the extraction of colour and phenolics into the wines. Chapters 4 contribute to a better understanding of the relationship between ripening levels, grape and wine phenolics and cell wall components. Firstly, a clear vintage effect, probably as a consequence of the different climatic conditions between 2015 and 2016, was found to influence the grape composition. Seasons with extremely high temperatures close to véraison may alter the pectin fraction in the grape skins and the synthesis of different phenolics compounds, leading to wines with lower phenolic levels. In Chapter 5 we highlighted the influence of grape ripening level on depectination during the course of fermentation. In theory the greater the cell wall deconstruction and "opening-up" of the skin pomace, the greater the amount of certain phenolics extracted into the wines. However, this level of depectination can also lead to wine with lower levels of phenolics in certain cases due to two reasons. As described by Bindon *et al.* (2014), the cell wall porosity which is strongly related to the conformation of the pectin layer, can lead to the encapsulation and the subsequent retention of phenolic compounds, especially condensed tannins. Secondly we can hypothesise that making wine from berries with a greater depectination level could lead to an increase in cell wall polysaccharides leaching into the wines and reacting with phenolic compounds, thereby lowering phenolic concentrations in the wine due possible precipitation. Future research could be directed to not only better elucidate the relationship between phenolics and cell wall components, but also to the development of techniques to evaluate "in situ" the cell wall composition of the grapes while still on the vine. More knowledge of the berry skin cell wall composition could help the winemaker towards a better phenolic management during the winemaking process.

The objective for the last part of this work (Chapter 6) was to assess the impact of oxygen in a Wine Like system (WL) containing three different anthocyanin/tannin ratios over time obtained by adding different amounts of seeds. As expected, these different seed levels had a clear impact on the total amount of phenolics, especially tannins and their development over time. . In addition, our results have showed that subsequent polymerisation reactions are influenced by the phenolic proportions, irrespective of the presence or absence of oxygen. However, polymer formation was enhanced by the oxygen addition, probably due to the formation of ethyl-bridged compounds as a consequence of the oxidative process (Timberlake & Bridle, 1976; Dallas, *et al.*, 1996; Picariello, *et al.*, 2017). It

seems that under our conditions the oxygen favoured the polymerisation between tannins, especially in the presence of seed phenolics, leading to more free anthocyanins in the solution. Additionally, it also increased the formation of polymeric pigments in some cases as also recently reported by Picariello *et al.* (2017). Thus, keeping in mind the potential negative taste and mouth-feel attributes such as excessive bitterness resulting from the presence of seeds, they could have a positive indirect role on the presence of anthocyanins in the wines. Winemaking decisions such as the déléstage may thus also affect the colour of the wines. Different results may have probably been observed with a gradual oxidation, which was not employed in our study.

The second objective of this last chapter was to investigate the precipitate formed over time in the WL system. The use of untargeted analysis permitted us to evaluate the precipitate formed and identify the main compounds driving the main differences between treatments and WL and precipitate. The excess of seeds influenced the phenolic stability. Oxygen (although the OPLSA model was not significant) also influenced the precipitation of certain compounds, especially in their glucoside form. In relation with the previous chapters, the addition of grape polysaccharides could be interesting to evaluate their reactions with phenolics and if these influences the specific composition of the precipitate formed. This could help to predict colour and phenolic stability made from more depectinated berries or as a consequence of techniques like extended maceration. Further investigations regarding the formation of precipitate is necessary to understand the formation of phenolic derived precipitates in red wines.

7.2. CONCLUDING REMARKS

The present work, through all four research chapters, have shown the importance of understanding phenolic extractability as the colour and phenolic composition of young wines seems to influence the ageing potential of the wines. Factors such as grape ripeness, can influence the grape phenolic extractability altering not only the phenolic proportions in young wines but also its evolution over time. The study in a wine-like system therefore further helped to obtain a better understanding of the impact of specific anthocyanin/tannin ratios during ageing. Determining the specific impact of different vineyard management practices and grape ripening on the wine composition is a difficult task as numerous factors can influence this. Due to the large variability existing between different vineyards, the use of the same vineyard during the entire study has helped to exclude most of these other external variables.

For the first part of the project (Chapter 3), a better sampling to strategy would have been more ideal in 2014, as the ºBrix differences between certain grapes treatments may have influenced the phenolic profile of the wines to a certain extent. Due to unforeseen circumstances, a change in some

of the sensorial panel members during the two sensory evaluations in 2014 also did not allow us to establish a real evolution of the wines.

The impact of vintage, ripening and alcoholic fermentation on the phenolic and cell wall associated polysaccharides was successfully assessed in Chapter 4 and 5. However, the additional information which we could have obtained from the quantification of non-extracted individual and groups of phenolic compounds in the fermenting skins from 23ºBrix and 25ºBrix grapes would have been very valuable. In addition, although it was not part of the present project, the analysis of the polysaccharide composition in the wines would have helped to reach a better understanding of the relationship between the cell wall breakdown and the release of phenolic compounds.

Nonetheless, the current study makes a valuable contribution to research in grape and wine and to the South African wine industry. The colour and phenolic composition of young wines seems to influence the ageing potential of the wines. From this study, a relationship between berry intactness and final phenolic content in wines was also seen. In future work, a better assessment of the impact of the different climatic conditions on the cell wall composition can be very relevant for the winemaker. There are currently still a lot of unknowns regarding the specific relationship between cell wall polysaccharides and phenolic compounds, but with the use of more advanced and powerful analytical techniques a better understanding of phenolic and polysaccharide derived compounds will be reached in future.

In addition, the results in Chapter 6 have highlighted the influence of seed phenolics and oxygen on the wine polymerisation over time. A more progressive oxidation under the same conditions will be ideal to confirm the impact of the seed tannins in a wine evolution. Assessing the phenolic evolution within the first three months of our study would probably have also been more ideal, as large changes occurred between T0 and 3M months ageing. However, the evolution of phenolic compounds present at different ratios under different storage conditions can also be an interesting factor to investigate in future. The large volumes of grape seeds required for such a study could be a limitation which can influence the number of variables to study.

The use of other red cultivars or vineyards could have yielded different results from those obtained in our study, but the general aims set out in Chapter 1 has been achieved to a large degree. However, we consider that the overall findings from the present work will contribute to knowledge on colour and phenolic compounds and factors affecting their evolution in Shiraz grapes and wines.

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Appendix

APPENDIX CHAPTER 3

Appendix Table 3.1. List of aroma, taste and mouth-feel attributes and their corresponding standards for the sensory evaluation of 2014 wines (6M and 12M).

	Descriptors	Standards	6M 2014	12M 2014
Aroma	Dark berries	Fresh/frozen mix of dark berries (*Hillcrest Berry Orchads*)	x	x
	Red berries	Fresh/frozen mix of red berries (*Hillcrest Berry Orchads*)	x	
	Cherries	Cherry syrup		x
	Prunes	Prunes(Safari)	x	x
	Raisins	Raisins (Safari)	x	x
	Dry peaches	Dried peaches (Safari)	x	
	Vanilla/Caramel	Vanilla essence	x	x
	Tobacco	Tobacco (Domingo)	x	x
	Black pepper	(Robertson) black pepper	x	x
	Liquorice	Liquorice candy (Allsorts)	x	x
	Pencil shavings	Pencil shavings (Staedtler)	x	x
	Toasted wood	Medium toasted French oak chips	x	
	Elderflower	Elderflower syrup (Blütensirup, Holunderblüte)	x	x
	Floral	Le nez du vin		
	Soy sauce	Soy sauce (Vital)	x	x
	Bovryl/Meaty	Beefy bovryl	x	x
	Leather	New leather stripe		x
	Roasted coffee	Roasted coffee beans	x	
	Herbaceous	Mint	x	
	Fresh vegetative	Cut grass		x
	Eucalyptus	Fresh eucalyptus		x
	Dry herbs	Mixed herbs (Robertson)		x
	Cooke veg.	Asparagus/cauliflower	x	x
	Musk/Animal	Le nez du vin	x	
Taste and mouth-feel				
	Sweetness	Different concentrations of sucrose (Sigma-Aldrich)	x	x
	Acidity	Different concentrations of tartaric acid (Sigma-Aldrich)	x	x
	Bitterness	Different concentrations of caffeine (caffeine tablet)	x	x
	Astringency	Alum Crystals B.P. (Alpha Pharm) (0, 0.25, 0.5, 1 g/L)	x	x
	Body	Carboxymethyl cellulose (CMC) solution (0.5/1/2 g/L)	x	x
	Alcohol burn	96% Ethanol (13%, 15%)	x	x

Appendix Table 3.2. List of aroma, taste and mouth-feel attributes and their corresponding standards for the sensory evaluation of 2015 wines (6M and 12M).

	Descriptors	Standards	6M 2015	12M 2015
Aroma	Dark berries	Fresh/frozen mix of dark berries (*Hillcrest Berry Orchads*)	x	x
	Red berries	Fresh/frozen mix of red berries (*Hillcrest Berry Orchads*)	x	x
	Plum	Fresh plums	x	
	Cherries	Cherry syrup		x
	Prune/Raisins	Prunes/raisins (Safari)	x	x
	Vanilla/Caramel	Vanilla essence	x	x
	Sweet spices	Cinnamon (Robertson) Cloves (Robertson)	x	
	Aniseed	Star aniseed (Robertson)	x	
	Liquorice	Liquorice candy (Allsorts)	x	x
	Floral	Le nez du vin	x	x
	Elderflower	Elderflower syrup (Blütensirup, Holunderblüte)		x
	Muscat	Le nez du vin		x
	Cooked veg	Asparagus/cauliflower	x	x
	Herbaceous/Mint	Fresh mint leaves	x	
	Fresh vegetative	Cut grass		x
	Soy sauce	Soy sauce (Vital)	x	x
	Meaty	Beefy bovryl	x	x
	Coriander seeds	Crushed coriander seeds	x	x
	Black pepper	Black pepper (Robertson)	x	x
	Pencil shavings	Pencil shavings (Staedtler)	x	x
	Leather	New leather stripe	x	
	Tobacco	Tobacco (Domingo)	x	x
	Roasted coffee	Roasted coffee beans		x
	Humus/Earthy	Wet earth	x	
Taste and mouth-feel				
	Sweetness	Different concentrations of sucrose (Sigma-Aldrich)	x	x
	Acidity	Different concentrations of tartaric acid (Sigma-Aldrich)	x	x
	Bitterness	Different concentrations of caffeine (caffeine tablet)	x	x
	Astringency	Alum Crystals B.P. (Alpha Pharm) (0, 0.25, 0.5, 1 g/L)	x	x
	Body	Carboxymethyl cellulose (CMC) solution (0.5/1/2 g/L)	x	x
	Alcohol burn	96% Ethanol (13%, 15%)	x	x

Appendix Table 3.3. Univariate test of significance for grape tannins, anthocyanins and TP in 2014.

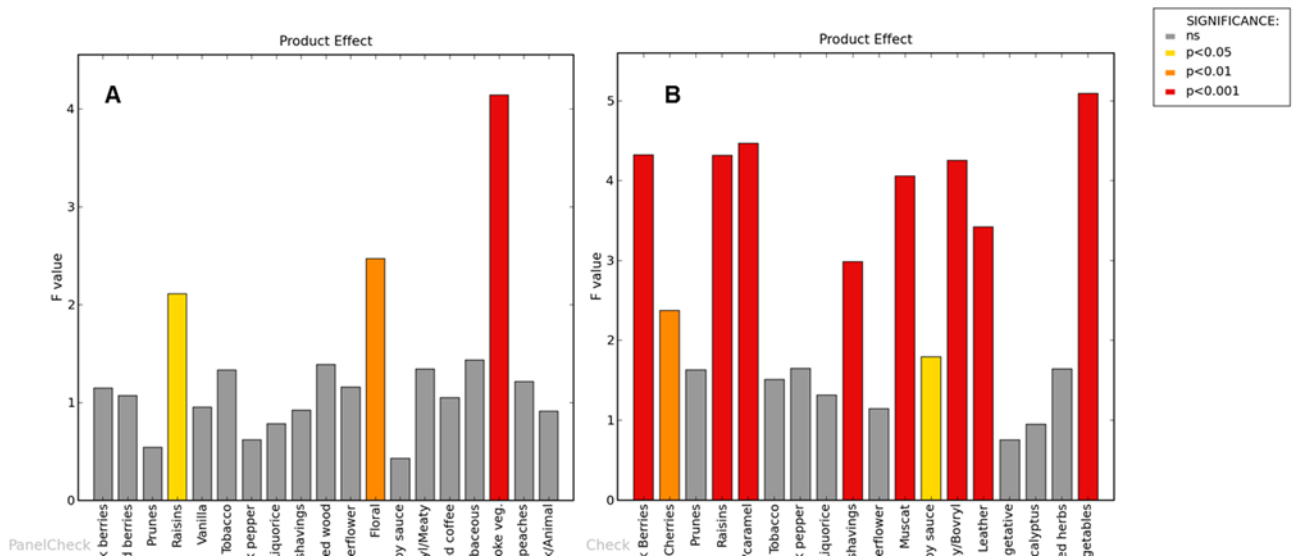
	Tannins		Anthocyanins		TP	
	F	p	F	p	F	p
Training system	25.050	0.000	39.028	0.000	2.319	0.147
Vigour	1.129	0.304	3.875	0.067	73.354	0.000
Brix	1.096	0.311	81.765	0.000	8.773	0.009
Training system *vigour	7.206	0.016	0.232	0.636	1.413	0.252
Training system *brix	1.218	0.286	55.279	0.000	16.228	0.001
Vigour*brix	3.904	0.066	0.121	0.732	0.066	0.800
Training system *vigour*brix	0.098	0.758	4.896	0.042	38.643	0.000

Appendix Table 3.4. Univariate test of significance for tannins. TRP and TP in 2014 wines at bottling (T0).

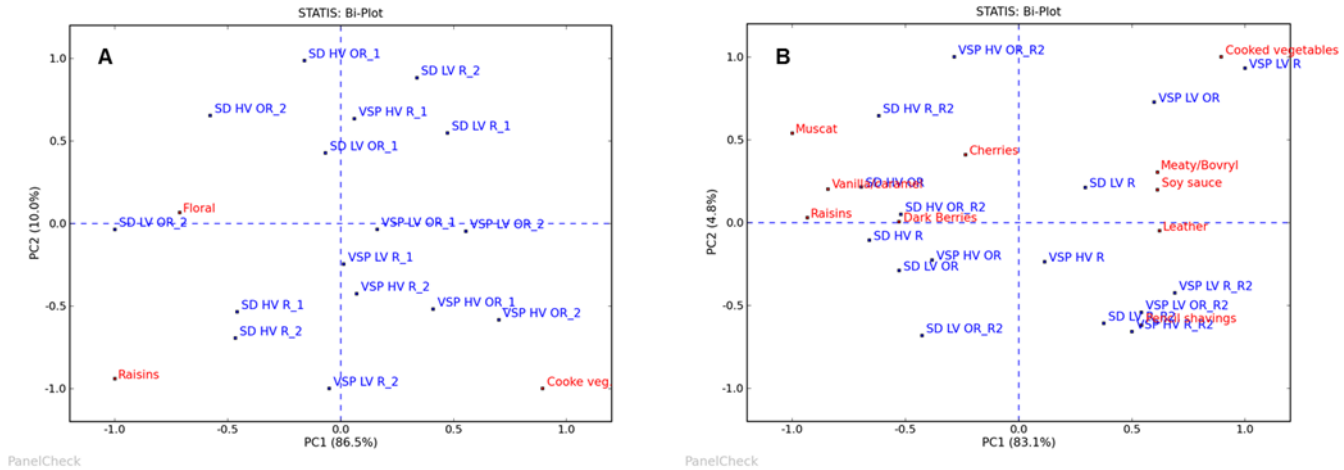
	Tannins		TRP		TP	
	F	p	F	p	F	p
Training system	11.714	0.003	79.066	0.000	15.737	0.001
Vigour	5.847	0.028	39.189	0.000	62.009	0.000
Brix	26.259	0.000	46.717	0.000	0.051	0.824
Training system *vigour	9.831	0.006	1.121	0.305	16.422	0.001
Training system *brix	0.669	0.425	4.130	0.059	7.143	0.017
Vigour*brix	0.006	0.940	1.017	0.328	1.791	0.200
Training system *vigour*brix	2.434	0.138	3.836	0.068	0.098	0.758

Appendix Table 3.5. Multivariate test of significance (Wilks test) for the impact of the different variables in the individual phenolic composition of all 2014 wines over time.

	F	p
Training system	13.183	0.000
Vigour	5.962	0.000
Ripening	53.010	0.000
Time	28.321	0.000
Training system*Vigour	3.748	0.001
Training system*Ripening	13.053	0.000
Vigour*Ripening	2.204	0.031
Training system*Time	4.114	0.000
Vigour*Time	2.491	0.000
Ripening*Time	4.732	0.000
Training system*Vigour*Ripening	5.032	0.000
Training system*Vigour*Time	1.242	0.169
Training system*Ripening*Time	2.455	0.000
Vigour*Ripening*Time	1.263	0.151
Training system*Vigour*Ripening*Time	2.175	0.000



Appendix Figure 3.1. Significant aroma attributes for 2014 wines after 6 months (A) and 12 months in bottle.



Appendix Figure 3.2. Bi-plots illustrating the distribution of the different wine samples after 6 (A) and 12 months (B) in bottle according to the significant aroma attributes.

Appendix Table 3.6. Intensity values (0-100) for the taste and mouth-feel attributes of 2014 wines evaluated after 6 months of storage. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

Wine 6M	SD HV R	SD LV R	SD HV OR	SD LV OR	VSP HV R	VSP LV R	VSP HV OR	VSP LV OR
Acidity	55.33 ± 14.64 ^{ab}	56.55 ± 14.11 ^{a}	50.79 ± 15.10 ^{bc}	48.29 ± 14.01 ^{b}	53.77 ± 14.17 ^{ab}	50.65 ± 15.82 ^{bc}	47.14 ± 14.55 ^{c}	46.59 ± 14.20 ^{c}
Sweetness	28.27 ± 11.64 ^{bc}	25.83 ± 11.44 ^{c}	31.42 ± 14.03 ^{ab}	33.61 ± 14.27 ^{a}	29.17 ± 13.32 ^{bc}	29.62 ± 13.87 ^{b}	30.20 ± 13.23 ^{ab}	28.79 ± 13.25 ^{bc}
Body	38.02 ± 10.96 ^{c}	37.38 ± 11.80 ^{c}	42.91 ± 11.62 ^{a}	43.70 ± 11.31 ^{a}	39.41 ± 11.98 ^{bc}	42.06 ± 11.91 ^{ab}	43.58 ± 12.62 ^{a}	43.20 ± 11.15 ^{a}
Alcohol burn	44.95 ± 14.86 ^{b}	45.64 ± 15.66 ^{b}	47.82 ± 14.94 ^{ab}	50.80 ± 14.25 ^{a}	46.33 ± 13.96 ^{ab}	45.98 ± 16.55 ^{b}	50.59 ± 12.90 ^{a}	48.20 ± 15.37 ^{ab}
Astringency	42.77 ± 15.96 ^{c}	46.47 ± 14.90 ^{abc}	45.88 ± 14.64 ^{bc}	44.17 ± 15.39 ^{bc}	43.92 ± 15.73 ^{bc}	45.32 ± 15.61 ^{bc}	50.47 ± 15.41 ^{a}	47.35 ± 14.84 ^{ab}
Bitterness	44.06 ± 17.32 ^{c}	43.26 ± 16.21 ^{c}	49.38 ± 15.84 ^{b}	51.95 ± 19.49 ^{ab}	43.88 ± 16.73 ^{c}	50.23 ± 16.96 ^{ab}	54.82 ± 15.59 ^{a}	54.17 ± 16.58 ^{ab}

Appendix Table 3.7. Intensity values (0-100) for the taste and mouth-feel attributes of 2014 wines evaluated after 12 months of storage. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

Wine 12M	SD HV R	SD LV R	SD HV OR	SD LV OR	VSP HV R	VSP LV R	VSP HV OR	VSP LV OR
Acidity	47.79 ± 1.62^{c}	52.93 ± 1.32 ^{ab}	47.83 ± 1.32 ^{c}	49.31 ± 1.47 ^{bc}	55.68 ± 1.35 ^{a}	49.71 ± 1.53 ^{bc}	46.55 ± 1.44 ^{c}	51.95 ± 1.19 ^{ab}
Sweetness	45.99 ± 1.61 ^{b}	39.54 ± 1.40 ^{c}	51.13 ± 1.67 ^{a}	47.65 ± 1.53 ^{ab}	37.04 ± 1.31 ^{c}	37.64 ± 1.61 ^{c}	46.06 ± 1.59 ^{b}	39.06 ± 1.53 ^{c}
Body	47.50 ± 1.55 ^{bc}	44.41 ± 1.63 ^{cde}	56.84 ± 1.43 ^{a}	56.14 ± 1.33 ^{a}	40.89 ± 1.66 ^{e}	42.45 ± 1.52 ^{de}	51.73 ± 1.54 ^{b}	46.20 ± 1.64 ^{cd}
Alcohol burn	45.29 ± 1.88 ^{cd}	45.13 ± 1.66 ^{cd}	51.38 ± 1.34 ^{ab}	55.54 ± 1.51 ^{a}	40.10 ± 1.58 ^{e}	41.26 ± 1.58 ^{de}	51.24 ± 1.70 ^{ab}	48.15 ± 1.72 ^{bc}
Astringency	45.08 ± 2.02 ^{ab}	47.84 ± 2.00 ^{a}	46.48 ± 1.62 ^{a}	46.88 ± 1.63 ^{a}	43.64 ± 2.13 ^{ab}	40.25 ± 1.92 ^{b}	48.75 ± 2.09 ^{a}	47.35 ± 1.74 ^{a}
Bitterness	34.96 ± 1.89 ^{d}	37.98 ± 1.86 ^{d}	46.73 ± 1.87 ^{ab}	51.74 ± 2.38 ^{a}	35.14 ± 2.04 ^{d}	39.40 ± 2.05 ^{cd}	44.76 ± 2.50 ^{bc}	43.93 ± 1.92 ^{bc}

Appendix Table 3.8. Test of significance (Test of SS Whole model vs SS residual) for the colour and phenolics (spectrophotometric results) of 2015 wines at AF.

	F	p
Colour Density	8.976	0.006
Hue	3.712	0.061
MCD	8.761	0.007
TP	12.697	0.002
TRP	4.798	0.034
% TRP	5.075	0.029
TANNINS	1.924	0.204
SO2 RESISTANT	123.970	0.000
Copigments	4.207	0.046

Appendix Table 3.9. Test of significance (Test of SS Whole model vs SS residual) for the individual phenolic compounds of 2015 wines at AF.

	F	p
Gallic acid	1.373	0.319
Catechin	1.979	0.196
B1	0.735	0.560
Polymeric phenols	6.768	0.014
Total hydroxycinnamic acids	29.804	0.000
Total flavonols	6.237	0.017
Total glucosylated anthocyanins	12.925	0.002
Total acetylated	12.183	0.002
Total coumaroylated	29.854	0.000
Polymeric pigments	4.506	0.039

Appendix Table 3.10. Multivariate test of Significance (Wilks test) for colour and phenolics (measured by spectrophotometric methods) in all 2015 wines made from different grape ripeness levels (21ºBrix. 23ºBrix. 24ºBrix and 25ºBrix) during 18 months of bottle ageing.

	F	p
Ripening	17.710	0.000
Time	30.940	0.000
Ripening*Time	3.510	0.000

Appendix Table 3.11. Univariate test of Significance for colour and phenolics (measured by spectrophotometric methods) in all 2015 wines made from different grape ripeness (21ºBrix. 23ºBrix. 24ºBrix and 25ºBrix) during 18 months of bottle ageing.

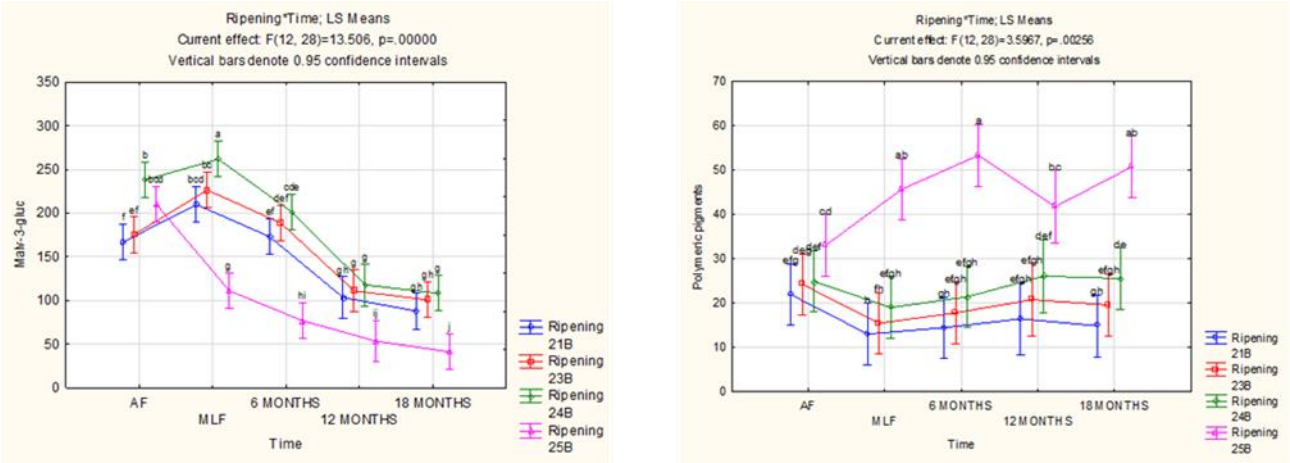
	CD F	CD p	Hue F	Hue p	MCD F	MCD p	TP F	TP p
Ripening	66.522	0.000	18.89	0.000	53.909	0.000	20.682	0.000
Time	39.558	0.000	69.19	0.000	38.296	0.000	11.401	0.000
Ripening*Time	0.630	0.804	3.63	0.001	3.380	0.002	1.016	0.453
	TRP F	**TRP p**	**Tannins F**	**Tannins p**	**SO_2 resistant F**	**SO_2 resistant p**	**Copigments F**	**Copigments p**
Ripening	21.661	0.000	44.623	0.000	92.068	0.000	1.305	0.285
Time	70.650	0.000	3.434	0.016	130.053	0.000	19.528	0.000
Ripening*Time	1.829	0.076	3.429	0.002	11.921	0.000	4.151	0.000

Appendix Table 3.12. Multivariate test of Significance (Wilks test) for individual phenolic compounds in all 2015 wines made from different grape ripeness (21ºBrix. 23ºBrix. 24ºBrix and 25ºBrix) during 18 months of bottle ageing.

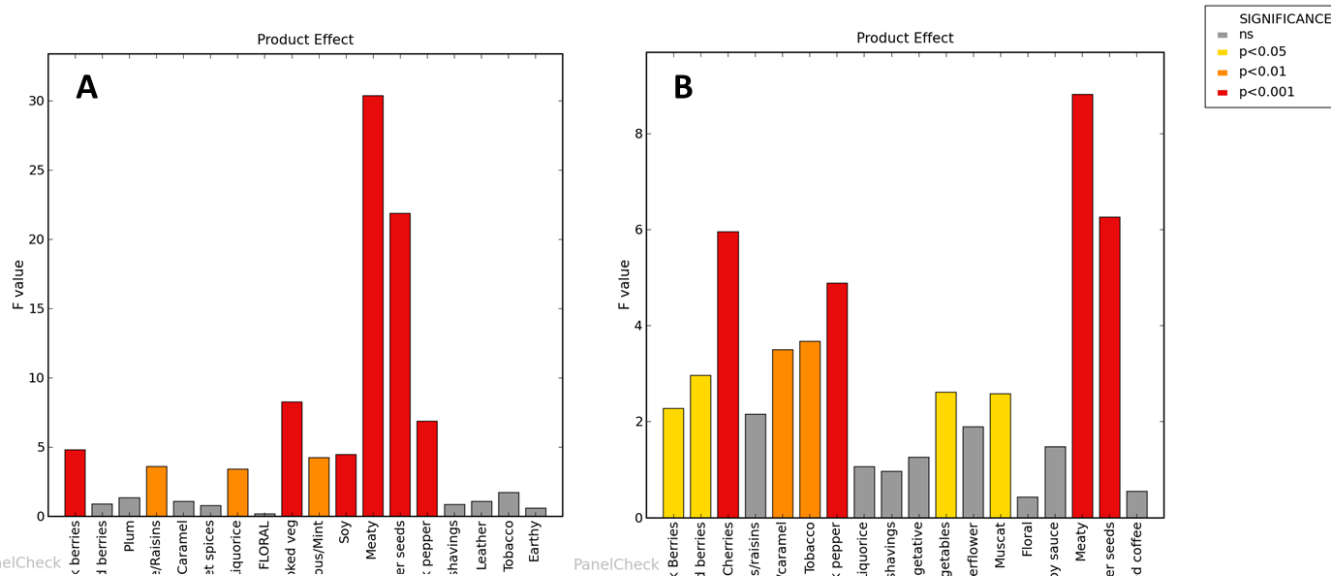
	F	p
Ripening	29.544	0.000
Time	15.418	0.000
Ripening*Time	2.592	0.000

Appendix Table 3.13. Univariate test of significance for the individual and group of phenolic compounds in 2015 wines made from grapes at different ripening levels (21ºBrix. 23ºBrix. 24ºBrix and 25ºBrix) during 18 months of bottle ageing.

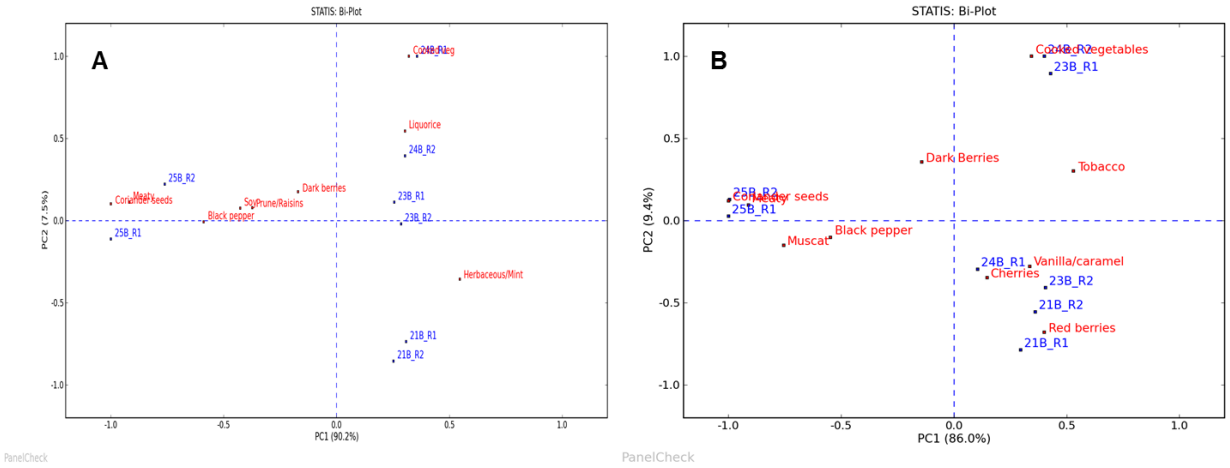
	Gallic acid F	Gallic acid p	Catechin F	Catechin p	B1 F	B1 p	Pol. phenols F	Pol. phenols p	Hydroxy. Ac. F	Hydroxy. Ac. p
Ripening	40.640	0.000	1.071	0.374	1.345	0.275	167.903	0.000	41.740	0.000
Time	112.610	0.000	2.061	0.106	1.689	0.174	1.665	0.180	51.730	0.000
Ripening * Time	3.540	0.002	4.383	0.000	3.655	0.001	4.648	0.000	1.430	0.198
	Flavonols F	**Flavonols p**	**Gluc. anth. F**	**Gluc. anth. p**	**Acyl. anth. F**	**Acyl. anth. p**	**Coum. anth. F**	**Coum. anth. p**	**Pol. pigment F**	**Pol. Pigment p**
Ripening	7.693	0.000	58.245	0.000	64.849	0.000	54.959	0.000	64.590	0.000
Time	66.120	0.000	106.509	0.000	148.839	0.000	151.668	0.000	0.945	0.449
Ripening * Time	1.596	0.137	8.461	0.000	11.426	0.000	13.101	0.000	2.568	0.014



Appendix Figure 3.3. Changes occurred over time in the concentration of malvidine-3-glucoside (mg/L) and polymer pigments (mg/L) in all 2015 wines made from different grape ripening levels (21ºBrix. 23ºBrix. 24ºBrix and 25ºBrix) during 18 months of bottle ageing. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.



Appendix Figure 3.4. Significant aroma attributes for 2015 wines made from different ripening levels (21ºBrix. 23ºBrix. 24ºBrix and 25ºBrix) after 6 months (A) and 12 months (B) of bottle ageing.



Appendix Figure 3.5. Bi-plots with the sample distribution of 2015 wine made from different ripening levels (21ºBrix. 23ºBrix. 24ºBrix and 25ºBrix) after 6 (A) and 12 months (B) of bottle ageing. Sample distribution is shown according to the corresponding significant aroma attributes.

Appendix Table 3.14. Test of significance (Test of SS Whole model vs SS residual) for the taste and mouth-feel attributes in 2015 wines after 6 months of storage.

	Acidity		Sweetness		Body		Alcohol burn		Astringency		Bitterness	
	F	p	F	p	F	p	F	p	F	p	F	p
Ripening	2.976	0.059	3.726	0.030	20.379	0.000	47.244	0.000	14.592	0.000	29.788	0.000
Time	1.424	0.278	9.352	0.022	2.644	0.155	0.011	0.918	0.000	0.988	0.000	0.987
Ripening*Time	14.400	0.000	9.919	0.000	2.212	0.122	2.599	0.084	3.664	0.032	2.671	0.078

APPENDIX CHAPTER 4

Appendix Table 4.1. Multivariate test of significance (Wilk test) for the monosaccharide composition of fresh grapes from three ripening levels (21ºBrix. 23ºBrix and 25ºBrix) during two consecutive seasons (2015 and 2016).

	F	p
Ripening	6.28	0.146
Year	92.42	0.080
Ripening*Year	19.70	0.049

Appendix Table 4.2. Multivariate test of significance (Wilk test) for the overall monosaccharide composition of fresh grapes from three ripening levels (21ºBrix. 23ºBrix and 25ºBrix) in 2015.

2015	F	p
Ripening	31.238	0.031

Appendix Table 4.3. Multivariate test of significance (Wilk test) for the overall monosaccharide composition of fresh grapes from three ripening levels (21ºBrix. 23ºBrix and 25ºBrix) in 2016.

2016	F	p
Ripening	6.891	0.133

Appendix Table 4.4. Multivariate test of significance (Wilks test) for the CoMPP epitopes extracted in CDTA. Data comprising the analysis in fresh grapes from three ripening levels (21ºBrix. 23ºBrix and 25ºBrix) during two consecutive seasons (2015 and 2016).

	F	p
Ripening	3.905E+13	0.000
Year	4.829E+26	0.000
Ripening*Year	4.611E+13	0.000

Appendix Table 4.5. Multivariate test of significance (Wilks test) for the CoMPP epitopes extracted in NaOH. Data comprising the analysis in fresh grapes from three ripening levels (21ºBrix. 23ºBrix and 25ºBrix) during two consecutive seasons (2015 and 2016).

	F	p
Ripening	61.350	0.000
Year	242.012	0.000
Ripening*Year	68.786	0.000

Appendix Table 4.6. Multivariate test of significance (Wilks test) for the phenolic parameters measured in the "soft extraction" in fresh grapes from three ripening levels (21ºBrix. 23ºBrix and 25ºBrix) during two consecutive seasons (2015 and 2016).

	F	p
Ripening	7.477	0.000
Year	12.030	0.001
Ripening*Fermentation stage	9.367	0.000

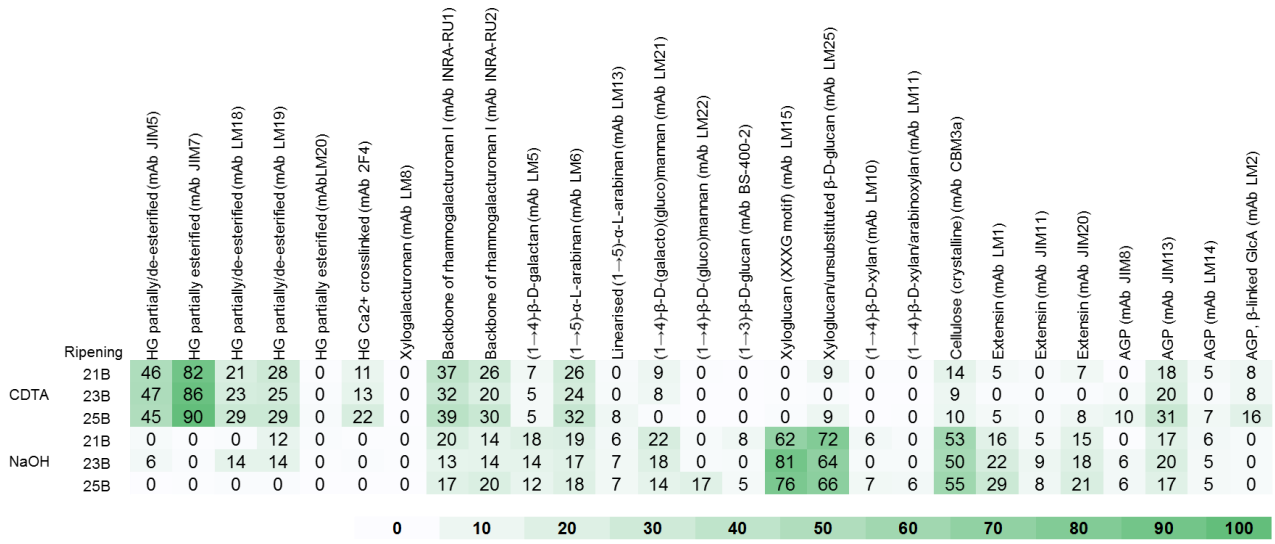
Appendix Table 4.7. Multivariate test of significance (Wilks test) for individual phenolic compounds measured with HPLC in fresh grapes from three ripening levels (21ºBrix. 23ºBrix and 25ºBrix) during two consecutive seasons (2015 and 2016).

	F	p
Ripening	4.633	0.033
Year	14.875	0.024
Ripening*Year	5.075	0.026

APPENDIX CHAPTER 5

Appendix Table 5.1. Multivariate test of significance (Wilk test) for the monosaccharide composition of fermenting pomace of Shiraz grapes from three different ripening levels.

	F	p
Ripening	1.7	0.080
Fermentation stage	5.3	0.000
Ripening*Fermentation stage	1.5	0.025



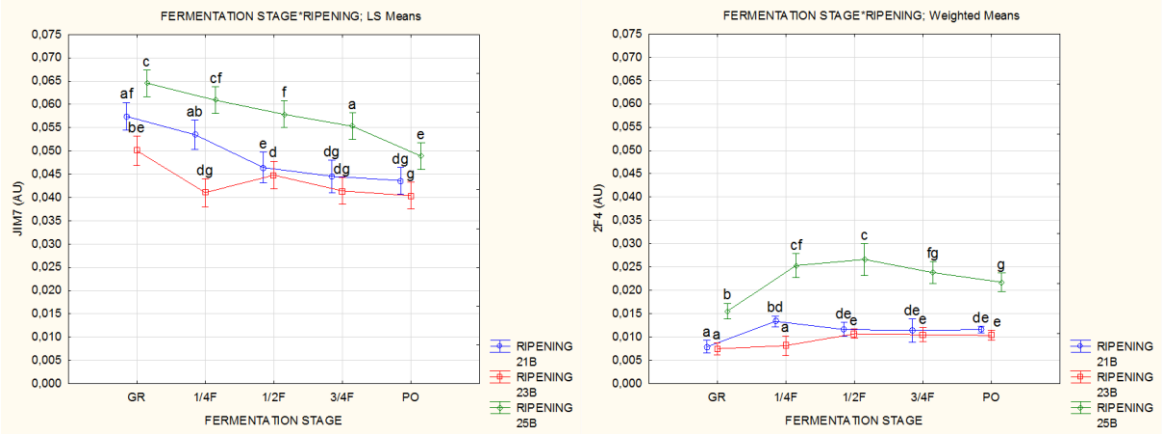
Appendix Figure 5.1. Heatmap of the epitope abundance (CDTA and NaOH extract) of fresh grapes from three grape ripening levels (21. 23 and 25Brix). The values are average of three biological repeats. Values lower than 5 are indicated as 0.

Appendix Table 5.2. Multivariate test of significance (Wilks test) for the CoMPP epitopes extracted in CDTA. Data comprising the analysis in fresh grapes from three different ripening levels (21ºBrix. 23ºBrix and 25ºBrix) and the evolution of their fermented skins at different stages of the alcoholic fermentation (¼F. (½ F. ¾ F and AF).

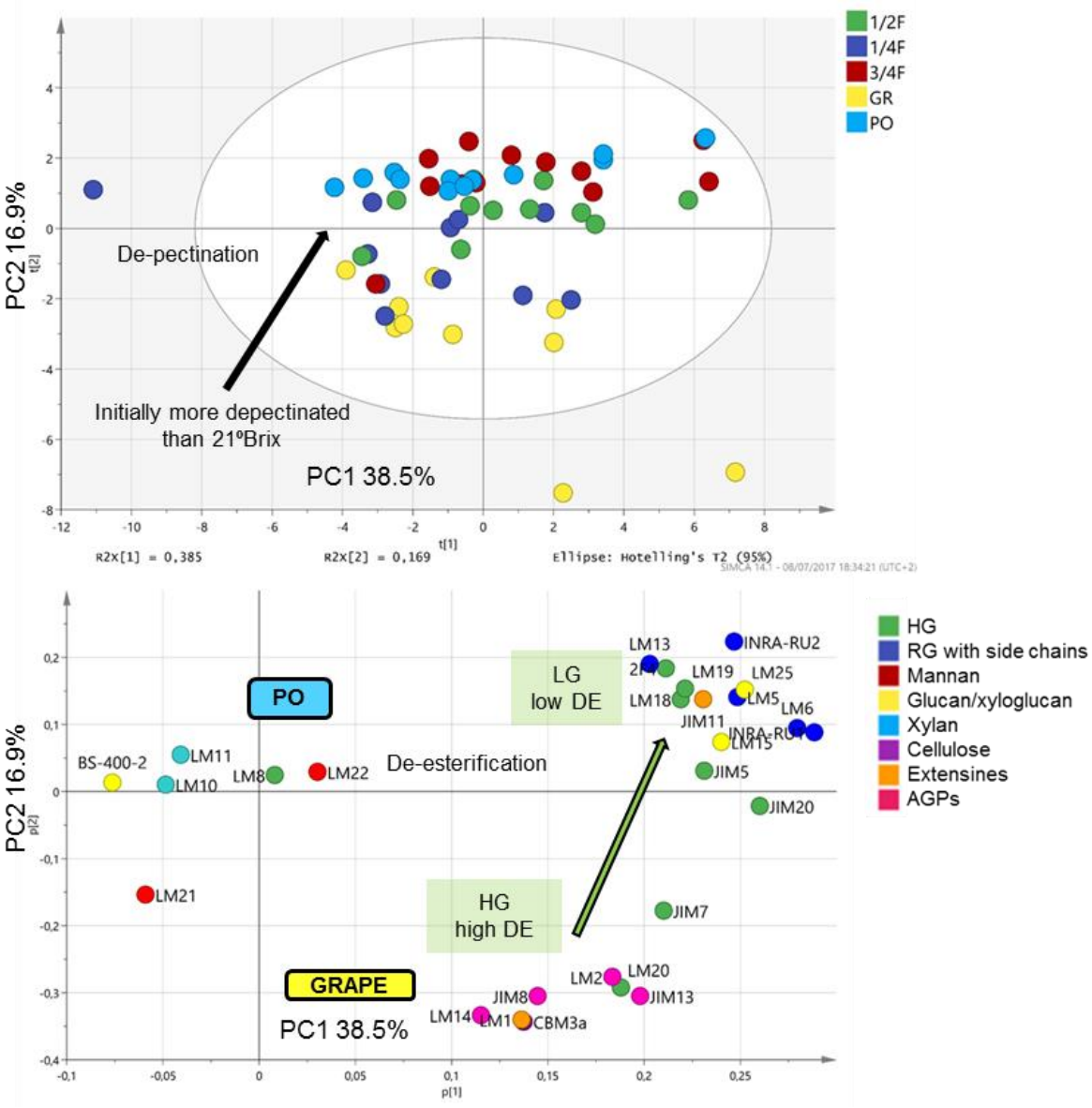
	F	p
Ripening	100.493	0.000
Fermentation stage	7.570	0.000
Ripening*Fermentation stage	3.764	0.000

Appendix Table 5.38. Multivariate test of significance (Wilks test) for the CoMPP epitopes extracted in NaOH. Data consists of analysis in fresh grapes from three different ripening levels (21ºBrix. 23ºBrix and 25ºBrix) and the evolution of their fermented skins at different stages of the alcoholic fermentation (¼F. (½ F. ¾ F and AF).

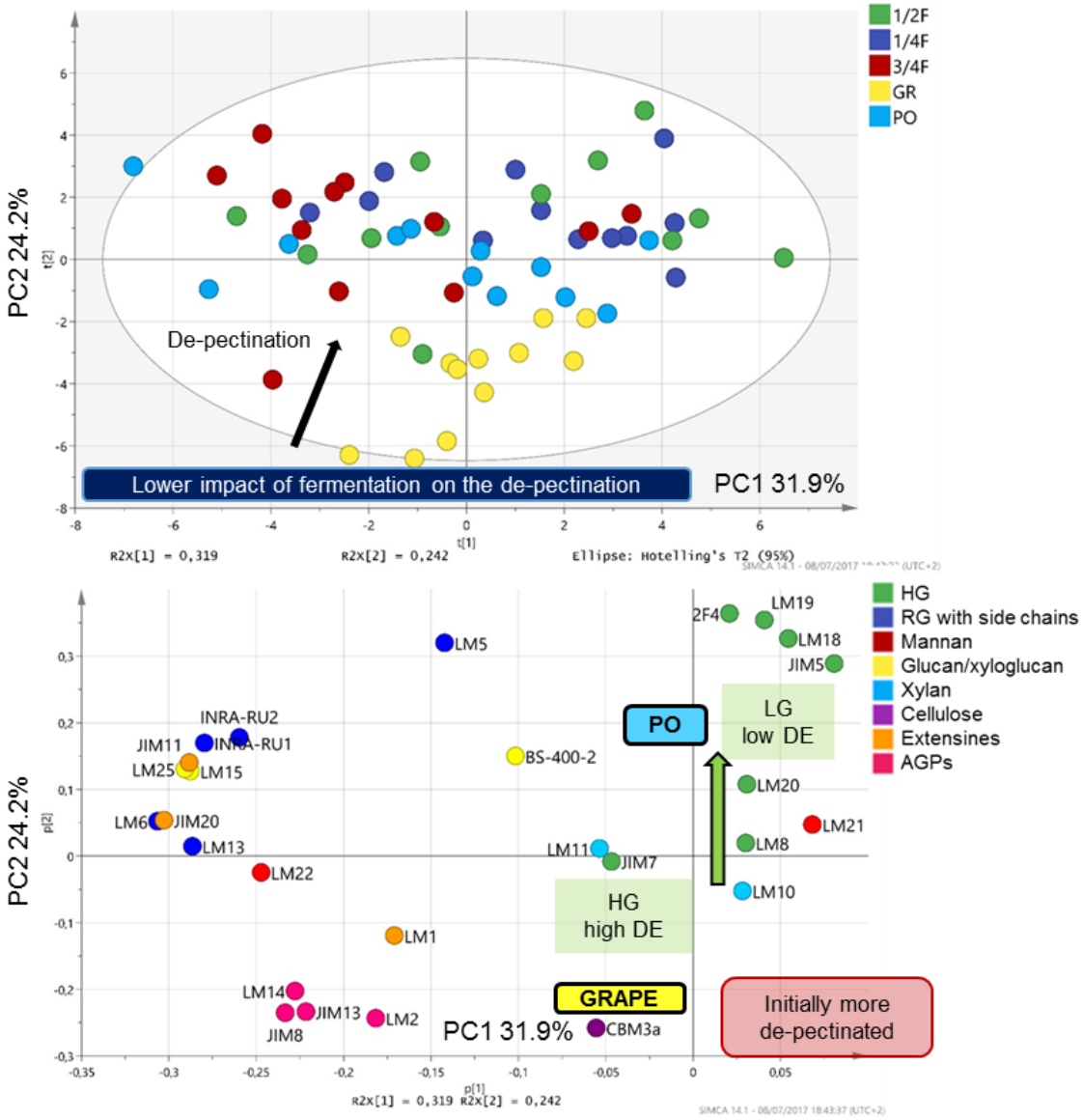
	F	p
Ripening	457.879	0.000
Fermentation stage	11.199	0.000
Ripening*Fermentation stage	4.819	0.000



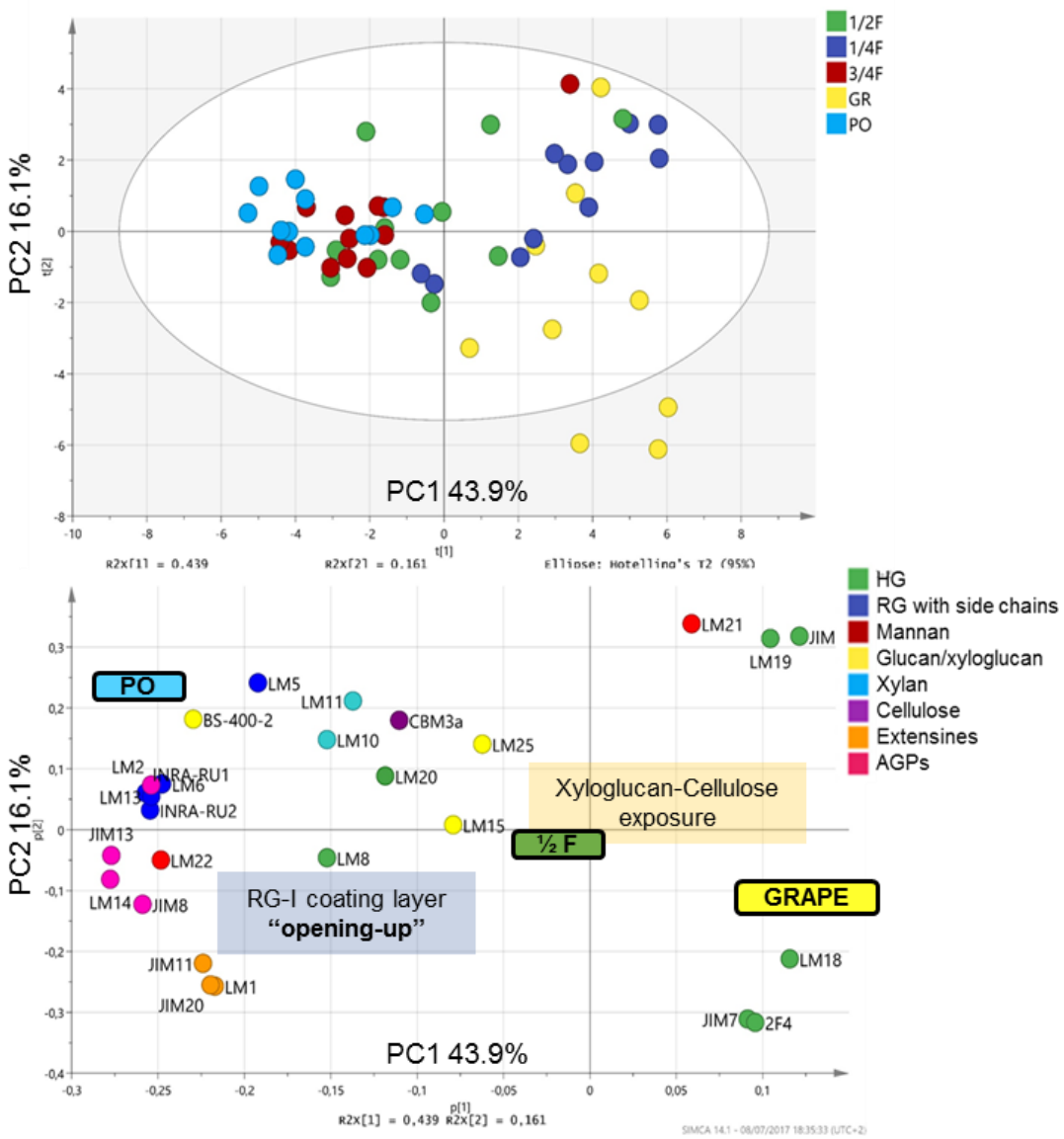
Appendix Figure 5.2. Evolution of the extraction of JIM7 and 2F4 epitopes in CDTA extract in the grape pomace during alcoholic fermentation of Shiraz grape from three ripening levels during fermentation. Values are displayed in absorbance units (AU). The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments (ripening and fermentation stage).



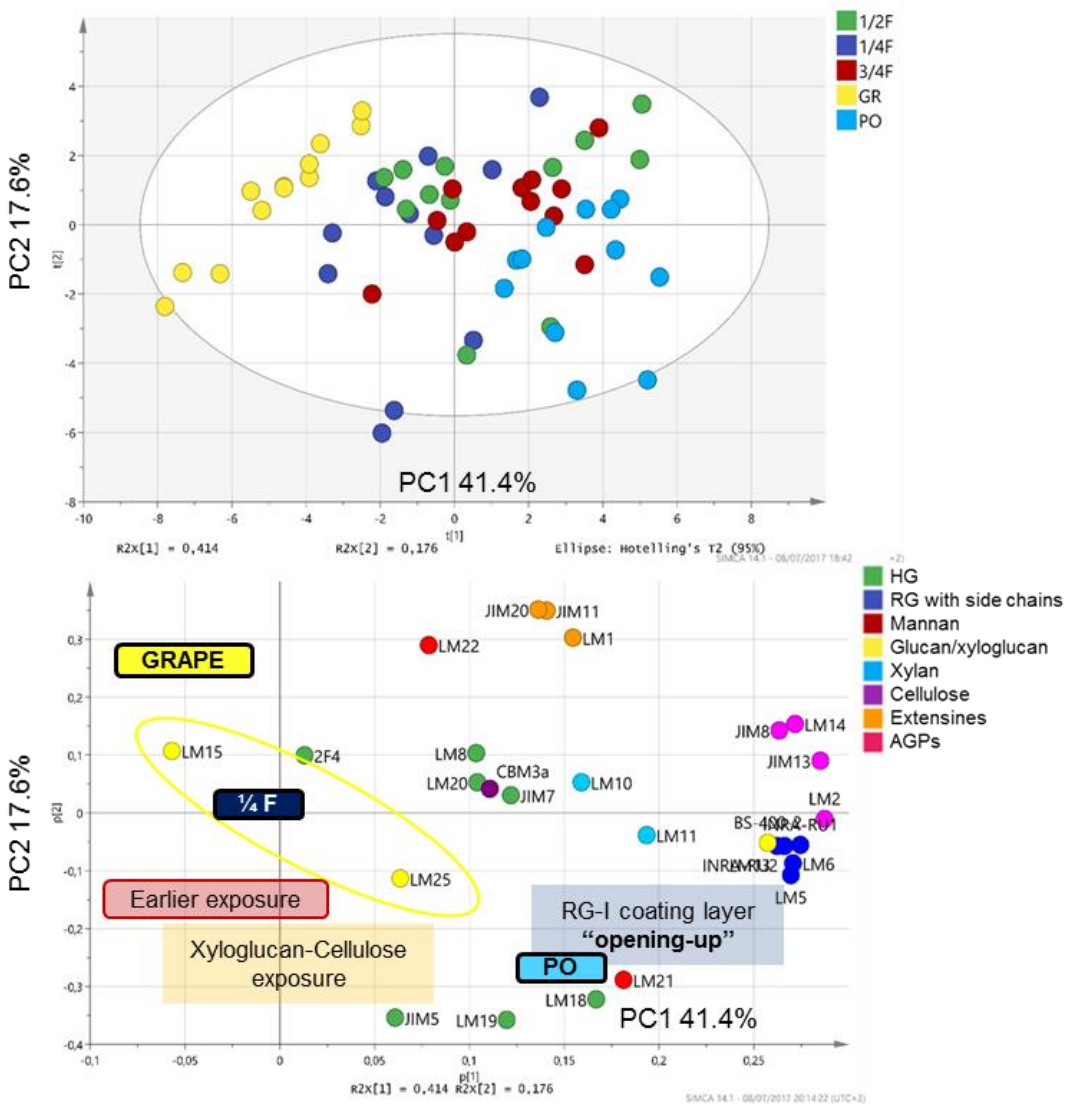
Appendix Figure 5.3. PCA score and loading score with the CoMPP results of the CDTA extract (pectin rich) of 23ºBrix treatment. The samples are coloured according to the alcoholic fermentation stage (GR. ¼ F. ½ F. ¾ F and PO – fermented pomace) and the epitope category (loading plot). The following abbreviation represents: Homogalacturonans (HG). rhamnogalacturonan-I (RG) and arabinogalactoproteins (AGPs).



Appendix Figure 5.4. PCA score and loading score with the CoMPP results of the CDTA extract (pectin rich) of the 25ºBrix treatment. The samples are coloured according to the alcoholic fermentation stage (GR. ¼ F. ½ F. ¾ F and PO – fermented pomace) and the epitope category (loading plot).



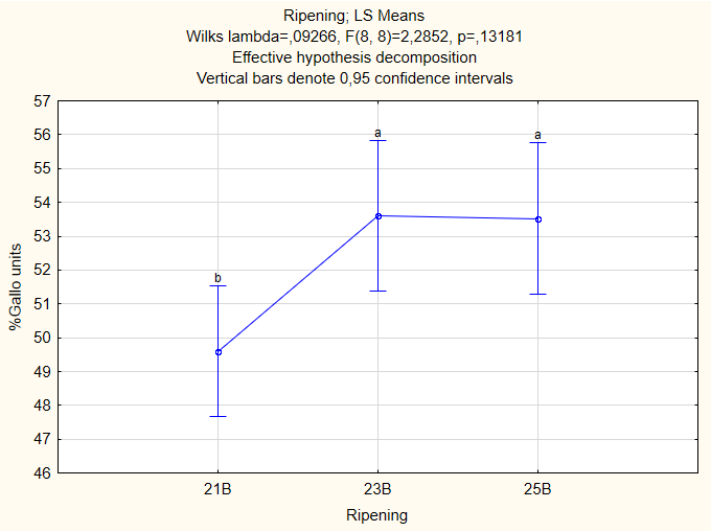
Appendix Figure 5.5. PCA score and loading score with the CoMPP results of the NaOH extract (hemicellulose rich) of the 23ºBrix treatment. The samples are coloured according to the alcoholic fermentation stage (GR. ¼ F. ½ F. ¾ F and PO – fermented pomace) and the epitope category (loading plot).



Appendix Figure 5.6. PCA score and loading score with the CoMPP results of the NaOH extract (hemicellulose rich) of the 25ºBrix treatment. The samples are coloured according to the alcoholic fermentation stage (GR. ¼ F. ½ F. ¾ F and PO – fermented pomace) and the epitope category (loading plot).

Appendix Table 5.49. Multivariate test of significance (Wilks test) for the phloroglucinolysis analysis performed on the fermenting pomace of Shiraz grape from three different ripening levels.

	F	p
Ripening	2.923	0.001
Fermentation stage	4.658	0.000
Ripening*Fermentation stage	2.071	0.000



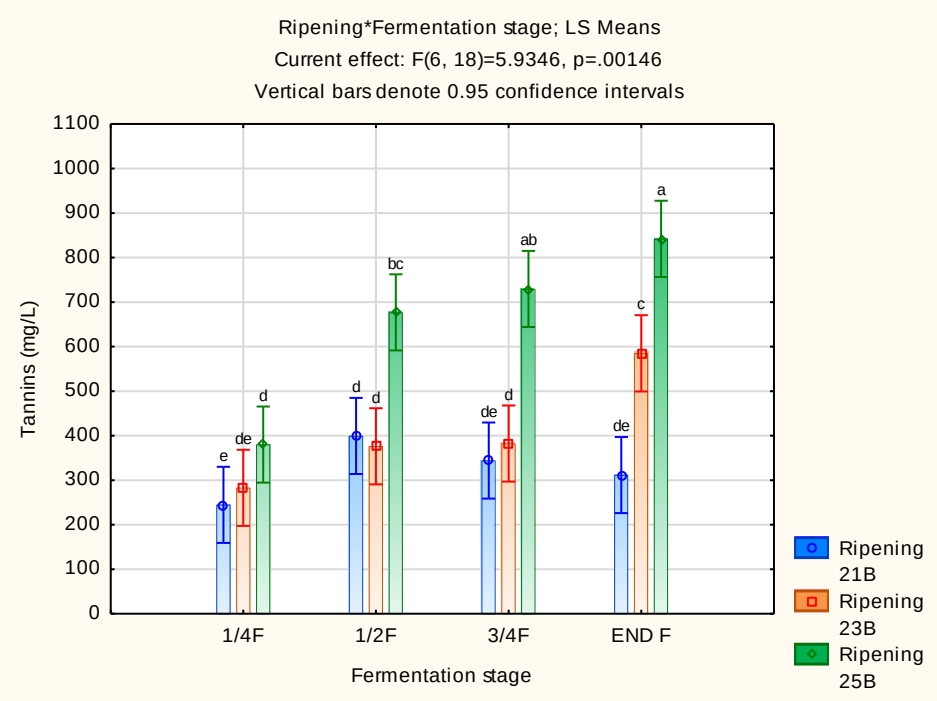
Appendix Figure 5.7. % Gallo units in fresh Shiraz grapes from three different ripening levels (21ºBrix. 23ºBrix and 25ºBrix). The different letters indicate significant differences (ANOVA, $p < 0.05$) between the ripening levels.

Appendix Table 5.5. Evolution of the extension and terminal subunits during fermentation of Shiraz grapes for three ripening levels (21ºBrix. 23ºBrix and 25ºBrix). The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments (ripening and fermentation stage).

		21ºBrix					23ºBrix					25ºBrix				
		GR	**¼ F**	**½ F**	**¾ F**	**PO**	**GR**	**¼ F**	**½ F**	**¾ F**	**PO**	**GR**	**¼ F**	**½ F**	**¾ F**	**PO**
Extension units	Average	3.8 ^{ab}	3.0 ^{cb}	2.3 ^{ced}	1.3 ^{e}	1.8 ^{ef}	4.2 ^{a}	3.4 ^{abd}	2.1 ^{ce}	2.7 ^{cdf}	2.0 ^{ce}	3.9 ^{ab}	3.0 ^{cb}	2.5 ^{cdfg}	2.0 ^{ce}	1.5 ^{eg}
	St. dv	0.6	0.2	1.4	0.1	0.2	0.1	0.1	0.1	0.3	0.3	0.5	0.1	0.3	0.1	0.1
EGC-P%	Average	49.6 ^{ab}	50.3 ^{ab}	42.7^{fdg}	38.3 ^{hg}	42.6 ^{fe}	53.6 ^{a}	47.1 ^{cbd}	42.7 ^{fdg}	42.3 ^{feg}	38.6 ^{fh}	53.5 ^{a}	48.3 ^{cb}	44.7 ^{cde}	40.1 ^{fh}	35.8 ^{h}
	St. dv	0.5	1.5	4.9	3.6	1.0	1.3	0.3	1.0	1.3	1.8	1.0	0.3	0.8	1.3	1.0
C-P%	Average	4.4 ^{cdb}	1.8 ^{cd}	4.1 ^{cdb}	10.2 ^{a}	7.0 ^{ab}	1.8 ^{d}	2.4 ^{cd}	3.5 ^{cd}	3.2 ^{cd}	4.1 ^{cdb}	1.8 ^{d}	2.6 ^{cd}	3.1 ^{cd}	4.4 ^{cdb}	5.6 ^{cb}
	St. dv	2.0	0.2	1.7	1.0	2.5	0.1	0.0	0.2	0.2	0.4	0.1	0.2	0.3	0.5	0.3
EC-P%	Average	42.0 ^{e}	43.5 ^{ed}	48.1 ^{ad}	46.6 ^{db}	46.1 ^{dc}	41.2 ^{e}	45.8 ^{dc}	48.8 ^{abc}	49.4 ^{abc}	51.6 ^{a}	41.0 ^{e}	44.7 ^{de}	47.6 ^{db}	50.1 ^{ab}	52.2 ^{a}
	St. dv	1.4	1.3	2.8	2.2	1.5	1.2	0.4	0.6	1.0	2.2	0.7	0.1	0.3	0.6	0.8
ECG-P%	Average	4.0 ^{ef}	4.4 ^{edg}	5.1 ^{bcd}	5.0 ^{bcd}	4.2 ^{eg}	3.4 ^{f}	4.7 ^{be}	5.0 ^{bcd}	5.1 ^{bcd}	5.7 ^{ac}	3.7 ^{fg}	4.5 ^{ed}	4.6 ^{ed}	5.4 ^{bc}	6.4 ^{a}
	St. dv	0.2	0.5	0.4	0.6	0.3	0.0	0.1	0.2	0.2	0.1	0.2	0.2	0.3	0.3	0.2
Terminal units	Average	0.3 ^{ab}	0.3 ^{acb}	0.2 ^{c}	0.2 ^{cd}	0.3 ^{ac}	0.4 ^{e}	0.4 ^{e}	0.3 ^{ab}	0.3 ^{b}	0.2 ^{cdf}	0.3 ^{abd}	0.3 ^{abf}	0.3 ^{abd}	0.3 ^{ab}	0.2 ^{cd}
	St. dv	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
C %	Average	28.9 ^{bd}	30.7 ^{bd}	49.8 ^{a}	37.5 ^{b}	27.9 ^{bd}	26.2 ^{dc}	22.3 ^{d}	26.4 ^{dc}	28.3 ^{bd}	36.3 ^{bc}	32.8 ^{bd}	25.5 ^{dc}	30.4 ^{bd}	33.3 ^{bc}	37.8 ^{ab}
	St. dv	2.5	1.9	2.7	7.4	1.7	4.2	1.1	1.1	1.3	2.9	3.5	2.0	2.6	5.7	6.9
EC %	Average	3.5 ^{e}	3.4 ^{ecd}	3.8 ^{eb}	6.8 ^{a}	6.7 ^{a}	2.9 ^{e}	5.5 ^{abc}	6.7 ^{a}	6.8 ^{a}	5.9 ^{abd}	3.0 ^{ec}	4.6 ^{ae}	4.4 ^{ae}	6.5 ^{ab}	5.5 ^{abc}
	St. dv	0.5	0.3	1.3	1.4	0.8	0.5	0.7	0.6	0.6	1.2	0.7	0.5	0.7	0.1	1.7
ECG %	Average	0.7 ^{bc}	0.7 ^{bdc}	0.2 ^{d}	0.5 ^{bde}	0.7 ^{bcf}	1.5 ^{a}	1.4 ^{a}	0.9 ^{c}	0.5 ^{bd}	0.3 ^{df}	0.5 ^{bdc}	0.8 ^{bc}	0.8 ^{bc}	0.8 ^{ce}	0.6 ^{bdc}
	St. dv	0.1	0.2	0.1	0.0	0.0	0.1	0.2	0.0	0.2	0.0	0.1	0.2	0.2	0.1	0.2

Appendix Table 5.10. Evolution of the CIELab parameters (L*. a*. b*. Cab* and hab*) during the alcoholic fermentation of Shiraz grapes with three ripening levels (21ºBrix. 23ºBrix and 25ºBrix). The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments (ripening and fermentation stage).

		21ºBrix	23ºBrix	25ºBrix
.L*	¼ F	19.38±1.13 ^{a}	15.83 ±3.40 ^{bc}	15.32 ±1.65 ^{bc}
	½ F	14.09±0.57 ^{bcd}	9.90 ±0.58 ^{ef}	9.48 ±0.26 ^{efg}
	¾ F	12.51±2.67 ^{cde}	6.18 ±2.03 ^{gh}	11.93 ±1.96 ^{de}
	AF	16.25 ±1.38 ^{ab}	5.34 ±2.24 ^{h}	7.31 ±2.67 ^{fgh}
a*	¼ F	51.67±0.98 ^{a}	47.65 ±3.55 ^{abc}	47.39 ±1.88 ^{abc}
	½ F	46.00±0.38 ^{bc}	40.71 ±0.61 ^{de}	40.20 ±0.18 ^{de}
	¾ F	43.92±4.27 ^{cd}	35.65 ±3.16 ^{f}	43.93 ±2.41 ^{cd}
	AF	48.66 ±1.52 ^{ab}	34.54 ±3.57 ^{f}	37.45 ±4.08 ^{ef}
b*	¼ F	35.86±2.47 ^{bcd}	34.19 ±1.46 ^{d}	34.76 ±4.53 ^{cd}
	½ F	39.11±1.73 ^{abc}	36.79 ±1.27 ^{bcd}	36.81 ±2.11 ^{bcd}
	¾ F	38.58±3.36 ^{abcd}	34.18 ±3.03 ^{d}	39.73 ±0.87 ^{ab}
	AF	42.16 ± 1.76 ^{a}	34.57 ±3.55 ^{cd}	37.01 ±3.54 ^{bcd}
C_{ab}*	¼ F	62.92±1.60 ^{ab}	58.69 ±2.65 ^{bcd}	58.86 ±2.82 ^{abcd}
	½ F	60.39±1.31 ^{abc}	54.88 ±0.39 ^{cde}	54.52 ±1.30 ^{de}
	¾ F	58.46±5.31 ^{bcd}	49.39 ±4.38 ^{ef}	59.26 ±1.34 ^{abcd}
	AF	64.38±2.22 ^{a}	48.87 ±5.03 ^{f}	52.65 ±5.35 ^{ef}
H_{ab}*	¼ F	34.74±1.94 ^{f}	35.72 ±2.68 ^{f}	36.17 ±3.96 ^{f}
	½ F	40.35±1.12 ^{e}	42.10 ±1.42 ^{cde}	42.45 ±1.77 ^{abcde}
	¾ F	41.32±1.15 ^{de}	43.79 ±0.26 ^{abcd}	42.15 ±2.10 ^{bde}
	AF	40.90 ±0.63 ^{de}	45.03 ±0.19 ^{ab}	44.69 ±0.79 ^{ac}



Appendix Figure 5.8. MCP tannins (mg/L) extracted during the alcoholic fermentation of Shiraz grapes with three different grape ripening levels. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments (ripening and fermentation stage).

APPENDIX CHAPTER 6

Appendix Table 6.1. Fixed effect test for significance analysis of the wine extracts phenolic parameters (TP. tannin concentration. TRP and copigments).

	F	p
TP (AU)		
Treatment	121.552	0.000
Ratio	400.067	0.000
TIME	15.088	0.001
Treatment*Ratio	6.334	0.005
Treatment*TIME	0.974	0.410
Ratio*TIME	19.434	0.000
Treatment*Ratio*TIME	1.029	0.438
Tannins (mg/L)		
Treatment	68.951	0.000
Ratio	318.603	0.000
TIME	20.512	0.000
Treatment*Ratio	11.091	0.000
Treatment*TIME	17.671	0.000
Ratio*TIME	7.797	0.004
Treatment*Ratio*TIME	4.322	0.027
TRP (AU)		
Treatment	63.072	0.000
Ratio	21.536	0.000
TIME	317.065	0.000
Treatment*Ratio	8.447	0.001
Treatment*TIME	9.487	0.005
Ratio*TIME	23.234	0.000
Treatment*Ratio*TIME	6.595	0.007
Copigments		
Treatment	10.682	0.002
Ratio	23.567	0.000
TIME	25.337	0.000
Treatment*Ratio	3.006	0.063
Treatment*TIME	42.085	0.000
Ratio*TIME	2.703	0.092
Treatment*Ratio*TIME	5.820	0.011

Appendix Table 6.2. Fixed effect test for significance analysis of the wine extracts colour components (CD. 420nm. 520nm. 620nm and Hue).

	F	p
CD (AU)		
Treatment	46.141	0.000
Ratio	12.868	0.000
TIME	19.074	0.000
Treatment*Ratio	2.743	0.079
Treatment*TIME	36.288	0.000
Ratio*TIME	2.953	0.075
Treatment*Ratio*TIME	3.173	0.063
420 nm		
Treatment	170.411	0.000
Ratio	8.266	0.001
TIME	9.893	0.004
Treatment*Ratio	3.967	0.028
Treatment*TIME	28.493	0.000
Ratio*TIME	1.130	0.395
Treatment*Ratio*TIME	2.563	0.103
520 nm		
Treatment	0.011	0.913
Ratio	32.782	0.000
TIME	32.815	0.000
Treatment*Ratio	3.045	0.061
Treatment*TIME	55.152	0.000
Ratio*TIME	5.589	0.012
Treatment*Ratio*TIME	4.371	0.026
620 nm		
Treatment	0.166	0.686
Ratio	6.769	0.003
TIME	25.030	0.000
Treatment*Ratio	1.218	0.308
Treatment*TIME	31.764	0.000
Ratio*TIME	6.069	0.009
Treatment*Ratio*TIME	3.044	0.069
Hue		
Treatment	3033.660	0.000
Ratio	397.872	0.000
TIME	58.687	0.000
Treatment*Ratio	90.326	0.000
Treatment*TIME	132.019	0.000
Ratio*TIME	21.279	0.000
Treatment*Ratio*TIME	4.644	0.022

Appendix Table 6.3. Colour and phenolic results over time of the three different anthocyanin/tannin ratios in the presence or absence of oxygen. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

		CONTROL			OXYGEN		
		SK	**SKSD**	**SK4SD**	**SK**	**SKSD**	**SK4SD**
.420 nm (AU)	3M	3.02 ± 0.27^{ef}	4.07 ± 0.33^{abcd}	3.78 ± 0.43^{cd}	4.02 ± 0.22^{bcd}	3.98 ± 0.19^{bcd}	3.80 ± 0.28^{cd}
	6M	2.58 ± 0.13^{f}	2.84 ± 0.06^{ef}	2.81 ± 0.11^{ef}	4.12 ± 0.17^{abc}	4.51 ± 0.42^{a}	4.26 ± 0.41^{ab}
	9M	2.89 ± 0.26^{ef}	3.19 ± 0.12^{e}	2.92 ± 0.05^{ef}	3.90 ± 0.09^{bcd}	4.01 ± 0.26^{bcd}	3.65 ± 0.23^{d}
520 nm (AU)	3M	2.07 ± 0.20^{cdef}	3.00 ± 0.25^{a}	3.12 ± 0.37^{a}	1.90 ± 0.14^{efg}	2.06 ± 0.09^{cde}	2.28 ± 0.18^{cd}
	6M	1.70 ± 0.09^{g}	1.91 ± 0.04^{efg}	2.00 ± 0.07^{defg}	1.99 ± 0.05^{defg}	2.32 ± 0.20^{c}	2.61 ± 0.25^{b}
	9M	1.77 ± 0.15^{fg}	2.03 ± 0.11^{def}	1.86 ± 0.05^{efg}	2.00 ± 0.05^{defg}	2.11 ± 0.17^{cde}	2.13 ± 0.13^{cde}
620 nm (AU)	3M	0.56 ± 0.10^{cdef}	0.97 ± 0.16^{a}	0.96 ± 0.27^{a}	0.52 ± 0.06^{cdef}	0.58 ± 0.03^{bcdef}	0.71 ± 0.08^{b}
	6M	0.48 ± 0.03^{def}	0.52 ± 0.01^{cdef}	0.52 ± 0.02^{cdef}	0.56 ± 0.03^{cdef}	0.66 ± 0.07^{bc}	0.73 ± 0.08^{b}
	9M	0.46 ± 0.07^{ef}	0.55 ± 0.10^{cdef}	0.43 ± 0.04^{f}	0.58 ± 0.03^{bcdef}	0.63 ± 0.06^{bcd}	0.59 ± 0.07^{cde}
CD (AU)	3M	5.65 ± 0.56^{efg}	8.04 ± 0.73^{a}	7.86 ± 1.06^{a}	6.45 ± 0.41^{cde}	6.63 ± 0.30^{c}	6.79 ± 0.53^{bc}
	6M	4.76 ± 0.24^{g}	5.26 ± 0.11^{fg}	5.33 ± 0.20^{fg}	6.67 ± 0.25^{bcd}	7.48 ± 0.68^{ab}	7.60 ± 0.74^{a}
	9M	5.13 ± 0.48^{fg}	5.76 ± 0.33^{def}	5.21 ± 0.14^{fg}	6.49 ± 0.16^{cde}	6.76 ± 0.48^{bc}	6.36 ± 0.43^{cde}
Hue	3M	1.46 ± 0.03^{g}	1.36 ± 0.00^{h}	1.21 ± 0.01^{i}	2.11 ± 0.04^{a}	1.93 ± 0.02^{bc}	1.67 ± 0.02^{de}
	6M	1.52 ± 0.00^{g}	1.48 ± 0.01^{g}	1.41 ± 0.01^{h}	2.07 ± 0.03^{a}	1.94 ± 0.06^{bc}	1.63 ± 0.02^{e}
	9M	1.63 ± 0.02^{e}	1.57 ± 0.03^{f}	1.57 ± 0.02^{f}	1.95 ± 0.00^{b}	1.90 ± 0.06^{c}	1.71 ± 0.01^{d}
TRP (AU)	3M	4.66 ± 0.23^{de}	5.78 ± 0.34^{c}	5.65 ± 0.24^{c}	4.91 ± 0.27^{d}	6.46 ± 0.23^{b}	8.10 ± 0.80^{a}
	6M	3.47 ± 0.28^{hij}	3.70 ± 0.32^{ghi}	3.78 ± 0.19^{fghi}	3.38 ± 0.27^{hij}	4.29 ± 0.28^{ef}	3.89 ± 0.42^{fgh}
	9M	3.30 ± 0.12^{ij}	2.98 ± 0.01^{jk}	2.66 ± 0.47^{k}	3.85 ± 0.27^{fghi}	4.08 ± 0.27^{fg}	3.61 ± 0.18^{ghu}
TP (AU)	3M	19.99 ± 0.43^{k}	27.03 ± 1.36^{gh}	37.15 ± 1.66^{bc}	23.16 ± 1.04^{ij}	31.31 ± 1.08^{ef}	44.45 ± 1.87^{a}
	6M	21.12 ± 1.46^{jk}	25.10 ± 2.72^{hi}	32.78 ± 0.71^{de}	22.90 ± 2.42^{ij}	31.44 ± 1.56^{ef}	38.92 ± 1.47^{b}
	9M	22.69 ± 1.57^{ijk}	24.97 ± 1.26^{hi}	30.66 ± 2.16^{ef}	25.06 ± 0.92^{hi}	29.38 ± 1.31^{fg}	34.91 ± 0.70^{cd}
Tannins (mg/L)	3M	475.48 ± 26.52^{ijk}	792.58 ± 40.32^{ef}	1079.68 ± 30.71^{b}	345.23 ± 62.78^{k}	818.72±19.28^{def}	1408.07± 166.20^{a}
	6M	498.51 ± 44.79^{hij}	623.94 ± 100.19^{gh}	923.53 ± 142.67^{cde}	706.17 ± 63.79^{fg}	951.26 ± 48.09^{bc}	1424.69 ± 87.92^{a}
	9M	402.36 ± 118.30^{jk}	616.26 ± 33.00^{ghi}	938.42 ± 27.98^{bcd}	519.34 ± 65.81^{hij}	711.19 ± 44.63^{fg}	1012.46 ± 75.25^{bc}

Appendix Table 6.4. Univariate test of significance for the CIELab parameters of the different A/T treatments.

	F	p
L*		
Ratio	0.96	0.392
Treatment	52.12	0.000
Time	62.33	0.000
Ratio*Treatment	0.37	0.691
Ratio*Time	2.97	0.033
Treatment*Time	3.11	0.057
Ratio*Treatment*Time	2.40	0.068
a*		
Ratio	5.25	0.010
Treatment	3.39	0.074
Time	4.37	0.020
Ratio*Treatment	0.82	0.448
Ratio*Time	1.81	0.149
Treatment*Time	2.88	0.069
Ratio*Treatment*Time	0.52	0.720
b*		
Ratio	17.82	0.000
Treatment	10.88	0.002
Time	70.17	0.000
Ratio*Treatment	13.79	0.000
Ratio*Time	4.14	0.007
Treatment*Time	2.88	0.069
Ratio*Treatment*Time	1.03	0.404
C_{ab}*		
Ratio	14.62	0.000
Treatment	1.49	0.230
Time	23.63	0.000
Ratio*Treatment	8.84	0.001
Ratio*Time	1.41	0.249
Treatment*Time	0.16	0.852
Ratio*Treatment*Time	0.73	0.579
H_{ab}*		
Ratio	8.55	0.001
Treatment	27.81	0.000
Time	98.64	0.000
Ratio*Treatment	8.62	0.001
Ratio*Time	6.53	0.000
Treatment*Time	10.57	0.000
Ratio*Treatment*Time	1.22	0.320

Appendix Table 6.5. Evolution of the CIELab parameters (L*. a*. b* C_{ab}* and H_{ab}*) of the different A/T treatments over time. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments.

		CONTROL			OXYGEN		
		SK	**SKSD**	**SK4SD**	**SK**	**SKSD**	**SK4SD**
.L*	3M	34.46 ± 2.71^{de}	33.54 ± 0.82^{e}	29.24 ± 0.34^{f}	28.72 ± 3.73^{f}	27.17 ± 0.83^{f}	28.76 ± 0.79^{f}
	6M	41.24 ± 2.04^{ab}	37.87 ± 0.75^{bcd}	37.85 ± 0.56^{bcd}	37.08 ± 1.26^{bcde}	34.68 ± 0. 21de	36.43 ± 0.87^{cde}
	9M	39.51 ± 1.08^{bc}	40.30 ± 0.18^{b}	44.74 ± 0.53^{a}	34.14 ± 0.46^{de}	35.02 ± 0.32^{de}	35.06 ± 1.30^{de}
a*	3M	47.44 ± 1.48^{abcde}	50.33 ± 0.65^{a}	49.73 ± 0.78^{ab}	43.95 ± 2.83^{e}	48.14 ± 0.98^{abcd}	45.99 ± 2.04^{cde}
	6M	43.76 ± 3.20^{e}	48.75 ± 1.36^{abc}	48.33 ± 0.80^{abc}	45.32 ± 1.24^{cde}	46.46 ± 0.83^{bcde}	47.36 ± 0.70^{abcde}
	9M	44.61 ± 0.58^{de}	46.57 ± 1.08^{bcde}	44.56 ± 0.44^{de}	46.53 ± 0.48^{bcde}	46.11 ± 1.33^{bcde}	44.03 ± 0.67^{e}
b*	3M	40.34 ± 2.65^{i}	46.77 ± 2.15^{gh}	46.08 ± 0.72^{h}	48.65 ± 4.30^{fgh}	51.23 ± 0.91^{efg}	48.46 ± 0.82^{fgh}
	6M	49.16 ± 2.27^{efgh}	51.96 ± 1.01^{def}	58.50 ± 0.76^{bc}	56.12 ± 0.56^{bcd}	56.60 ± 0.65^{bcd}	54.46 ± 1.41^{cde}
	9M	48.98 ± 1.40^{fgh}	58.22 ± 1.36^{bc}	66.93 ± 0.47^{a}	56.49 ± 0.58^{bcd}	58.51 ± 0.99^{bc}	60.29 ± 2.46^{b}
C_{ab}*	3M	62.30 ± 2.79^{h}	68.76 ± 1.09^{defg}	67.80 ± 1.02^{efg}	65.58 ± 5.08^{gh}	70.30 ± 1.25^{cdefg}	66.82 ± 1.98^{fgh}
	6M	65.93 ± 0.43^{fgh}	71.25 ± 1.66^{bcdef}	75.88 ± 1.06^{ab}	72.16 ± 0.45^{bcde}	73.22 ± 1.01^{bcd}	72.19 ± 0.94^{bcde}
	9M	66.27 ± 1.00^{fgh}	74.58 ± 1.26^{bc}	80.41 ± 0.50^{a}	73.20 ± 0.37^{bcd}	74.52 ± 1.07^{bc}	74.67 ± 2.38^{bc}
H_{ab}*	3M	40.29 ± 1.12^{e}	42.85 ± 1.63^{e}	42.82 ± 0.23^{e}	47.79 ± 0.75^{d}	46.78 ± 0.40^{d}	46.54 ± 0.84^{d}
	6M	48.35 ± 3.39^{cd}	46.84 ± 0.25^{d}	50.44 ± 0.23^{bc}	51.09 ± 1.02^{bc}	50.63 ± 0.24^{bc}	48.97 ± 1.00^{cd}
	9M	47.65 ± 0.99^{d}	51.34 ± 0.92^{b}	56.34 ± 0.29^{a}	50.52 ± 0.51^{bc}	51.77 ± 0.97^{b}	53.82 ± 0.69^{a}