UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA, ALIMENTARIA Y
DE BIOSISTEMAS

Use of non-*Saccharomyces* yeasts to enhance the quality of red wines

Tesis Doctoral

Carlos Escott Pérez
Licenciado en Ciencias Químicas

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AGRADECIMIENTOS

Agradezco, en primer lugar, a mi tutor y director de tesis, Prof. Antonio Morata Barrado por la confianza depositada en mí al momento de aceptar dirigir este trabajo doctoral. Han sido tres años que me han dejado satisfacciones personales y profesionales y, además, me han devuelto la pasión por la investigación.

Dra. Laura Romero Robles, mi reconocimiento a la profesional que eres. Mi cariño y agradecimiento por siempre darme un voto a favor.

Agradezco al CONACYT México por la beca otorgada para la realización de este doctorado. Sin ella no habría sido capaz de realizar este logro personal.

Agradezco a los profesores del Departamento de Tecnología de Alimentos por compartir conocimientos y buenos momentos. José Antonio, María Antonia, Wendu, María Jesús, Carmen, Karen, Felipe, Santiago, Fernando. Gracias también a Juan Antonio, Paco, José Antonio y, en particular, Cristian por el apoyo incondicional en todo momento.

Gracias al personal de la Facultad de Veterinaria de la UCM que nos permitió el uso del equipo de Luz Pulsada para realizar ensayos de esterilización de uva. Por compartir vuestra experiencia y conocimientos.

Agradezco al Profesor Jorge M. Ricardo da Silva del Instituto Superior de Agronomía de la Universidad de Lisboa por recibirme durante tres meses para la realización de una estancia de investigación. Gracias también a la gente maravillosa que conocí en el Edificio Ferreira Lapa y al resto de profesionales de la facultad con especial mención a Daniel, Joana, Diana, Sofía, Julianne, Diana, Ana Catarina, Joao, Gonçalo, Luis, Santiago, Francisco. Gracias por vuestra amistad.

Gracias a mis compañeros de aventuras y despacho, Iris y Juan Manuel, habéis sido una parte importante en todo momento durante estos años.

A mis amigos, los nuevos, los que siempre han estado, los que están aquí, los que están desde la distancia conmigo: Laura, Michelle, Jorge, Susana, Yesica, Sara, Marta, Marina, Álvaro O,

Álvaro L, Theano, Julia, Güicha, Lila, Karina, Anwar, Chivis, Ramón, Nayeli. Han recorrido este camino junto conmigo.

Gracias familia, os quiero. Sé que os ha costado preocupaciones y desvelos también. Adán, Martha, Gabriela y Alejandro sois y seréis siempre mi motor. Os dedico este trabajo.

RESUMEN

El uso controlado de levaduras no-*Saccharomyces*, tanto en fermentaciones mixtas como en fermentaciones secuenciales, representa una alternativa biotecnológica en la búsqueda de parámetros organolépticos de calidad, y a la vez diferenciadores, en vinos tintos. El metabolismo microbiano de las distintas especies de levaduras favorece la producción de metabolitos fermentativos que no solo contribuyen al enriquecimiento del perfil aromático de los vinos, sino que, al mismo tiempo, favorecen reacciones de condensación y polimerización de polifenoles, incluidos los antocianos, así como la formación de compuestos del tipo vitisinas y vinilfenólicos.

La selección microbiológica, de la mano de buenas prácticas enológicas durante la maceración, la vinificación y el envejecimiento del vino, favorecerá dicha mejora biotecnológica. Las tecnologías emergentes para el tratamiento de la conservación de los alimentos se han convertido en alternativas tecnológicas en el control de microorganismos durante los distintos procesos productivos y, al mismo tiempo, favorecen la protección del perfil aromático y una mayor extracción de pigmentos en el vino.

Esta tesis presenta el estudio realizado con diversas levaduras no-*Saccharomyces* con el objetivo de evaluar su potencial en la formación de pigmentos del tipo pyranoantociánico y su capacidad en la formación de pigmentos poliméricos mediante el uso de técnicas analíticas avanzadas. Por otra parte, se evalúa el impacto que tienen dichas levaduras en los demás parámetros organolépticos a través del análisis sensorial de los vinos.

Palabras clave:

Vino tinto, no-*Saccharomyces*, pyranoantocianos, pigmentos poliméricos, acetaldehído, fermentación secuencial, HPLC-MS, vinilfenólicos, flavanoles, taninos

ABSTRACT

The use of non-*Saccharomyces* yeasts in controlled mixed and sequential fermentations is, nowadays, a biotechnological alternative in the production of wines with optimized organoleptic parameters. This optimization would become a differentiator of wines for the costumers. The metabolism of the diverse yeasts species allows the production of fermentative volatiles able to enrich que aroma profile of wines; in the same time, these yeasts contribute to the polymeric reactions and condensation of polyphenols, including anthocyanins, just as they promote the formation of vitisins and vinyl phenolics.

Microbiological selection of yeasts, together with good oenological practices during maceration, wine making and wine ageing, will enhance such biotechnological practice. Emerging technologies used in food preservation and conservation, have become useful in the control of microorganisms during the different productive stages of wine making; these technologies would enrich aroma profile, pigments extraction and complexity of wines as an overall result.

This thesis work explores the use of non-*Saccharomyces* yeasts with interest in the formation of pyranoanthocyan and polymeric pigments through pure culture and sequential fermentations. The assessment of the potential these yeasts may have, is achieved by instrumental analysis techniques. On the other hand, sensory analysis is also used to evaluate their impact on the organoleptic parameters of the experimental wines produced.

Keywords:

Red-wine, non-*Saccharomyces*, pyranoanthocyanins, polymeric-pigments, acetaldehyde, sequential-fermentation, HPLC-MS, vinyl phenolics, flavanols, tannins

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(LSD TEST) 123

1. INTRODUCTION

To talk about red wine quality parameters is to talk in the same time about its sensory attributes in terms of colour, aroma profile and body or complexity. When opening a bottle of wine, we could describe it after the variety it is made from, the ageing process it went through or the terroir and the unique characteristics of the region it comes from. Wine has been mentioned in literature over the years and has evolved into a beverage that marry meals and has been attributed beneficial properties for health.

1.1 Colour of red wine: anthocyanins

The colour of red wines is coming from polyphenolic compounds named anthocyanins found in the skin of red grape berries from the *Vitis vinifera* spp. Anthocyanins belong to the phytochemicals flavonoids which are widely spread in nature among several species of flowers and fruits (Delgado-Vargas, Jiménez, & Paredes-López, **2000**). Anthocyanins, as well as the rest of flavonoid phytochemicals, have a C6 – C3 – C6 structure where two aromatic rings are linked by three carbon atoms forming an heterocycle in the middle with aromatic properties as well (Wolfe & Liu, **2008**). There are several anthocyanin structures. The difference between anthocyanins lies in the hydroxyl and metoxy groups attached to the main skeleton; the number, position and type of sugar and the number as well as the nature of acids bonded to those sugars also contribute to differentiate the anthocyanins; in this way and according with Wallace and Giusti (**2015**), there are up to 700 anthocyanin structures out of which 27 are found in nature and 6 are the most commonly distributed in fruits and plants: pelargonidin, delphinidin, cyanidin, petunidin, peonidin and malvidin (see **Figure 1**).

Figure 1 Anthocyanin-3-O-glucosides main skeleton.

The substituents define the anthocyanin's nature. In the case of the 6 more common anthocyanins the substituents for the structure in **Figure 1** are: pelargonidin 3-*O*-glucoside (R1:H, R2:H), cyanidin-3-*O*-glucoside (R1:OH, R2:H), petunidin-3-*O*-glucoside (R1:OCH₃, R2:H), delphinidin-3-*O*-glucoside (R1:OH, R2:OH), peonidin-3-*O*-glucoside (R1:OCH₃, R2:OH), malvidin-3-*O*-glucoside (R1:OCH₃, R2:OCH₃). In all the cases beforementioned the positions R3 and R4 have H as substituent.

1.1.1 *Vitis vinifera* L. cv. Tempranillo

The most frequent anthocyanin glucosides previously described are found in *Vitis vinifera* varieties in different proportion. These characteristics would eventually make possible to differentiate varieties between each other. Arozarena et al. (2002) have described the profile of anthocyanins in three different varieties, Tempranillo, Garnacha and Cabernet Sauvignon; the relative percentage of non-acylated glucosides comprising delphinidin, cyanidin, petunidin, peonidin and malvidin glucosides, is approximately 92% in Tempranillo and 95.7% in Garnacha vs. 68% from the variety Cabernet Sauvignon, while the percentage of acylated glucosides is 3.7% and 2.2% vs. 30.3% respectively. Considering total free anthocyanin content in the skins (mg/kg of berries) we can see in **Table 1** that Tempranillo is having lower concentration of anthocyanins after considering Cabernet Sauvignon variety. Differences in values given for same variety may be related to climate differences got in cultivars where the samples were taken from. Differences between varieties may be due, among other factors, to the size of the berry; Cabernet Sauvignon berries are smaller in size than those of Tempranillo 103g/100berries vs. 134g/100berries respectively (Sergio Navarro et al., 2008).

Table 1 Comparison of free anthocyanin content in berries of Tempranillo and Cabernet Sauvignon varieties.

Tempranillo	Cabernet Sauvignon	Reference
595.7	1198.4	Arozarena et al. (2002) ¹
710.3	836.3	Ryan and Revilla (2003) ²
1700	2350	Navarro et al. (2008) ^{1,3}
1363.5	-	Diago et al. (2012) ¹

¹At time of harvest

²Average concentration at day 40 after the onset of veraison

³Concentration given in mg/L

1.1.2 Water stress and climate

Weather has shown to have an influence on the concentration of anthocyanins in Tempranillo red grapes as Ryan and Revilla (2003) have studied. The effect of a rainy year has increased the weight of the berries reducing the total anthocyanins measured with HPLC, although the sugar content of the must has remained constant for similar ripening time (days after veraison) of berries from other years. On the contrary, in the case of regulated deficit irrigation during veraison, the size of the berries is smaller than those cultivated under conventional irrigation yielding, as a result, an increased content of total anthocyanins as a consequence of the size of the berry (Santesteban, Miranda, & Royo, 2011); when comparing two regulated deficit irrigation regimes from the same study, the first regime having one deficit period and the second one having two deficit irrigation periods, the increase in anthocyanin content may be explained by an overexpression of the genes responsible of the synthesis of anthocyanins. Intrigliolo and Castel (2009) have shown, in this matter, that irrigation regimes even before veraison may induce higher concentration of anthocyanin and total phenolics without having a collateral effect on the Tempranillo berries' quality. This is an effect also expected on varieties different from Tempranillo when growing berries due to an excess or lack of water in their structure during berry ripening.

On the other hand, the effect that weather may have on the evolution of pigments in the skin could be explained by insolation or, explained in other words, the amount of sun radiation that the berries receive. Leaf defoliation has demonstrated to also have an impact on the amount of anthocyanins per weight of berry with an overall larger amount of anthocyanins at the time of harvest (Diago, Ayestarán, Guadalupe, Poni, & Tardáguila, 2012). According to that research work, there is a positive effect on performing the defoliation mechanically vs. manually whilst there is no significant difference on doing it at pre-blooming or during fruit-set.

1.1.3 Maceration and anthocyanins extraction

Anthocyanins are extracted during maceration and it is the ripening stage of the berries a determining factor for the extraction-rate taking place. Hernández-Hierro et al. (2014) studied the maturity of the skin as to evaluate the degree of extraction of anthocyanins during maceration of berries from variety Tempranillo. The results showed that the anthocyanins,

both non-acylated and acylated, are extracted in greater extent when the grapes are over-ripened and, in the same time, the insoluble residue from the cell wall from the skin decreased due to the softening and skin loosening. During red winemaking process, if the wine is lacking phenolic constituents, as tannins, the maceration with stems improves organoleptic properties and ensure colour stability over time (Basile, Alba, Suriano, Savino, & Tarricone, **2018**).

Carbonic maceration is the process that takes places inside grape berries when they are left under anaerobic conditions (Navarro et al., **2000**). The process requires grapes without crushing or destemming treatments and no yeast inoculation for fermentation (Park et al., **2004**). The colour extracted through carbonic maceration seems to be less than the traditional maceration process from contact with pomace's skin but, the concentration of proanthocyanidins extracted is larger than traditional maceration (Spranger et al., **2004**).

The maceration process may be enhanced by the use of emerging technologies that have a disruptive effect on grapes' skin cells to increase anthocyanins diffusion from skins to the must. One of these technologies is pulsed electric fields (PEF) that was used to permeabilize cellular tissues of grapes *Vitis vinifera* L. cv Mazuelo, Garnacha and Graciano (López, Puértolas, Condón, Álvarez, & Raso, **2008a**). The results were better for the variety Mazuelo. This and other emerging technologies, used for grape processing and antimicrobial treatments, will be described in the next section.

1.1.4 Nonthermal pasteurization technologies and anthocyanins extraction

Emerging technologies such as high hydrostatic pressure (HHP), e-beam irradiation (EB irradiation), pulsed electric fields (PEF) and pulsed light are being considered for antimicrobial applications in food processing (Bañuelos et al., **2016**; Morata, Bañuelos, et al., **2015**; Morata, Loira, et al., **2015**; Puértolas, López, Condón, Raso, & Álvarez, **2009**). They are called nonthermal (cold pasteurization) technologies because, even though temperatures could punctually increase several hundred degrees at molecular level, the temperature of the food products do not increase as to modify its organoleptic properties and composition (Morata, Loira, et al., **2015**).

High hydrostatic pressure (HHP) technology uses working pressure between 400 and 600 MPa during short periods of time (~3 to 10 min) (Buzrul, **2012**). Since HHP does not have the ability to break down covalent bonds, the molecules responsible for colour, aroma and flavour in the different food products are preserved (Considine, Kelly, Fitzgerald, Hill, & Sleator, **2008**). HHP has been tested on the reduction of wild yeasts, moulds and bacteria on food products (Norton & Sun, **2008**) and, it can also be done at low temperatures or under refrigeration having the ability to avoid contamination of spoilage yeasts such as *Brettanomyces/Dekkera* spp. in winemaking (Morata, Benito, González, et al., **2012**).

Morata et al. (**2015**) evaluated the effect of HHP in grape structure, pigment extraction and wine quality. They have observed that after different HHP treatments, the structure of the grape is not altered and, they also observed diffusion of anthocyanins from the skin to the pulp and into the seeds (**Figure 2**). The extraction of acylated anthocyanins could be enhanced after performing HHP (Corrales, García, Butz, & Tauscher, **2009**) and, in general polyphenols, with the exception of flavan-3-ols, were extracted to larger extent with this technology (Chen et al., **2012**).

Figure 2 Effects of HHP on shape and appearance of grapes (A), anthocyanin diffusion from skin to pulp (B) and anthocyanin adsorption by seeds (C). (Morata, Loira, et al., **2015**)

The pulsed electric fields (PEF) are based on electroporation or electropermeabilization, which is understood as the production of pores in the cell membranes when electric pulses with short duration and high electric fields (nanoseconds to milliseconds and, 100 to 300 V/cm) generate up to 300 kV/cm (Parniakov et al., **2015**). The use of electric fields with 500-700 V/cm during 40-100 ms on grapes *Vitis vinifera* L. cv. Merlot promoted an increase on the extraction of both, anthocyanins and polyphenols (Delsart et al., **2012**). The possibility of performing PEF at low temperatures (5° C) may preserve food product quality ensuring

organoleptic properties. PEF have been also used to treat Cabernet Sauvignon grape pomace at different maceration times leading to having wines with larger amounts of anthocyanins and tannins and greater colour intensity (López, Puértolas, Hernández-Orte, Álvarez, & Raso, **2009**). Other studies have shown that the use of PEF with different energy intensities and duration enhance the extraction of polyphenolic compounds among several other compounds (Corrales, Toepfl, Butz, Knorr, & Tauscher, **2008**), colouring matter or anthocyanins (López et al., **2008a**; Puértolas, López, Saldaña, Álvarez, & Raso, **2010**) and reduce microbial populations of wine spoilage microorganisms from the genera *Brettanomyces* and *Lactobacillus* with a reduction rate of 99.9% (López, Puértolas, Condón, Álvarez, & Raso, **2008b**; Puértolas et al., **2009**).

The pulsed light (PL) technology uses intense and short pulses from a broad-spectrum light source that also includes UV light (Barbosa-Canovas, Schaffner, Pierson, & Zhang, **2000**). The light source are inert-gas flash lamps that have been used since the 1970's in Japan, where the microbial inactivation technology began (Oms-Oliu, Martín-Belloso, & Soliva-Fortuny, **2010**). The wavelength spectrum of the light goes from 170 to 2600 nm, in other words, from the ultraviolet (UV) to the near infrared (NIR) spectra. The antimicrobial activity is mainly related to the high energy short wavelengths in the UV spectra (Morris, Brody, & Wicker, **2007**). This lethal high energy wavelengths pulses have effect on microorganisms and on grape skins' cells. In that matter, continuous irradiation with UV-C light (~254 nm) has shown to break down epidermal cells in the skin of *Vitis labrusca* L. (Fava et al., **2011**). This effect may be responsible to increase anthocyanin extraction during maceration.

The electron beam irradiation (EB irradiation) is probably the less studied technology, from the ones already described, in terms of improving anthocyanins extraction. One drawback of this technology may be the lower penetration energy compared to other technologies, but it is more affordable than gamma or x-ray radiations (Morris et al., **2007**). An electron accelerator is used to irradiate food products mostly to extend the shelf-life of fresh products offered to sale in packaging (Boynton et al., **2005**). The energy of the ionizing radiation should not exceed 1.0 kGy according to the Food and Drug Administration on fruits and vegetables.

The use of these technologies may not only reduce initial microbial populations and enhance anthocyanins extraction, but it could also reduce the amount of SO₂ additions during wine

making (Morata et al., **2017**). The fact of reducing SO₂ may avoid allergic problems and also may reduce colour loss in the different stages of wine's life.

1.1.5 Oak barrel and bottle ageing

Wines experience colour evolution while ageing in barrels and during their life span in the bottle. Anthocyanins, as flavonoid molecules, are subjected to chemical reactions that will eventually transform them into oligomeric or polymeric structures and, that will modify the colour of wine from bright red to brick-red tonality. The process that polyphenols in general, and anthocyanins in particular, suffer during wine ageing are set by factors such as copigments, acetaldehyde and other yeast metabolites, temperature, pH, oxygen and SO₂ levels, etc. (Fulcrand, Cheynier, Oszmianski, & Moutounet, **1997**; Haslam, **1980**; Loira et al., **2013**; Morata, Calderón, González, Gómez-Cordovés, & Suárez, **2007**; Morata, Gómez-Cordovés, Calderón, & Suárez-Lepe, **2006**).

Wine ageing in oak barrels is a practice that benefits wines from two technical points of view: it improves wine quality by transferring compounds from the wood into the wines such as ellagitannins, furfural compounds, guaiacol, lactone and eugenol; and it also allows oxygenation in a controlled way, which produces change in colour and modification of sensory parameters (Tao et al., **2016**). Oak barrel ageing allows small amounts of oxygen to favour reactions for vitisin formation (Lee, Swinny, Asenstorfer, & Jones, **2004**) but, the age of the barrel is important since the older the barrel the less oxygen than can diffuse into the wine due to deposits formed in the inner surface (Nevares & del Álamo, **2008**). Micro-oxygenation (MO) is comparable to oak barrel ageing in terms of anthocyanin evolution although the vitisin formation is higher with MO but the stability of polymeric pigments is lower over time, probably due to the presence of ellagitannins and wood aldehydes extracted from the wood during the ageing process (Cano-López, López-Roca, Pardo-Minguez, & Gómez Plaza, **2010**). The use of high hydrostatic pressure (HHP), may result in reducing the amount of SO₂ needed for controlling microbial population during ageing period in barrels, thus promoting the condensation of anthocyanins with flavanols and free acetaldehyde. The HPP, applied for 2 hours accelerate ageing in red wine, and was regarded as an alternative to oak barrel ageing since this technology would help to avoid traditional dangers such as potential microbiological contamination and other drawbacks like cost and volume occupied

by the barrels (Tao et al., **2012**). This technology might be recommended for wines with low ageing potential.

In the bottle, the most evident consequence over time is the decay of monomeric anthocyanins. A total phenolics follow up of wines from three different grape varieties for 26 months have demonstrated that wines suffered a markedly decrease of total anthocyanins from 43% for Tempranillo wine to 66% decrease for Cabernet Sauvignon wines (Monagas, Gómez-Cordovés, & Bartolomé, **2006**). In the same time low polymerized-polyphenols (LPP) also decreased their concentration and the content of catechins and proanthocyanidins increased suggesting an increase in condensation reactions and a structural transformation of proanthocyanidins during ageing respectively. The decrease rate of anthocyanins content in wines is proportional to the amount of acetaldehyde present. In port wines, the anthocyanins loss was five times higher for wines containing high concentration of acetaldehyde (200 mg/L) than wines having a minimal acetaldehyde content (Bakker, **1986**).

1.2 Red wine colour stability

The colour of red wines may be affected by diverse factors. Negative factors affecting red wine colour are pH variations, storage temperature, the use of SO₂ as microbicide while; these factors may contribute to colour variations and mostly to colour reduction. On the other hand, the copigmentation phenomena, the formation of pyranoanthocyanin-type pigments and the polymerization may promote the production of more stable pigment structures with different colour profile but prone to lasting longer.

1.2.1 Effect of pH variation and temperature.

Under acidic conditions, or low pH values, the flavylium cation is the main species found in solution. The flavylium cation has a positive charge in the heteroaromatic ring which is expressed as a maximum wavelength of absorption between 515 and 530 nm of the visible light spectrum. Nonetheless, at wine pH (~3.6), anthocyanins are expected to be in their noncolored hemiacetal form after interacting with water molecules (Oliveira, Mateus, Silva, & De Freitas, **2009**) in equilibrium with small amounts of chalcones. This effect, is overcome in red wines by copigmentation phenomena, self-association interactions and complexation mechanisms that protect the chromophore avoiding the formation of such hemiacetal units (Goto & Kondo, **1991**). If the conditions change and the pH value increases towards neutral or basic values, the flavylium salt is then transformed into the quinoidal base form by proton transfer reactions (Leydet et al., **2012**); quinoidal bases have maximum wavelength at high pH values reason why the colour perceived is purple and blue-violet (>540 nm).

To summarize the equilibrium reactions previously described, Leydet et al. (**2012**) have proposed a simple equilibrium reaction diagram (**Figure 3**). It can be observed that the flavylium cation (AH⁺) could undergo two different yet parallel reactions. The first one, and faster, is the proton transfer to form the quinoidal base (A) towards left side in the figure, and the second reaction, also slower reaction, happens when interacting with water molecules to form the hemiacetal (B2) and the further formation of chalcones (Cc and Ct) in either way, *cis* or *trans* conformations. These three last molecular conformations (B2, Cc, Ct) are thermodynamically more stable than structure A at this pH and therefore the quinoidal base would tend to totally or partially disappear over time. The equilibrium constants are totally dependent on pH values.

Figure 3 Chemical equilibrium reactions of malvidin-3-O-glucoside taken from Leydet et al. (2012).

On the other hand, temperature may also have an influence on the formation and the stability of the pigments in red wines. Lower temperatures seem to promote the formation of vitisin A according to Romeo and Bakker (2000) as seen through experimental design using wine model solutions; the formation kinetics slowed down as the temperature of the media raised. In terms of stability of already formed vitisins, it was also observed that these pigments are less stable at higher temperatures, mainly above 32 °C, when simulating storage conditions for wines (Baranowski & Nagel, 1983).

1.2.2 Use of SO₂

Sulphur dioxide (SO₂) is an antimicrobial used in wine production to avoid undesired spoilage yeast and bacteria and, also used to stabilize finished wines microbiologically. The addition of SO₂ to wines should consider its interaction with acetaldehyde to forecast its antimicrobial ability. Total SO₂ comprises the bound and the free forms, and only the free form is having antimicrobial properties (Liu & Pilone, 2000). The free SO₂ is mainly present as bisulphite anion (HSO₃⁻) and a small fraction of molecular SO₂ (SO₂·H₂O) and sulphite anion (SO₃⁻²). The bound SO₂ is forming a complex molecule with carbonyl compounds; acetaldehyde is the one in larger concentration in wines. The complex is named hydroxy-sulphonate and it accounts for the majority of SO₂ added to wines. Bound SO₂ has minimal antimicrobial properties.

The concentration of SO₂ in wines may be critical to wines for two reasons: it reduces the polymerization reaction between acetaldehyde and polyphenols and, it contributes to the decolourizing effect that it has on anthocyanins (Ribéreau-Gayon, Pontallier, & Glories, 1983).

The decolourizing effect, also known SO₂ bleaching of anthocyanins (Morata et al., 2006), occurs when the flavylium cation of anthocyanins reacts with SO₂ in its bisulphite anion form. The flavylium cation is sensitive to nucleophilic addition of bisulphite anion. Position C4 in the pyran ring of anthocyanins is the most electrophilic position and it is here where the reaction takes place. SO₂ gas reacts with water and produces sulphurous acid that releases bisulphite anion after dissociation. Bisulphite anion is a strong nucleophilic reagent able to break the aromaticity of the pyran ring after bonding to position C4. The pyran ring loses the possibility to absorb light and it is transformed into a colourless molecule (Morata et al., 2007). This reaction is reversible, at the beginning there is a colour loss, but colour is slowly recovered to its initial level. A schematic representation of the nucleophilic attack of SO₂ to a malvidin-3-*O*-glucoside molecule is shown in **Figure 4**.

In this way, acetaldehyde plays an important role in colour stability and polymerization reactions involving anthocyanins, proanthocyanidins and condensed tannins, as it reduces the negative impact of having SO₂ added to wines. Although having large concentrations of free acetaldehyde may also cause instability. Colour instability is produced by the precipitation of complexes formed after the condensation of anthocyanins with catechin and tannins and, precipitation of colloidal polymers formed with acetaldehyde and catechin (Saucier, Little, & Glories, 1997; Somers, 1998).

Figure 4 Anthocyanin's bleaching by nucleophilic attack of SO_2

1.3 Copigmentation

Despite the fact that anthocyanins were extracted with alcohol from blue cornflower (*Centaurea cyanus*) in ca. 1850 and, the blue pigment also found in other flowers such as violets and iris, the pigments were not characterized until several decades after. The name cyanidin was designated to those pigments (Willstätter & Everest, **1913**) and, the variations in colour observed in these pigments were then associated to different pH media.

Years after, the pigment was renamed to protocyanin and the structure has been lately described as a complex produced from the interaction of anthocyanin and flavone with metal ions Fe^{3+} and Mg^{2+} ; Ca^{2+} is also essential for the formation of these structure (Shiono, Matsugaki, & Takeda, **2005**). The molecule, which crystal structure has been determined by these authors, contains six cyanidin-3-*O*-(6-*O*-succinylglucoside)-5-*O*-glucoside molecules and six other flavone molecules. The anthocyanins are coordinated to one Fe^{3+} and one Mg^{2+} ions while, the flavones are coordinated to two Ca^{2+} ions. **Figure 5** shows the crystal structure of the protocyanin obtained by X-ray crystallographic analysis and, it is the answer to the question why roses are red and cornflowers are blue if they both have the same anthocyanin (Takeda, **2006**).

The molecular associations or complexes formed from the interaction of anthocyanins and usually non-coloured organic molecules is known as copigmentation phenomena (Boulton, **2001**). These phenomena produce an increase on the colour exhibited by either plants or fruits and it is a phenomenon also observed in young red wines. Half of the colour of young red wines is accounted to copigmentation phenomena.

Hydrophobic interactions between the planar polarisable nuclei of the flavylium cation, coloured form of anthocyanins, and other molecules explain the copigmentation phenomenon (González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón, & Santos-Buelga, **2009**). The sandwich configuration adopted by this interaction protects the chromophore from nucleophilic attack of water and other agents, avoiding the formation of colourless structures.

Figure 5 Stereo view of the crystal structure of protocyanin. Three-fold axis (top) and side view (bottom). From Takeda (2006). Anthocyanins are shown in blue and flavones in yellow.

Therefore, copigmentation of anthocyanins (**Figure 6**) from this type of interaction could be intermolecular with a diversity of organic molecules or self-association from the interaction between anthocyanin molecules. A third interaction, as already seen, is the one coming from the interaction with metal ions forming metallic complexes.

Figure 6 Copigmentation interactions: intermolecular interaction between caffeic acid and anthocyanin (top right), self-association between anthocyanin molecules (bottom right) and metal complex formation (bottom left)

In red wines, the more promising cofactors prone to participating in the intramolecular copigmentation phenomena are flavonoids and hydroxycinnamoyl derivatives (González-Manzano et al., 2009); the concentration of such molecules vary in function of the red grape variety; the flavanols are far more abundant in grapes than flavonols and hydroxycinnamoyl derivatives for instance although their copigment strength seems weaker than these two last molecules. The charge-transfer complex (**Figure 7**) produced by the interaction of the flavylium cation with other organic molecules with electron density, is a way to stabilize anthocyanins electron deficiency and thus preserve the colour of the cationic salt (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). As a result of this copigmentation process, it is observed an hyperchromic effect (increase in colour intensity) and a bathochromic shift (displacement of higher maximum wavelength of absorption toward blueish tonality).

Figure 7 Stabilization of anthocyanin (flavylium cation) through the formation of charge-transfer complex. Taken from Castañeda-Ovando (2009)

The vertical stacking of anthocyanins was proposed back in 1982 (Hoshino, Matsumoto, Goto, & Harada, **1982**) for the self-association interaction of anthocyanins as an evidence of hydrophobic and $\pi - \pi$ interaction forces taking place. The self-association can be demonstrated as a positive deviation of the Beer's law as the concentration of anthocyanins increases (González-Manzano, Santos-Buelga, Dueñas, Rivas-Gonzalo, & Escribano-Bailón, **2008**). This interaction, even though is similar to the one observed by intramolecular copigmentation with organic molecules other than anthocyanins, is yet different on the maximum wavelength of absorption measured. Self-associated anthocyanins produce an hypsochromic shift (Hoshino, Matsumoto, & Goto, **1981**) and is experimentally measured between 4 to 6 nm displacement (González-Manzano et al., **2008**).

With regards to the metal complex structure, the ability of forming such interactions seemed to be related to the nature of the anthocyanin and the possibility of having an ortho-dihydroxyl arrangement on the pyran ring (Harborne, **1958**). Cyanidin, delphinidin and

petunidin glucosides might be prone to forming these complexes while, pelargonidin, peonidin and malvidin glucosides might not. Metal complexing is responsible for the blue colour mainly observed in plants. Delphinidin glucoside needs Mg^{2+} ions to produce blue colour while cyanidin requires Fe^{3+} (Yoshida, Kitahara, Ito, & Kondo, **2006**). Despite this fact, it has been observed that also Al^{3+} can produce the formation of blue complexes although the molecular structure of this complex is not yet clearly defined. It has been proposed that aluminium coordinate with the ortho-dihydroxyl group of the pyran ring and a carboxylate and hydroxyl group from an ester residue from the cofactor (Kondo, Toyama-Kato, & Yoshida, **2005**). In this way, delphinidin glucoside forms metallic complexes with aluminium and without flavonoid molecules.

1.4 Pyranoanthocyanins and polymeric pigments.

There are a series of pigments more stable than the non-acylated and the acylated anthocyanins naturally found in red grapes. The stability of these pigments is due to the fact that there is a second pyran ring protecting position C4 in the anthocyanin skeleton reducing, in this way, the possibility of suffering nucleophilic attacks and increasing the number of stable forms by resonance. Three main families of stable pigments described hereafter are vitisins and vinylphenolics, both pyranoanthocyanins, and polymeric pigments.

1.4.1 Vitisins

Although vitisins have been observed during the fermentation of red grape musts according to Rentzsch, Schwarz, Winterhalter and Hermonsín-Gutiérrez (2007) mainly due to the fact that the concentration of their precursors is at its maximum, specially pyruvic acid (Asenstorfer, Markides, Iland, & Jones, 2003; Morata, Gómez-Cordovés, Colomo, & Suárez, 2003); Amic, Davidovic-Amic, Beslo and Trinajstić (2000) have observed their formation during wine ageing as well and Romero and Bakker (2000) have found them in small quantities in matured porto wines and analysed their formation in wine model solutions. Furthermore, Oliveira, de Freitas and Mateus (2009) have followed the formation of vitisin B through the reaction of vinyloxy-tri-methylsilane with malvidin-3-*O*-glucoside and malvidin-3-*O*-*p*-coumaroylglucoside derivative yielding the formation of pigments with a fourth ring in model wine solutions. The vitisins are a form of pyranoanthocyanins produced through the interaction between anthocyanins and fermentative metabolites such as acetaldehyde and pyruvic acid and have shown to have whole or partial stability against bleaching and preserve colour up to pH values of 7 (Bakker & Timberlake, 1997). The most common vitisin-type pigments are described hereafter.

Pyruvic acid is one of the intermediates in the alcoholic fermentation and it can react with malvidin-3-*O*-glucoside to yield vitisin A production (Rentzsch, Schwarz, & Winterhalter, 2007) (**Figure 8**). Vitisin A is formed before day 6 (Morata et al., 2006) since, at this time the must is still rich in nutrients and the pyruvic acid is excreted from the yeast cytosol to the exterior but, when nutrients become scarce, the yeast may use this pyruvic acid for further metabolic processes. This vitisin-type pigment is also known as 5-carboxypyrananthocyanin

since it has a carboxyl group attached to the newly formed pyran ring. Vitisin A has proven to be more stable than vitisin B (Rentzsch, Schwarz, & Winterhalter, 2007).

Figure 8 Formation of vitisins from glycolytic metabolites. Taken from Benito et al. (2011)

Vitisin B is the pigment produced from the reaction between malvidin-3-O-glucoside and acetaldehyde (**Figure 8**), and it has been isolated from port wine (Rentzsch, Schwarz, & Winterhalter, 2007) and later from red wines (Monagas, Núñez, Bartolomé, & Gómez-Cordovés, 2003; Morata et al., 2003; Revilla, Pérez-Magariño, González-SanJosé, & Beltrán, 1999). This pigment has only hydrogen substituents on the newly formed pyran ring and therefore it is the simplest possible structure among the pyranoanthocyanin-type structures. Vitisin B is greatly formed towards the end of the fermentation since the production of acetaldehyde is directly linked to the quantity of sugars consumed or fermented by the yeasts (Morata et al., 2006). These pigments have an hypsochromic effect on the maximum absorbance wavelength from that of the anthocyanins they are formed from; the maximum

absorbance observed for these pigments is between 490 and 492 nm (Oliveira, de Freitas, et al., 2009).

A third type of vitisin-like pigments are the methylpyranoanthocyanins firstly observed through the condensation reaction of anthocyanins with acetone as an artefact when extracting pigments (Rentzsch, Schwarz, & Winterhalter, 2007); they were named methylpyranoanthocyanins due to the methyl group located at C5 in the new pyran ring formed. This last pigment is not considered naturally happening unlike the one found in port wines after the condensation reaction between malvidin-3-*O*-glucoside and acetoacetic acid (Jingren, Santos-Buelga, Silva, Mateus, & De Freitas, 2006) and according to these authors the hypsochromic effect observed in this pigment is even larger than the one observed in the other two vitisin-type pigments, A and B; the maximum wavelength of absorbance observed in the methylpyranoanthocyanin pigments isolated from port wines is 478 nm.

The molecular structure of the three pyranoanthocyanins previously described is summarized in **Figure 9**. The simplest structure corresponds to vitisin B (**Fig. 9A**) with just hydrogen substituents; the presence of a carboxyl substituent is observed in vitisin A (**Fig. 9B**) and, there is a methyl substituent in the methylpyranoanthocyanin (**Fig. 9C**) after the cycloaddition of acetaldehyde, pyruvic acid and acetoacetic acid respectively between positions C4 and C5 of the malvidin-3-*O*-glucoside structure.

Figure 9 Pyranoanthocyanin pigments found in red wines. A) vitisin B, B) vitisin A or 5-carboxypyrananthocyanin and C) methylpyrananthocyanin

The cycloaddition or cyclization is the mechanism that vitisins, as well as other pyranoanthocyanin-like pigments, follow. This reaction mechanism takes place between carbon 4 (C4) and the hydroxyl group located at carbon 5 (C5) of the anthocyanin's structure (Vivar-Quintana, Santos-Buelga, Francia-Aricha, & Rivas-Gonzalo, 1999) producing the

formation of the characteristic second pyran ring seen in these chemical structures. This reaction may also take place with the double bond of vinylphenols as it will be described in the next topic below.

Vitisins, as well as vinylphenolic pyranoanthocyanins have better stability than anthocyanins towards pH variations, hydration and to bleaching from the use of SO₂ as bacteriostatic agent. Bakker and Timberlake (1997) refer the stability to the saturation of carbon 4 (C4) due to the formation of the second pyran ring. If electrophilic position C4 is saturated and therefore blocked by the second pyran ring, nucleophiles may not attack promoting bleaching by SO₂ (Figure 10). Besides the protection against SO₂ bleaching, vitisins may not undergo hydration reactions towards the production of hemiacetal forms often seen in anthocyanins; this explains the retention of colour in pyranoanthocyanins at higher pH values and therefore, the higher stability that these pigments have in comparison with monomeric anthocyanins (Oliveira, Mateus, & De Freitas, 2014).

Figure 10 Inhibition of bleaching by SO₂ in vitisins and pyranoanthocyanins.

1.4.2 Vinylphenolics

Vinylphenolic pyranoanthocyanins are reaction products formed by the reaction of malvidin-3-*O*-glucoside with 4-vinylphenols or hydroxycinnamic acids (Quaglieri, Jourdes, Waffo-Teguo, & Teissedre, **2017**). The 4-vinylphenols are produced as a consequence of the decarboxylation of the corresponding cinnamic acids during red wine production due to enzymatic activity (**Figure 11**). The enzymatic activity is known as hydroxycinnamate decarboxylase (HCDC).

Figure 11 Formation of 4-vinylphenols from the decarboxylation of hydroxycinnamic acids. Taken from Vanbeneden et al. (2008).

The 4-vinyl derivatives produced in beverages under fermentation, such as wine and beer, may produce ethyl-phenols in the presence of *Brettanomyces/Dekkera* spp. yeasts (Vanbeneden, Gils, Delvaux, & Delvaux, **2008**). The aroma profile of 4-ethyl phenols resembles that of animal phenolic odours at rather low concentrations of 425 µg/L, or even smell of barnyard and stable at higher concentrations (Chatonnet, Dubourdieu, & Boidron, **1995**); this it is not desired in wines thus, the use of yeasts with (+) HCDC activity is desired.

The 4-vinylphenols and the hydroxycinnamic acids may react with anthocyanins from the red wine producing vinylphenolic pyranoanthocyanins (**Figure 12**). These pigments, also named pinotins since they were firstly observed in pinotage wines, were formed due to the high concentration of caffeic acid in *Vitis vinifera* L. cv. Pinotage (Schwarz & Winterhalter, **2003**).

Figure 12 Formation of vinylphenolics from enzymatic hydroxycinnamate decarboxylase activity. Taken from Benito et al. (2011).

The stability in vinylphenolic pyranoanthocyanins is also coming from the saturation of carbon 4 (Morata, González, & Suárez-Lepe, 2007). The second pyran ring blocks position C4 from nucleophilic attacks and stabilizes the structure. This characteristic reduces the loss of colour intensity as a consequence of the bleaching by SO₂ (Figure 10). Vinylphenolic pyranoanthocyanins, as well as vitisins, have a maximum absorption wavelength between 498 nm and 515 nm in the visible spectra producing a hue more orange than grape anthocyanins (Morata et al., 2007).

Finally, it is important to mention that there is other type of pyranoanthocyanin-like pigments in certain red wines, yet in smaller quantity, but contributing to colour stability; these pigments are portisins originally found in Port wines (De Freitas & Mateus, 2011) or pyranoanthocyanin-flavanyl adducts. This last type of pyranoanthocyanin formation will be covered in the following section.

1.4.3 Polymeric pigments

The polymerization of anthocyanins is another process through which the colour of red wines can be preserved. It usually involves flavonoid molecules and acetaldehyde for it to take place. Some wine making practices that were thought to improve this process included the increase in acetaldehyde production by means of aeration, as a way to promote the polymerization of anthocyanins with catechins and tannins. Not only the colour of red wines can be benefited from polymerization reactions but also, from a sensory point of view, the polymerization could reduce the tannic character of wines having a direct impact on the astringency and the bitterness (Ribéreau-Gayon et al., **1983**).

The reaction products are wide and diverse. Anthocyanins and derivatives may condense with flavanols, the most abundant flavonoid found in red wines, in order to form oligomeric and polymeric structures. The rapid condensation of these organic molecules has been documented by Timberlake and Bridle (**1976**) after adding acetaldehyde to fermenting model media. The simplest molecular structure comprises an anthocyanin and a molecule of (epi)catechin. The dimerization between flavanol and anthocyanin happens in the same time that the dimerization between two flavanol units takes place (Es-Safi, Fulcrand, Cheynier, & Moutounet, **1999b**) although the flavanol-ethyl-flavanol structure is less stable than the flavanol-ethyl-anthocyanin dimer. **Figure 13** schematizes the process in which anthocyanins begin to form dimeric and trimeric structures through condensation with flavanols and acetaldehyde. The dimers are shown by structures *a*, *b* and *e* while, the trimers are represented by the structures *b* and *d*.

Structures *a* and *b* are the reaction product of the condensation of one (epi)catechin molecule with an anthocyanin in the absence and the presence of acetaldehyde respectively. The reaction happens faster with epicatechin than with catechin (Es-Safi, Fulcrand, Cheynier, & Moutounet, **1999a**). The condensation reaction to produce flavanol-anthocyanin dimers (*a*) undergoes a slower mechanism than the followed for ethyl-linked units (*b*). Eglinton et al. (**2004**) demonstrated that these oligomers could be formed before and after fermentation in a slower rate than in the presence of fermenting yeasts, as well as in a lower production yield associated to the lack of yeast metabolites (acetaldehyde). In the presence of acetaldehyde and constant SO₂ levels, the condensation reaction for the production of polymeric pigments ethyl-linked (*b*) is enhanced.

Figure 13 Dimeric and trimeric conformation of anthocyanins through polymeric condensation: a) dimer flavanol-anthocyanin, b) dimer flavanol-ethyl-anthocyanin, c) trimer flavanol-ethyl-flavanol-ethyl-anthocyanin, d) trimer anthocyanin-ethyl-flavanol-ethyl-anthocyanin and e) dimer pyranoanthocyanin-ethyl-flavanol (Portisin A type).

Es-Safi et al. (1999b) observed that a polymer moiety stops the polymerization reaction when two anthocyanin molecules are located at both ends of the oligomer. Structures *c* and *d* are examples of growing oligomeric pigments.

Dimer *e* (Figure 13), also known as portisin-type pigment, are produced through the nucleophilic attack of the vinyl group from the 8-vinyl-(epi)catechin to the position C10 of a pyranoanthocyanin (Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & De Freitas, 2003); the dimeric pigment presents bathochromic shift with a maximum absorption wavelength of 570

nm (He et al., **2012**) and a blue-purple colour as a consequence of this shift. The reaction is mediated by a molecule of acetaldehyde, the responsible of the formation of the 8-vinyl-flavanol adduct (Mateus, Oliveira, Haettich-Motta, & de Freitas, **2004**). If the reactions take place with a molecule of vitisin A the portisin formed is named portisin A (Mateus, Oliveira, Santos-Buelga, Silva, & De Freitas, **2004**); if it happens through the nucleophilic attack of an hydroxycinnamic acid or a vinylphenol, the molecule is named portisin B (Mateus et al., **2006**; Oliveira, De Freitas, Silva, & Mateus, **2007**). These pigments are called portisins since they were firstly discovered in port wines and, they are usually found in aged wines, although, unlikely other dimeric pigments, these can also be present in young wines.

1.5 Non-*Saccharomyces* yeasts

Spontaneous fermentations begin with the implantation of native non-*Saccharomyces* yeasts that contribute to enrich wines complexity. The effect of having such yeasts species during wine fermentation apparently depends on the inoculum ratio of *Saccharomyces* to non-*Saccharomyces* yeasts (Comitini et al., **2011**). The ratio would emulate natural or simultaneous fermentative conditions. The contribution of the non-*Saccharomyces* yeasts to the wine's colour and to its aromatic profile is described below.

1.5.1 Effect on the colour

Metabolically wise, some yeast species may increase the production of intermediates and precursors promoting the formation of stable pigments. Such is the case of the species *Schizosaccharomyces pombe* (Morata, Benito, Loira, et al., **2012**; Suárez-Lepe, Palomero, Benito, Calderón, & Morata, **2012**) which increases the concentration of pyruvic acid excreted out of the cytosol and, as a consequence, increase the formation of vitisin A in wines fermented with these strains. Benito et al. (**2012**) have also seen that parallel to this effect, the strains of *Schizosaccharomyces pombe* also reduced urea production.

A strategy to promote the formation of vinylphenolic pyranoanthocyanins is the use of yeast species with high hydroxycinnamate decarboxylase activity (HCDC); *Saccharomyces cerevisiae* with 50% activity and *Pichia guilliermondii* with 90% activity were used to ferment grape must enriched with natural pigments as to enhance the formation of vinylphenolics (Benito, Morata, Palomero, González, & Suárez-Lepe, **2011**).

Loira et al. (**2014**) have also evaluated the capacity of increasing vinylphenolic pyranoanthocyanins in sequential fermentations with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* as a parameter of improving wine quality. The outcome showed that there was larger concentration of vinylphenolic pyranoanthocyanins in pure culture fermentations with *Saccharomyces cerevisiae* while on the fermentations with *Torulaspora delbrueckii* in sequential fermentation with *Saccharomyces cerevisiae* the concentration was lower; the strain of *Saccharomyces cerevisiae* showed more enzymatic activity in that case.

Acetaldehyde plays an important role in colour increase in port wines (Bakker & Timberlake, **1986**). The concentration of acetaldehyde in port wines is higher than in red or white wines due to the addition of brandy during the fortification process. Free aldehyde may interact with anthocyanins and polyphenols yielding large molecular weight colour molecules. Non-*Saccharomyces* yeasts may produce considerable amounts of acetaldehyde among other “non-desired” volatiles and, could eventually have chemical deviations with negative impact in organoleptic profile of wines (Suárez-Lepe et al., **2012**). *Schizosaccharomyces pombe*, traditionally observed as wild or spoilage yeast to wine, is one of this non-*Saccharomyces* yeasts able to increase acetaldehyde production. Mixed fermentations having this yeast species increases complexity and aromatic typicity of wines (Garde-Cerdán & Ancín-Azpilicueta, **2006**) and, may eventually contribute to colour stabilization by enhancing condensation reactions with acetaldehyde.

1.5.2 Effect on the aroma profile

Mixed fermentations having both, non-*Saccharomyces* and *Saccharomyces* yeasts, are considered superior wines in the Old World compared to pure culture fermentations. Non-*Saccharomyces* yeasts could have effects on the sensorial profile of wines (Viana, Gil, Genovés, Vallés, & Manzanares, **2008**; Zott et al., **2011**). The use of non-*Saccharomyces* yeasts in controlled mixed fermentations can lead to modify the wine profiles in terms of polysaccharides production, glucosidase and esterase activity and purity of fermentation (Comitini et al., **2011**). As a consequence, compounds such as 2-phenyl ethanol significantly increases when using *Torulaspora delbrueckii* and *Lachancea thermotolerans* in accordance with these authors favouring the presence of rose aroma in wines; volatile acidity decreased when using *Metschnikowia pulcherrima*, *Lachancea thermotolerans* and *Torulaspora delbrueckii* in the same time that *Lachancea thermotolerans* increased the amount of lactic acid. Renault et al. (**2009**) also documented the reduction in volatile acidity when fermenting with *T. delbrueckii* and an increase in glycerol and volatile aromatic compounds.

In terms of esters production, the use of non-*Saccharomyces* yeasts may increase the concentration of ethyl acetate, isoamyl acetate and 2-phenyl ethyl acetate (Rojas, Gil, Piñaga, & Manzanares, **2001**). Yeasts from the genera *Hanseniaspora* and *Pichia* may produce larger concentration of these volatile molecules than *Saccharomyces cerevisiae* under aerobic and anaerobic conditions. *H. guilliermondii* is able to produce 2-phenyl ethyl acetate with flowery

(roses) aroma; *P. anomala* is able to produce isoamyl acetate with banana aroma; but, on the other hand, these two yeasts are also able to produce extremely high concentrations of ethyl acetate which aroma is not desired in wines.

Other parameter that may be optimized by the use of non-*Saccharomyces* yeasts is the total acidity and in particular, the increase of freshness in wines from the increase of (+) L-lactic acid production (Gobbi et al., **2013**). The species *Lachancea thermotolerans* has shown to be an acidifying agent due to this metabolic characteristic. Mixed fermentations of *Lachancea thermotolerans* and *Saccharomyces cerevisiae* have produced wines with lower pH values, larger amounts of (+) L-lactic acid and, as a consequence, higher total acidity. In the same time, mixed fermentations of *L. thermotolerans* and *S. cerevisiae* have increased the concentration of glycerol and 2-phenyl ethanol. The use of *Lachancea thermotolerans* in mixed fermentations with *Schizosaccharomyces pombe* is promising in terms of microbiological stabilization as an alternative to malolactic fermentation with lactic bacteria (Benito, Calderón, Palomero, & Benito, **2015**). Total consumption of (+) L-malic acid with enhanced production of (+) L-lactic acid to overcome the loss in acidity is the result of such fermentations; in the same time, the production of acetic acid and biogenic amines diminished.

In other example, mixed fermentations of *Torulaspora delbrueckii* with *Saccharomyces cerevisiae* have produced wines with lower 2,3-butanediol but larger amounts of diacetyl, ethyl lactate and 2-phenyl acetate (I. Loira et al., **2014**).

The benefit of performing mixed fermentations with non-*Saccharomyces* and *Saccharomyces* yeasts in terms of improving aroma profile is not always achieved. Pure culture fermentation with *Candida zemplinina* produced a positive increase of lactones and terpenes yet, the effect observed when the same strain was used in mixed fermentations with *Saccharomyces cerevisiae* was the opposite (Sadoudi et al., **2012**). **Table 2** summarizes the use of some non-*Saccharomyces* yeasts in pure and mixed fermentations and their contribution to aromatic profile complexity of wines.

Table 2 Non-*Saccharomyces* yeasts able to ferment grape must and their contribution to volatile profile of wines.

Yeast	Property	Reference
<i>M. pulcherrima</i>	Less volatile acidity (VA)	<i>Comitini et al., 2011</i>
<i>T. delbrueckii</i>	2-phenyl ethanol, less VA	<i>Comitini et al., 2011</i>
	Ethyl lactate, diacetyl	<i>Loira et al., 2014</i>
	Isoamyl alcohol	<i>Renault et al., 2009</i>
<i>L. thermotolerans</i>	(+) L-lactic acid production, glycerol	<i>Gobbi et al., 2013</i>
	2-phenyl ethanol	<i>Comitini et al., 2011</i>
<i>H. guilliermondii</i>	2-phenyl acetate	<i>Rojas et al., 2001</i>
<i>C. zemplinina</i>	Terpenes and lactones	<i>Sadoudi et al., 2012</i>
<i>S. pombe</i>	Malic acid reduction	<i>Benito et al., 2015</i>
<i>P. anomala</i>	Isoamyl acetate	<i>Rojas et al., 2001</i>

The alcohol esterification potential, the acidification enhancement, etc., of non-*Saccharomyces* yeasts species has been demonstrated and may contribute to enrich profile aroma and complexity of red wines. On the other hand, there is still the problem with the control of other populations of non-*Saccharomyces* yeasts such as *Brettanomyces/Dekkera* spp. and *Saccharomyces ludwigii* that may be considered spoilage species or contaminant to musts. Control of these non-desired species could avoid having “animal/farmyard/mousy” aromas or high amounts of ethyl acetate respectively (Jolly, Augustyn, & Pretorius, 2006). *Saccharomyces ludwigii* also has the ability of surviving to high sulphated musts due to their high resistance to SO₂; due to this characteristic, this yeast may spoil wine.

1.6 Thesis objectives

General Objective

To evaluate the formation of stable pigments in red wines through sequential fermentations with non-*Saccharomyces* yeasts and, to assess the effect on the organoleptic profile of experimental wines with the aim of improving quality parameters during wine making.

Particular Objectives

There were a series of experiments performed during this doctoral work to select the most promising non-*Saccharomyces* yeasts and to achieve the beforementioned general objective. The individual objectives of each experimental set up are shown hereafter.

Experiment 1: to study the effect of several non-*Saccharomyces* yeasts on the formation of both pyranoanthocyanins and polymeric pigments during pure culture fermentations as a way to produce higher amount of stable pigments in red wines.

Experiment 2: to assess the production of stable pigments through sequential fermentations using non-*Saccharomyces* yeasts together with the yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. To evaluate the influence of both yeasts in the pigment's stability after the addition of three different flavanols to fresh musts (catechin, procyanidin B2 and procyanidin C1).

Experiment 3: to study the formation of polymeric pigments by following up the condensation reaction of malvidin-3-*O*-glucoside with 4 flavanols with different molecular structure. To assess the microbial contribution to the polymerization reaction in three different fermentation scenarios: 1) pure culture with *Saccharomyces cerevisiae*, 2) sequential fermentation with *Lachancea thermotolerans* and *Saccharomyces cerevisiae*, and 3) sequential fermentation with *Lachancea thermotolerans* and *Schizosaccharomyces pombe*.

Experiment 4: to assess the ability of phenolic aldehydes in either acting as nucleophiles and forming pyranoanthocyanin structures or, taking place in anthocyanin-flavanol condensation through covalent bridge formation. Media wine solution with commercial anthocyanin was used to evaluate five different food-grade phenolic aldehydes.

Experiment 5: to evaluate the potential that pulsed light (PL) has in controlling wild microorganisms, both bacteria and yeasts, in grapes; the reduction of microorganisms in grape skins may facilitate the growth and development of yeast starter in wine production. To assess the repercussion of grape processing with PL in red wine's quality: colour and aroma profile.

2. MATERIALS AND METHODS

2.1 Fermentation media

The liquid media used in each of the experimental set ups met the requirements needed for each evaluation. In the Experiments 1 and 2 the fermentation media used was Tempranillo pressed must obtained from red grapes got from Bodegas Comenge (**Figure 14**), a winery located in Curiel de Duero (Valladolid, Spain), in the DO Ribera del Duero, from the vintage 2012; Tempranillo grapes from the same winery but from the vintage 2016 were used in Experiment 5 after pulsed light treatment; experimental set up in article 3 used corrected white must and, Experiment 4 used hydro-alcoholic solution as model wine solution.

Figure 14 *Vitis vinífera* L. cv. Tempranillo from Bodegas Comenge vineyard. Curiel de Duero, Valladolid, Spain.

The conditions and preparation procedure for each of these fermentation media is detailed hereafter:

2.1.1 Tempranillo pressed must

Red grapes must, *Vitis vinífera* L. cv. Tempranillo was prepared from the vintage 2012. The grapes were crushed in a 3L Erlenmeyer flask and the must was obtained by pressing the crushed grapes manually using a sterile gauze to avoid the seeds and skins to get into the must (**Figure 15**). The must had a density of 1101 g/dm³ and pH was adjusted to a value of 3.5

after adding 2.4 g of L(+)-tartaric acid (Panreac Química; Barcelona, Spain). The alcohol potential for this must was 14.1% v/v according to its sugar content.

Figure 15 Must preparation. From left to right: frozen Tempranillo grapes; crushed grapes in Erlenmeyer flask; sterile gauze to press and filter; manual press of crushed grapes.

The must was sterilized in autoclave at 100° C during 1 min in 1L containers. Microbiological control analysis showed no presence of yeast colonies (100 in 100 μ l) and 7 FCU/mL of mesophilic bacteria in the must prior the inoculation of any yeast strain. The sterile must has been used according to each experimental design set up and are described below:

Experiment 1

The must was divided into three sets of 125 ml in Erlenmeyer flasks; one was used as control and the other two had added flavanols. The flavanols used in this experimental design were (+)-Catechin (CAS: 154-23-4 and purity of 99%) and procyanidin B2 (CAS: 29106-49-8 and purity of 90%) (Cymit Química S.L., Barcelona, Spain). The dosage of these two flavanols used in the musts was 80 and 40 mg/L respectively.

Experiment 2

The must was divided into 3 Erlenmeyer flasks with capacity of 900 mL and a 4th one with 150 mL. Each flask corresponded with one treatment, and they were named after the flavanol used in each treatment as follows: Control, (+)-Catechin, ProcyanidinB2 and ProcyanidinC1. The flavanols were used at a constant concentration of 40 mg/L: (+)-catechin (CAS: 154-23-4 and purity of 99%), procyanidin B2 (CAS: 29106-49-8 and purity of 90%) and procyanidin C1 (CAS: 37064-30-5 and purity of >95%) (Cymit Química S.L., Barcelona, Spain).

Experiment 5

The grape must used in this experimental design was obtained from fresh Tempranillo grapes collected right before harvest time during vintage 2016. In this article, part of the Tempranillo grapes under study were treated with pulsed light (PL) while the rest were kept without treatment. After PL treatments, the vacuum-packed grapes were examined in a laminar flow cabinet; the grapes were gently crushed (**Figure 16**) and transferred to sterilized 250 mL Erlenmeyer flasks. Aliquots of 5 mL were taken to sample the wild microbial population found in the Tempranillo grapes skin.

Figure 16 Grapes manually crushed inside the vacuum package for later fermentation.

2.1.2 Concentrated must

Concentrated must from *Vitis vinifera* L. cv. Airén from the vintage 2016 was used in Experiment 3. The must was corrected in order to have a density of 1100 g/dm³ and a pH of 3.4; the pH was reached by adding 0.79 g/L of L(+)-tartaric acid (Panreac Química; Barcelona, Spain). In function to the 23.4 ° Brix obtained in the corrected must, the potential alcohol volume after fermentation is expected to be 13.8 % v/v. The must was poured in 3 L Erlenmeyer flask and then sterilized in autoclave at 100 °C during 1 min. The microbiological

analysis of the must prior the addition of malvidin and flavanols showed absence of both yeast colonies (100 in 100 μ l) and mesophilic bacteria.

The pigment studied in this article was malvidin-3-*O*-glucoside (CAS: 643-84-5) which was added to the sterile must in a laminar flux hut in order to have an initial concentration of 40 mg/L (**Figure 17**). The must was divided into 4 flasks in order to prepare the four different media for the experiment. Each media had one of the flavanols to be evaluated: (+)-Catechin (CAS: 154-23-4 and purity of 99%) (CAT), Procyanidin B2 (CAS: 29106-49-8 and purity of 90%) (PB2), Procyanidin C1 (CAS: 37064-30-5 and purity of >95%) (PC1) and procyanidin A2 (CAS: 41743-41-3) (PA2) (Cymit Química S.L., Barcelona. Spain). The dose of all the flavanols added was at a concentration of 20 mg/L.

Figure 17 Sterile must during the addition of malvidin-3-*O*-glucoside (left) and after homogenization (right).

2.1.3 Model wine solution

The hydro-alcoholic solution prepared as model wine solution was used in the experimental design followed in Experiment 4. Ultrapure water was mixed with ethanol up to 14% v/v and the pH was adjusted with L(+)-tartaric acid (Panreac Química; Barcelona, Spain) in order to get acidity values similar to those found in red wine (3.5). The must was coloured with natural grape powder – enocyanin – (EV-12 Red Shade, E-163) from the company Secna (Valencia, Spain). The dose of enocyanin used in the wine model solution was 100 mg/L.

The must was divided into five different flasks each containing one of the phenolic aldehydes to be tested. The aldehydes were used in this experimental design were: cinnamaldehyde 98+% A14689 (Alfa Aesar, Germany), phenylacetaldehyde 95% A14263 (Alfa Aesar,

Germany), 3-phenylpropionaldehyde P0217 (TCI, Germany), syringaldehyde S29672 (Merck, Germany), 4-hydroxy-3-methoxybenzaldehyde 99% (Aldrich, Germany). These five treatments had a molar ratio of 1:10 with respect to the powder pigment; the molar concentration of the enocyanin was calculated from the molecular weight of the malvidin-3-*O*-glucoside, the main component in the skin of *Vitis vinifera* grape varieties. Another set of samples with no addition of aldehydes was used as control to assess the evolution of pigments in such conditions.

The treatments were named after the aldehydes used as follows: 1) control (CTRL), 2) cinnamaldehyde (CINN), 3) phenylacetaldehyde (PHAC), 4) 3-phenylpropionaldehyde (PHPR), 5) syringaldehyde (SYRN) and 6) 4-hydroxy-3-methoxybenzaldehyde (HMBZ). The wine model solutions were kept in the dark at constant 20 °C in 100 mL brown glass sealed flasks for 1 month. One sample of 2 mL was taken weekly during this time to perform HPLC-DAD-ESI/MS and colour parameters determination with UV-Vis spectrophotometry. Samples had to be filtered with 45 mm methylcellulose membranes prior the analysis (**Figure 18**).

Figure 18 Media wine samples prepared for HPLC-DAD-ESI/MS analysis. 1) Control; 2) PHAC; 3) CINN; 4) HMBZ; 5) SYRN and 6) PHPR.

2.2 Yeast populations

All the non-*Saccharomyces* yeasts as well as the strain of yeast *Saccharomyces cerevisiae* used in the experimental designs during the doctoral thesis are summarized in **Table 3**. The yeasts were used in all experimental designs except the one in Experiment 4 where no fermentation was carried on.

Table 3 List of yeasts used in the experimental set up by experiment (+).

Yeast	Strain	Exp. 1	Exp. 2	Exp. 3	Exp. 5
<i>Saccharomyces cerevisiae</i>	7VA ¹	+	+	+	+
<i>Schizosaccharomyces pombe</i>	938 ²	+	+	+	
<i>Saccharomycodes ludwigii</i>	979 ²	+			
<i>Torulaspora delbrueckii</i>	7013 ³	+	+		
<i>Metschnikowia pulcherrima</i>	L1781 ⁴		+		
<i>Lachancea thermotolerans</i>	Kt 421 ⁵		+	+	

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The yeast strains we have worked with were grown and maintained in YEPD agar media in slants under constant 4 °C at the Laboratory of the Food Technology Department (UPM, Madrid, Spain). At the beginning of every experimental design it was needed to produce liquid cultures out of the pure yeast culture slant collections. The liquid cultures prepared with each of the beforementioned yeasts is similar to that used for yeast growing in slants except that it does not contain agar, therefore the media remains liquid. In this way, the standard media used to prepare the slants and the liquid media had 1% yeast extract (Laboratorios Conda; Madrid, Spain), 2% peptone (Laboratorios Conda; Madrid, Spain), 2% dextrose (Panreac Química; Barcelona, Spain) and 2.4% agar (Panreac Química; Barcelona, Spain) (just for slants or plates). Depending on the amount of must to be fermented, the liquid culture could be prepared in glass tubes (**Figure 19**) or in small volume Erlenmeyer flasks.

Figure 19 Liquid culture for yeast propagation. Yeast grown in glass culture tubes for 48h (left), liquid culture mixing for homogenization (middle) and culture ready for most inoculation (right).

The liquid media has been sterilized in autoclave at 121 °C for 15 min. With the help of an inoculation loop, yeasts were transferred from the slants into the liquid media and kept in the orbital mixer at constant 24 °C for 48 hours prior inoculation.

The yeasts species and the purity of the cultures were determined by optical microscopy and by selective lysin plate for non-*Saccharomyces* yeasts. The optical microscope allows the researcher to observe the morphology of the yeasts and assure the purity of the culture to be used. The yeast strains shown in **Figure 20** had different morphology, therefore it was possible to identify the species and to assess the purity of the cultures. *Saccharomyces cerevisiae* have rounded morphology and most of them has visible spores inside; the strain of *Schizosaccharomyces pombe* have elongated cylindrical morphology with visible septum formation previous binary fission (Suárez-Lepe et al., **2012**) in some cases; the strain of *Saccharomycodes ludwigii* have a typical apiculate morphology resembling lemon fruits, most cells have typical stationary phase which nucleoids appeared as discrete dots dispersed in the cytoplasm (Miyakawa, Nakahara, & Ito, **2012**).

The plate with lysine medium CM0191 (OXOID LTD.; Basingstoke, England) allows non-*Saccharomyces* yeasts to grow and inhibit, in the same time, *Saccharomyces cerevisiae* strains from growing. This methodology was used in Article 3 when sequential fermentations were on going and it was needed to count the population of both species.

Figure 20 Optical microscope images of (A) *Saccharomyces cerevisiae*, (B) *Schizosaccharomyces pombe* and (C) *Saccharomyces ludwigii* with 650 X objective.

The yeast populations were assessed by microscopy Thoma cell counting chamber and by plate counting. The Thoma cell counting chamber allows the researcher to select different measurement areas in function of the size of the yeast population to be observed by reducing the number of cells by area; in this way the measurement area could be adjusted to 1 mm², 0.04 mm² or 0.0025 mm². The yeast concentration is then calculated by the equation (Ec. 1) shown below.

$$C = (\text{Total yeast observed} / \text{No. squares measured}) \times N \quad (\text{Ec. 1})$$

C = yeast concentration

N = 10 000 (1 mm² squares); 250 000 (0.04 mm² squares); 4x10⁶ (0.0025 mm² squares)

The counting plate technique uses sterile YEPD agar poured into petri glasses while liquid. Once the agar has set and the media is solid, a known volume with an also known dilution is

spread all across the plate. The dilution should be enough for single cells to grow into colonies differentiable among them. One technique to assess this is by using serial dilution using the previous dilution as inoculum for the new dilution in order to promote a dilution of cells by exponentially growing factor. **Figure 21** is an example of a petri dish were a serial 10-fold dilution of saturated cultures from musts under fermentation were spotted on YPD agar.

Figure 21 Petri dish with 10-fold dilutions for yeast population determination.

2.3 Micro-fermenters

During the experimental designs, two different size of micro-fermenters were used (**Figure 22**). The larger volume allowed us to carry on sensory analysis whilst the smaller flasks were used only for comparison of analytical parameters.

Figure 22 Two different volume micro-fermenters used in the experimental fermentation set-ups. To the left the 10mL fermenter with needle and to the right, the 60mL fermenter with Müller valve.

The conditions in which these two fermenters were used during the span of the experimental trials are described hereafter:

Experiment 1

In this experimental design the musts were divided into 10 mL micro-fermenters by triplicate; the musts were inoculated with 100 μ l liquid culture of each strain previously grown in liquid media which populations were between 1.26E+07 and 1.56E+07 UFC/ml. The strains used were *Saccharomyces cerevisiae* (Sc) strain 7VA (Department of Food Science and Technology, UPM, Madrid, Spain), *Schizosaccharomyces pombe* (Sp) strain 938 (IFI-CSIC, Spain), *Saccharomycodes ludwigii* (Sl) strain 979 (IFI-CSIC, Spain) and *Torulaspora delbrueckii* (Td) strain 1880 (LYCC 7013). All the strains were evaluated in pure fermentation trials except for *Torulaspora delbrueckii* (Td), which was assayed in a sequential fermentation with *Saccharomyces cerevisiae* (Sc) in order to obtain similar alcohol volume by the end of the fermentation. In the case of this sequential fermentation, the second inoculation with the strain of Sc was carried out 4 days after the beginning of the fermentation. All micro-fermenters were placed in an oven at a constant 25°C after being weighed. The fermentation kinetics as well as the fermentative potential were followed by measuring the variations in weight for each vial until reaching a steady weight. Fermentations spanned 12 days.

Experiment 2

The musts were divided into 60 mL and 10 mL micro-fermenters for fermentation volumes of 50 and 10 mL and then added with 750 µL and 120 µL of inoculum of each non-*Saccharomyces* medium fermentative power yeast strain respectively and by triplicate (*Lachancea thermotolerans* (Lt) strain CH Kt 421 (Viniflora®, Chr-Hansen), *Metschnikowia pulcherrima* (Mp) strain LAMAP-USACH L1781 and *Torulaspora delbrueckii* (Td) strain 7013 (LYCC, Canada). Micro- fermenters were sealed with Müller valves or with needle (**Figure 22**) and placed at constant 25° C after being weight. After the 5th day a second inoculum with yeasts *Saccharomyces cerevisiae* (Department of Food Science and Technology, UPM, Madrid, Spain) or *Schizosaccharomyces pombe* strain 938 (IFI-CSIC, Spain) was added to all trials to finish fermenting residual sugars and to achieve higher alcohol volume. The fermentation kinetics was followed until steady weight was reached. Fermentations spanned up to 21 days.

Experiment 3

Micro-fermenters with capacity of 60 mL volume were used in this experimental set up. The model solutions were inoculated with 750 µL of either *Saccharomyces cerevisiae* (Sc) strain 7VA (trial 1) or *Lachancea thermotolerans* (Lt) strain Kt 421 (trials 2 and 3) once they were poured into the 60 mL flasks. All flasks were sealed with a Müller valve filled with sulphuric acid and kept in an oven at constant 25 °C. A sequential fermentation was done after the 7th day by inoculating 750 µL of liquid culture of Sc (trials 1 and 2) and *Schizosaccharomyces pombe* (Sp) strain 938 (trial 3) as to achieve similar alcohol volume at the end of the fermentation. The kinetics of the fermentation were followed up by registering the weight of the micro-fermenters since day 0 and until a steady weight was noticed. The fermentation period spanned 14 days for the pure culture fermentation (only *Saccharomyces cerevisiae*) while the sequential fermentations *Lachancea thermotolerans* – *Saccharomyces cerevisiae* and *Lachancea thermotolerans* – *Schizosaccharomyces pombe* spanned 21 days.

Experiment 5

This experimental set up used 250 mL Erlenmeyer flasks as fermenters. The flasks were closed with Müller valves filled with sulphuric acid as to allow CO₂ to escape from the fermenter and to avoid microbiological contamination. The must obtained from each vacuumed package was inoculated with a liquid culture of the yeast *Saccharomyces*

cerevisiae strain 7VA (Department of Food Science and Technology, UPM, Madrid, Spain). The yeast population was estimated in 10⁸ CFU/mL. The musts were fermented in the Erlenmeyer flasks isothermally at 22° C without any SO₂ addition. Parallel to the inoculated musts, there were controls for spontaneous fermentation due to wild yeast population in both scenarios, the grapes non-treated with PL and the grapes treated at medium (fme) and maximum (fME) energies with five and ten flashes; they were named after the treatments as 5fme, 10fme, 5fME and 10fME respectively. The PL treatment was performed in Facultad de Veterinaria (UCM, Madrid, Spain) with the use of a double xenon lamp SteriBeam SBS XeMatic-2L-A PL device (SteriBeam Systems, Kehl am Rhein, Germany) (**Figure 23**). The samples in the vacuumed plastic bags were irradiated on both faces.

Figure 23 Pulsed light equipment. The size allowed to irradiate 20 cm x 14cm size samples.

A follow up of the growth of yeast and bacteria populations was performed by taking samples at day 0 and at day 15, the end of fermentation. The populations were estimated by counting plates.

2.4 Anthocyanin determination (HPLC-DAD-ESI/MS)

All the experimental wines produced, as well as the wine media solutions evaluated, were filtered with a 45 µm methylcellulose membrane in order for them to be analysed with the high-performance liquid chromatograph (HPLC) coupled with a mass spectrometer as it is shown in **Figure 24**. The filtered samples were poured into 2 mL clear glass vials sealed with

9 mm PTFE/silicon septum caps (Phenomenex, Torrance, CA, USA). After anthocyanins determination, the samples were used for colour parameters determination with UV-Vis spectrophotometry.

Figure 24 Sample preparation for anthocyanin determination with HPLC.

The non-acylated monomeric anthocyanins, the derived acylated anthocyanins, the pyranoanthocyanins including vitisins and vinylphenolics as well as the oligomeric and polymeric pigments were identified in all experimental wines and media solutions by using an Agilent Technologies (Palo Alto, CA, USA) series 1100 HPLC chromatograph equipped with a diode array detector (DAD) and a quadrupole mass spectrometer with an electrospray interface (MS-ESI) (**Figure 25**). The solvents used for the pigments separation were A (water/formic acid, 95:5 v/v) and B (methanol/ formic acid, 95:5 v/v) with the following gradient: 0–2 min, 15% B; 2– 10 min, 15–50% B linear; 10–12 min, 50% B; 12–13 min, 50–15% B linear; and 13–15 min, re-equilibration; the working flow was 0.8 mL/min. The column used for the pigments separation was a reverse-phase Poroshell 120 C18 column (Phenomenex, Torrance, CA, USA) with the following dimensions (50 mm × 4.6 mm; particle size 2.7 µm). Detection was performed by scanning in the 400–600-nm range.

Figure 25 High-performance liquid chromatograph (HPLC) coupled with a mass spectroscopy module (MS) for anthocyanins identification.

To quantify anthocyanins and derived pigments we have done a comparison against malvidin-3-*O*-glucoside expressed as milligram per litre used as an external standard at a signal of 525 nm ($r^2 = 0.9999$). All the pigments were identified by comparison with literature of the retention time, their distinctive maximum absorption wavelength in the visible spectra and their ion mass. The mass spectrometry was performed in positive scanning mode (m/z from 100 to 1500, fragmentor voltage 150 V from 0 to 15min). The injection volume was set to 20 mL of the previously filtered samples. The detection limit was 0.1 mg/L.

Table 4 summarizes the anthocyanins and derived pigments found in red wine produced with Tempranillo grapes (Monagas et al., 2003) that was used as reference for monomeric anthocyanin glucosides and derivatives identification in experimental wines. Although the maximum wavelength of absorption ($\lambda_{\max}$) observed for most of the non-acylated anthocyanin glucosides during the experimental analysis was slightly larger than the ones reported by Monagas et al. (2003); the $\lambda_{\max}$ obtained in our analysis for the malvidin-3-*O*-glucoside was 528 nm, 518 nm for cyanidin-3-*O*-glucoside and 520 nm for peonidin-3-*O*-glucoside.

Table 4 Anthocyanins and anthocyanin derived pigments found in Tempranillo wines and identified by LC-ESI/MS by Monagas et al (2003).

Anthocyanin	$\lambda_{\max}$ nm	$[M]^+$ (<i>m/z</i>)	Fragments (<i>m/z</i>)
Malvidin-3- <i>O</i> -glucoside-(epi)catechin	530	781	
Delphindin-3- <i>O</i> -glucoside	524	465	303
Cyanidin-3- <i>O</i> -glucoside	515	449	287
Petunidin-3- <i>O</i> -glucoside	526	479	317
Peonidin-3- <i>O</i> -glucoside	516	463	301
Malvidin-3- <i>O</i> -glucoside	520	493	331
Delphinidin-3- <i>O</i> -(6''-acetylglucoside)	507	507	303
Malvidin-3- <i>O</i> -glucoside pyruvate	513	561	399
Cyanidin-3- <i>O</i> -(6''-acetylglucoside)	516	491	287
Malvidin-3- <i>O</i> -glucoside-ethyl-(epi)catechin	543	809	
Petunidin-3- <i>O</i> -(6''-acetylglucoside)	532	521	317
Malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside) pyruvate	513	707	399
Peonidin-3- <i>O</i> -(6''-acetylglucoside)	520	505	301
Delphinidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	532	611	303
Malvidin-3- <i>O</i> -(6''-acetylglucoside)	530	535	331
Peonidin-3- <i>O</i> -(6''-caffeoylglucoside)	524	625	301
Malvidin-3- <i>O</i> -(6''-caffeoylglucoside)	536	655	331
Cyanidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	527	595	287
Malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside) <i>cis</i>	537	639	331
Petunidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	532	625	317
Peonidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	524	609	301
Malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside) <i>trans</i>	535	639	331

The vinylphenolic pyranoanthocyanins were identified in accordance with Morata, González and Suárez-Lepe (2007). The most common compounds, also found in our experimental wines, are shown in **Table 5**.

Table 5 Vinylphenolic pyranoanthocyanins identified with HPLC-DAD-ESI/MS by Morata et al. (2007)

Vinylphenolic pyranoanthocyanin	$\lambda_{\max}$ nm	$[M]^+$ (m/z)	Fragments ¹ (m/z)
Malvidin-3- <i>O</i> -glucoside-4-vinylcatechol	514	625	463 / 187
Malvidin-3- <i>O</i> -glucoside-4-vinylguaiacol	504	639	477 / 187
Malvidin-3- <i>O</i> -glucoside-4-vinylphenol	504	609	447 / 187
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-4-vinylphenol		651	447 / 187
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-4-vinylguaiacol		681	477 / 187

¹Aglycone fragment / Pyranoanthocyanin core fragment

In the case of larger molecules, the oligomers and polymeric pigments were identified with regard to the information provided by Asenstorfer, Hayasaka and Jones (2001), He et al. (2012) and Remy, Fulcrand, Labarbe, Cheynier and Moutounet (2000). Some of the pigments identified by these authors as well as their $\lambda_{\max}$ and molecular ion and fragment ions are listed in **Table 6**.

Table 6 Oligomeric anthocyanins identified by Asenstorfer et al. (2001), He et al. (2012) and Remy et al. (2000).

Anthocyanin oligomeric compound	$\lambda_{\max}$ nm	$[M]^+$ (m/z)	Fragments (m/z)
Peonidin-3- <i>O</i> -glucoside-4-vinyl(epi)catechin	499	775	613
Delphinidin-3- <i>O</i> -glucoside-4-vinyl(epi)catechin	501	777	615
Peonidin-3- <i>O</i> -glucoside-8-ethyl-(epi)catechin		779	
Malvidin-3- <i>O</i> -glucoside-(epi)catechin		781	619
Petunidin-3- <i>O</i> -glucoside-4-vinyl(epi)catechin	502	791	629
Petunidin-3- <i>O</i> -glucoside-8-ethyl-(epi)catechin		795	633,505
Malvidin-3- <i>O</i> -glucoside-4-methyl-(epi)catechin		795	505
Malvidin-3- <i>O</i> -glucoside-4-vinyl(epi)catechin	503	805	643
Malvidin-3- <i>O</i> -glucoside-8-ethyl-(epi)catechin		809	657,331
Peonidin-3- <i>O</i> -(6''-acetylglucoside)-4-vinyl(epi)catechin	501	817	613

Delphinidin-3- <i>O</i> -(6''-acetylglucoside)-4-vinyl(epi)catechin	503	819	615
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-(epi)catechin		823	619,493,331
Petunidin-3- <i>O</i> -(6''-acetylglucoside)-4-vinyl(epi)catechin	504	833	629
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-4-vinyl(epi)catechin	508	847	643
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-8-ethyl-(epi)catechin		851	
Peonidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)-8-ethyl-(epi)catechin		925	635,617
Malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)-(epi)catechin		927	619,331
Malvidin-3- <i>O</i> -glucoside-cyanidin-3- <i>O</i> -glucoside		941	779,491
Malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)-4-vinyl(epi)catechin	503	951	643
Malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)-8-ethyl(epi)catechin		955	803,341
Malvidin-3- <i>O</i> -glucoside-peonidin-3- <i>O</i> -glucoside		955	793,331
Malvidin-3- <i>O</i> -glucoside-delphinidin-3- <i>O</i> -glucoside		957	795,331,303
Malvidin-3- <i>O</i> -glucoside-petunidin-3- <i>O</i> -glucoside		971	809,331,317
Malvidin-3- <i>O</i> -glucoside-malvidin-3- <i>O</i> -glucoside		985	823,331
Malvidin-3- <i>O</i> -glucoside-peonidin-3- <i>O</i> -(6''-acetylglucoside)		997	631,303
Malvidin-3- <i>O</i> -glucoside-delphinidin-3- <i>O</i> -(6''-acetylglucoside)		999	331,303
Malvidin-3- <i>O</i> -glucoside-petunidin-3- <i>O</i> -(6''-acetylglucoside)		1013	809,331
Malvidin-3- <i>O</i> -glucoside-malvidin-3- <i>O</i> -(6''-acetylglucoside)		1027	865,331
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-petunidin-3- <i>O</i> -(6''-acetylglucoside)		1055	521,317

Malvidin-3- <i>O</i> -glucoside-PC dimer	1069	907,781
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-malvidin-3- <i>O</i> -(6''-acetylglucoside)	1069	331
Malvidin-3- <i>O</i> -glucoside-cyanidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	1087	617,287
Malvidin-3- <i>O</i> -glucoside-4-divinyl(epi)catechin	1093	
Malvidin-3- <i>O</i> -glucoside-4-ethyl-PC dimer	1097	519
Malvidin-3- <i>O</i> -glucoside-peonidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	1101	793,331
Malvidin-3- <i>O</i> -glucoside-delphinidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	1103	795,331
Malvidin-3- <i>O</i> -glucoside-4-propyl-PC dimer	1111	959
Malvidin-3- <i>O</i> -glucoside-petunidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	1117	809,317
Malvidin-3- <i>O</i> -glucoside-malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	1131	823,331
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	1173	865,331
(Epi)catechin-cyanidin-3- <i>O</i> -glucoside-malvidin-3- <i>O</i> -glucoside	1229	1067,917
(Epi)catechin-peonidin-3- <i>O</i> -glucoside-malvidin-3- <i>O</i> -glucoside	1243	1081
(Epi)catechin-delphinidin-3- <i>O</i> -glucoside-malvidin-3- <i>O</i> -glucoside	1245	1083,795
(Epi)catechin-petunidin-3- <i>O</i> -glucoside-malvidin-3- <i>O</i> -glucoside	1259	1097,671
(Epi)catechin-malvidin-3- <i>O</i> -glucoside-malvidin-3- <i>O</i> -glucoside	1273	1111,949
Malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)-malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)	1277	661,639
Malvidin-3- <i>O</i> -glucoside-4-trivinyl(epi)catechin	1381	
Malvidin-3- <i>O</i> -(6''-acetylglucoside)-4-trivinyl(epi)catechin	1423	

Malvidin-3- <i>O</i> -(6''- <i>p</i> -coumaroylglucoside)-4-trivinyl(epi)catechni	1527
Malvidin-3- <i>O</i> -glucoside-4-tetravinyl(epi)catechin	1669

2.5 Fermentative volatiles (GC-FID)

The fermentative volatile compounds were identified and characterized by gas chromatography with flame ionization detector (GC-FID). The chromatograph used was an Agilent Technologies™ 6850 (Palo Alto, CA, USA) equipped with a column DB-624 which dimensions are (60 m x 250 μ m x 1.4 μ m) (**Figure 26**). The temperature of the injector was set at 250 °C while the temperature in the detector was set at 300 °C. The temperature ramp during the measurement went from steady 40 °C for 5 min to 250 °C with a gradient of 10 °C/min. The temperature was then kept for 5 more minutes. The carrying gas used in this technique was hydrogen with a flow of 2.2 L/min and a split ratio of 1:10.

Figure 26 Gas chromatograph Agilent Technologies 6850 with automatic sampler.

For the quantification of the fermentative volatiles, we have added 100 μ L of a solution of 4-methyl-2-pentanol with concentration of 500 mg/L into 1 mL of previously membrane-

filtered samples (**Figure 24**); this compound has been used as internal standard which calibration was performed following Abalos, Vejarano, Morata, González and Suárez-Lepe (**2011**). All other fermentative volatiles were also calibrated as suggested by these authors. The detection limit was 0.1 mg/L. The fermentative volatile compounds were identified with respect to their retention time (t_R) which order, and CAS numbers are shown in **Table 7**. The concentration of these compounds was calculated with the individual calibration curves and comparison with the internal standard.

Table 7 List of fermentative volatile compounds calibrated for GC-FID determination.

Volatile compound	CAS Number*	t_R (min)	Calibration R^2
Acetaldehyde	75-07-0	3.550	0.99973
Methanol	67-56-1	3.788	0.99917
1-propanol	71-23-8	8.044	0.99938
Diacetyl	431-03-8	8.613	0.99977
Ethyl acetate	141-78-6	8.870	0.99980
2-butanol	78-92-2	9.108	0.99954
Isobutanol	78-83-1	9.970	0.99941
1-butanol	71-36-3	10.940	0.99932
Acetoin	513-86-0	12.600	0.99949
2-methyl-1-butanol	137-32-6	12.700	0.99925
3-methyl-1-butanol	123-51-3	12.780	0.99958
Isobutyl acetate	110-19-0	13.050	0.99987
4-methyl-2-pentanol	108-11-2	13.130	1.00000
Ethyl butyrate	105-54-4	13.590	0.99987
Ethyl lactate	687-47-8 ⁺	14.470	0.99916
2-3-butanediol	513-85-9	14.720	0.99109
Isoamyl acetate	123-92-2	15.270	0.99987
Hexanol	111-27-3	15.580	0.99935
2-phenylethyl alcohol	60-12-8	20.620	0.99460
2-phenylethyl acetate	103-45-7	22.350	0.99933

t_R – Retention time

*Chemical Abstract Service (CAS) register number.

⁺L-isomer

2.6 Colour parameters (UV-Vis Spectrophotometry)

In all the articles the colour of the trials has been determined with the use of a UV-visible spectrophotometer 8453 from Agilent Technologies™ (Palo Alto, CA, USA) with a photodiode array detector (**Figure 27**) and the use of a 1 mm path length cuvette to avoid signal saturation; the absorption was corrected afterwards by multiplying by 10. With a program collecting data at fixed absorption wavelengths (420 nm, 520 nm and 620 nm) allowed us to compare colour intensity (CI) and hue (tonality) in all experimental wines and model media. From the same data it was possible to express the colour of the samples in terms of percentage of yellow (%Y), red (%R) and blue (%B) (Kulkarni et al., **2015**).

***Figure 27** UV-Vis spectrophotometer. Module arrangement (left) and 1 mm path cuvette for experimental wine characterization (right).*

In Experiment 4, besides the characterization performed with basic UV-Vis colour parameters, a characterization of the change in colour between samples and with respect to the control trial using CIE *Lab* coordinates, was done according to Gordillo, Rodríguez-Pulido, Escudero-Gilete, González-Miret and Heredia (**2012**). The colour differences, or colour change, were monitored with the ΔE_{ab} (Eq. 2) as the Euclidian distance between two points in the CIE *Lab* coordinates. The CIE *Lab* coordinates were used to evaluate the differences in L^* (lightness), and C^*_{ab} (chroma) quantitatively; they also allowed to measure

qualitative differences of h_{ab} (hue). All the CIE $L^*a^*b^*$ values were also obtained from the same Agilent Technologies™ spectrophotometer. The equations used for the beforementioned calculations are:

$$\Delta E_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (Eq. 2)$$

$$\Delta h_{ab} = [(\Delta E_{ab})^2 - (\Delta L)^2 + (\Delta C_{ab})^2]^{1/2} \quad (Eq. 3)$$

$$\% \Delta L = [(\Delta L)^2 / (\Delta E_{ab})^2] \times 100 \quad (Eq. 4)$$

$$\% \Delta C = [(\Delta C)^2 / (\Delta E_{ab})^2] \times 100 \quad (Eq. 5)$$

$$\% \Delta h = [(\Delta h_{ab})^2 / (\Delta E_{ab})^2] \times 100 \quad (Eq. 6)$$

2.7 Ethanol Quantification (LC-RI)

Ethanol was determined by liquid chromatography with refractive index detection (LC-RI) using a Waters e2695 apparatus (Milford, MA, USA) equipped with a 2414 Refractive Index Detector (**Figure 28**). The separation column is a Phenosphere XDB C18 column with the following dimensions (4.6 mm × 150 mm, 5-μm particle size) (Phenomenex, Torrance, CA, USA). The solvent used is ultrapure water in isocratic mode at a flow of 0.4 mL/min. The temperature of the column and the detector was set at 30 °C. The identification is done by retention time and the quantification was performed against an external standard calibration curve done with ethanol 99.9% (Panreac, Barcelona, Spain). The sample preparation is the same as the one followed for anthocyanins and fermentative volatile determination; the samples should be filtered with a methyl-cellulose membrane with 0.45 μm porous size (Teknokroma, Barcelona, Spain). The injection volume was 2 μL.

Figure 28 Liquid chromatograph with refractive index detector (LC-RI) used for ethanol determination.

2.8 Oenological parameters by FT-IR

Residual sugars (glucose and fructose), organic acids (L-malic acid, L-lactic acid), volatile acidity (acetic acid), the total acidity and pH were analysed using OenoFOSS™ analyser (**Figure 29**) that makes rapid analysis of wines and musts under fermentation. The analyser uses Fourier transform infrared (FTIR) technology which executes scanning in the near infrared spectra.

Figure 29 Oeno FOSS™ equipment used for rapid ethanol, acidity and sugar analysis in wines.

CO₂ should be removed from the wine samples in order to avoid interferences with the scan. To remove CO₂, all samples are stirred with the use of a vortex mixer for 1 min in test tubes to allow CO₂ release.

2.9 Sensory analysis

A sensory analysis of experimental wines was performed as part of the experimental design in Experiments 1, 2 and 5 through a wine tasting session. Each wine tasting session had a panel with at least 10 experts. The panels were composed by adult members of the Chemistry and Food Department of the Technology School of Agricultural, Food and Biosystems Engineering (UPM, Madrid, Spain) with over 5 years of experience in wine tasting. The panel had members from both genders with ages between 28 and 54 years old and without the use of any standard kit. Each evaluator had a tasting sheet for the assessment of 15 different attributes; these basic wine descriptors were previously agreed by consensus of the panel and rated with a 5-point scale from low perception (1) to high perception (5) except for the parameter hue which was evaluated from red/purple – young wines (1) to orange/brick red – aged wines (5).

The wine descriptors evaluated by the panellists per wine tasting sheet comprised: colour intensity, hue, aroma intensity, aroma quality, herbs, flowers, fruity, reduction, oxidation, body, astringency, bitterness, general acidity, aftertaste and overall impression (**Figure 30**).

Tester name:	Date:				
Sample code:					
	1	2	3	4	5
Colour intensity					
Hue/Tonality					
Aroma intensity					
Aroma quality					
Herbs					
Flowers					
Fruity					
Reduction					
Oxidation					
Body					
Astringency					
Bitterness					
General acidity					
Aftertaste					
Overall impression					

Figure 30 Wine tasting sheet with appearance, nose and palate attributes.

The wine tasting sessions took place in a testing room with white walls and 21 °C temperature located in ETSIAAB at Department of Food Technology (UPM, Madrid, Spain). Each evaluator had a white paper tablecloth to be able to compare colour intensity and hue of each sample. All the samples had an arbitrary code assigned as identifier which was unknown by the evaluators.

2.9 Statistical analysis

In all data presented in the articles, the means and standard deviations were estimated, and the differences examined using ANOVA and the least significant difference (LSD) test. In some cases, a principal component analysis (PCA) was carried out in order to obtain uncorrelated variables or principal components from a larger possible-correlated set of variables to explain the differences observed in the data obtained. All calculations were made using PC Statgraphics v.XI software (Graphics Software Systems, Rockville, MD, USA). The significance was set at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Oenological parameters

3.1.1 Experiment 1

Organic acids such as malic acid, lactic acid, tartaric acid (total acidity), acetic acid (volatile acidity) and residual sugars (glucose and fructose), as well as pH and the alcohol (% v/v) were determined for each wine after fermentation was completed with FTIR. The species *Schizosaccharomyces pombe* is capable of doing maloalcoholic fermentation (Suárez-Lepe et al., 2012) and in this case not only sugars but also malic acid are substrates for yeasts and they are transformed into ethanol during fermentation. Malic acid is rather low for the wine fermented with this fermentative yeast (**Figure 31**). Volatile acidity, on the other hand, has reported values as low as 0.2 g/L in sequential fermentations done with *Saccharomyces cerevisiae* (strain 7VA) and *Torulaspora delbrueckii* (strain 1880) in these experiments while authors like Comitini et al. (2011) have reported larger values between 0.37 and 0.63 g/L. In terms of total acidity, the effect of the demalication and a low volatile acidity production by *Schizosaccharomyces pombe* has resulted in lower total acidity expressed as tartaric acid equivalents.

Figure 31 Oenological parameters for experimental wines. Different letters indicate statistical differences between means ($p < 0.05$) ($n=3$).

The volume of alcohol (% v/v) at the end of the fermentation is similar in all experimental trials, around 14 % v/v although, statistically, there are differences found between fermentative yeasts (**Figure 32**). *Schizosaccharomyces pombe* has yielded the largest amount of ethanol among all other yeasts. As a consequence of the difference observed in malic acid concentration and total acidity, which values are lower for the fermentation done with the species *Schizosaccharomyces pombe* (strain 938), the pH value is higher for the wine fermented with this yeast species (pH slightly higher than 4).

Figure 32 Ethanol volume (% v/v) and pH values for experimental wines. Different letters indicate significant differences between means ($p < 0.05$) for each parameter.

3.1.2 Experiment 2

There is a slight, yet not significant, lower ethanol content for those fermentations done with *Mp* and *Td* in sequential fermentation with *Sp* (12.1-12.3 % v/v vs. >12.5 % v/v) respectively. The pair *Mp-Sp* has led to having the lowest malic acid content (0.3-0.4 g/L) and therefore the highest pH value (3.9) in all treatments (**Figure 33**) and the lowest total acidity value in each treatment (2.3-2.6 g/L).

Figure 33 pH values (A) and malic acid concentration (B) for all treatments per fermentative yeasts.

Suárez-Lepe et al. (2012) have documented the demalication of *Schizosaccharomyces* spp. through which malic acid is transformed into ethanol and CO₂. The lactic acid is present in small amounts in wines fermented with *Mp-Sp* (0.1 g/L) with significant difference in the Control and the (+)-Catechin treatment, while the other treatments with *Mp* had smaller amounts of lactic acid with no significant difference with the rest of the trials. In these trials, despite the results reported by Ciani, Comitini, Mannazzu and Domizio (2010), the fixed acidity (mainly L-lactic acid) was not high for fermentations where *Lt* was used. The amount of residual sugars is significantly higher for the sequential fermentation done with *Td-Sp* for the treatments ProcyanidinB2 and ProcyanidinC1 (5 g/L and 7 g/L respectively).

3.1.3 Experiment 3

The kinetics for the growth of the yeast populations were followed up in this experimental (Figure 34) together with the variations in acetaldehyde and pyruvic acid. There are three

different fermentation designs, one of them being the pure culture of Sc and two other sequential fermentations with Lt + Sc and Lt + Sp. It can be observed that within 3 days Sc as well as Lt have achieved maximum growth with 6.4×10^7 CFU/ml and 4.5×10^7 CFU/ml respectively but did not have stationary phase on either case as observed by Sadineni et al. (2012) for pure culture and co-fermentations of *Saccharomyces cerevisiae*; what it is observed is a rapid decrease for Sc populations after the 7th day to an average of 1×10^5 CFU/ml at day 9. In the case of the sequential fermentation with Sc there is a slightly increase and a steady growth observed in the treatments CAT and PB2, while in the rest of treatments and for Sp there is a slower decrease of yeasts populations over the time until reaching populations between 1×10^5 and 1×10^6 CFU/ml after day 13. This last effect is due to the fact that the increasing concentration of ethanol makes it difficult to surviving for *Lachancea* spp. as it is for several other non-*Saccharomyces* yeasts such as *M. pulcherrima*, *T. delbrueckii* and *C. zemplinina* (González-Royo et al., 2015).

The production of fermentative metabolites followed the kinetics growth having a maximum concentration after 72h although no increase in production of these metabolites was observed after sequential inoculation; pyruvic acid got the maximum concentration in all treatments for both yeasts in the initial phase: Sc got up to 116 mg/L and Lt up to 105 mg/L; regarding acetaldehyde production, the maximum concentration was observed in treatments CAT and PB2 for pure culture Sc (81.1 mg/L and 63.6 mg/L respectively) and steady amount for PC1 and PA2. The concentration of pyruvic acid increased after the inoculation of sequential yeasts Sc (>140 mg/L) and Sp (>110 mg/L) in all treatments except for the pure culture Sc where a steady phase and slight decrease was observed after 72h and over the rest of the fermentation time.

On the other hand, when using Lt, the concentration of acetaldehyde decreased during the initial fermentative phase probably due to the fact that the production of acetaldehyde is proportional to the sugars fermented (Morata et al., 2006) and Lt decrease its metabolism when increasing ethanol concentration. After the inoculation of the sequential yeasts (Sc and Sp); acetaldehyde production increased and got maximums at day 9 (**Figure 34**) with values ca. 40 mg/L after.

Figure 34 Yeast population, pyruvic acid and acetaldehyde concentrations. Analyses performed with GC-FID (acetaldehyde), enzymatic analyser (pyruvic acid) and plate counting (cells growth). Each point represents mean values ($n = 3$). Dotted lines indicate starting time of the sequential fermentation.

The rest of the oenological parameters were measured with FT-IR and LC-RID as described in the materials and methods section. The sequential fermentation done with the species Lt-Sp had residual sugar; both glucose and fructose together are above 4 g/L, which, lead to having slightly lower ethanol content (% v/v) than the rest of the fermentations. Even though there is no statistical difference observed for this parameter. The differences observed in acidic composition depend on the yeasts species used to carry on the fermentations and it is mostly independent of the flavanols used in each treatment (**Table 8**). Volatile acidity as well as total acidity is statistically higher for the fermentations done with Sc (>0.6 g/L) while the pair Lt-Sp had the lowest concentrations for both parameters; these values are similar to those observed by Sun, Gong, Jiang, and Zhao (2014), with more than 0.55 g/L, for pure culture fermentation with the strain of *S. cerevisiae* and mixed fermentation with a strain of *M.*

pulcherrima. On the other hand, the content of lactic acid is statistically higher for those fermentations where *Lachancea thermotolerans* was used as starter culture (Lt-Sc and Lt-Sp). Malic acid content is statistically lower for the pair Lt-Sp and the pH values are therefore higher in these fermentations. The demalication observed in these last trials was performed by *Schizosaccharomyces pombe*, through which malic acid is transformed into ethanol and CO₂ (Suárez-Lepe et al., 2012).

Table 8 Concentration of organic acids and pH values in experimental wine trials. Results are shown by treatment. The statistical analysis was performed by parameter (columns) and the difference in letters indicates significant statistical difference (LSD method with 95% confidence level). Means $\pm$ STD ($n = 3$).

Treatment	pH	Malic Acid	Volatile Acidity	Lactic Acid	Total Acidity
		g/L	g/L	g/L	g/L
<i>(+) - Catechin</i>					
Sc	3.6 $\pm$ 0.0 ^c	2.0 $\pm$ 0.1 ^{ab}	0.6 $\pm$ 0.0 ^{ab}	0.5 $\pm$ 0.1 ^b	5.1 $\pm$ 0.0 ^a
Lt-Sc	3.6 $\pm$ 0.0 ^c	1.7 $\pm$ 0.1 ^d	0.2 $\pm$ 0.0 ^c	0.8 $\pm$ 0.1 ^a	4.9 $\pm$ 0.1 ^{bc}
Lt-Sp	3.8 $\pm$ 0.0 ^a	0.7 $\pm$ 0.1 ^f	0.2 $\pm$ 0.0 ^c	0.8 $\pm$ 0.1 ^a	3.4 $\pm$ 0.1 ^d
<i>PB 2</i>					
Sc	3.6 $\pm$ 0.0 ^c	2.0 $\pm$ 0.1 ^{abc}	0.6 $\pm$ 0.0 ^b	0.6 $\pm$ 0.1 ^b	5.1 $\pm$ 0.1 ^{ab}
Lt-Sc	3.6 $\pm$ 0.0 ^c	1.7 $\pm$ 0.0 ^d	0.2 $\pm$ 0.0 ^c	0.7 $\pm$ 0.0 ^a	4.8 $\pm$ 0.1 ^c
Lt-Sp	3.8 $\pm$ 0.0 ^a	0.7 $\pm$ 0.0 ^f	0.2 $\pm$ 0.0 ^c	0.7 $\pm$ 0.0 ^a	3.4 $\pm$ 0.1 ^d
<i>PC 1</i>					
Sc	3.7 $\pm$ 0.0 ^{bc}	2.1 $\pm$ 0.1 ^a	0.6 $\pm$ 0.1 ^{ab}	0.5 $\pm$ 0.0 ^b	5.2 $\pm$ 0.1 ^a
Lt-Sc	3.6 $\pm$ 0.0 ^c	1.8 $\pm$ 0.2 ^{bcd}	0.2 $\pm$ 0.0 ^c	0.8 $\pm$ 0.1 ^a	4.8 $\pm$ 0.1 ^c
Lt-Sp	3.8 $\pm$ 0.0 ^{ab}	0.6 $\pm$ 0.1 ^f	0.2 $\pm$ 0.0 ^c	0.8 $\pm$ 0.1 ^a	3.4 $\pm$ 0.1 ^d
<i>PA 2</i>					
Sc	3.6 $\pm$ 0.0 ^c	2.0 $\pm$ 0.1 ^{abc}	0.7 $\pm$ 0.0 ^a	0.5 $\pm$ 0.0 ^b	5.2 $\pm$ 0.1 ^a
Lt-Sc	3.6 $\pm$ 0.0 ^c	1.7 $\pm$ 0.1 ^{cd}	0.2 $\pm$ 0.0 ^c	0.9 $\pm$ 0.0 ^a	4.9 $\pm$ 0.0 ^c
Lt-Sp	3.8 $\pm$ 0.0 ^a	0.7 $\pm$ 0.1 ^f	0.2 $\pm$ 0.0 ^c	0.9 $\pm$ 0.1 ^a	3.5 $\pm$ 0.1 ^d

3.1.4 Experiment 4

No fermentations took place in experiment 4 since the evaluation was performed on wine model media. Therefore, no data for this experiment are available.

3.1.5 Experiment 5

This experiment also included microbial population determination besides the determination of oenological parameters. The initial population of wild yeasts measured in the control grapes was rather low, less than 2 log CFU/mL. Expected initial yeas populations, from the experience in previous experiments, is between 2 and 4 log CUF/mL. Nonetheless, the treatment with pulsed light (PL) has reduced the populations after treatments. There is no direct correlation either with the number of pulses nor with the power (**Figure 35**) in the populations reduction. There is statistical difference observed between the control and trials with 5 pulses and medium power (5pPm) and 10 pulses with high power (10pPt) ($p<0.05$).

Figure 35 Comparison of initial yeast population in grape musts between control and PL treatments. Average and error ($n = 3$). Different letters denote a significant difference with 95% confidence level (LSD test).

An explanation to having these results may lie on the fact that grapes are rounded, and their geometrical shape may produce shadows avoiding the radiation from the PL to reach the complete surface area. The depth of penetration of the PL radiation may be limited to not reaching microorganisms deeper on the surface. The effect of the PL treatment seems less effective in this experiment due to the number of pulses and the rather low initial populations.

The use of 50 pulses at 7 J of energy have been enough to reduce populations of *Rhodotorula mucilaginosa* and *Candida lambica* up to 2.8 log while, the use of 100 pulses at 3 J have reduced 3.7 log the population of *Saccharomyces cerevisiae* (Elmnasser et al., 2007).

In terms of non-*Saccharomyces* yeasts grown in lysin media, the results observed are not different from those shown in **Figure 35** for total yeast populations. The observation is that there are no specific, nor greater effects, produced by pulsed light in non-*Saccharomyces* yeasts.

The effect of the PL produced in bacteria populations was slightly different. In this case, the use of greater power, regardless of the number of pulses, has reduced the initial population significantly. There is statistical difference ($p < 0.05$) between both samples treated with higher power (5pPt and 10pPt) and the control (**Figure 36**). Medium energy has also reduced initial bacteria populations but with no significant difference.

Figure 36 Comparison of initial bacteria population in grape musts between control and PL treatments. Average and error ($n = 3$). Different letters denote a significant difference with 95% confidence level (LSD test).

The effect of the UV-C light (~254 nm) on the inactivation of DNA due to the formation of dimers (Gómez-López, Ragaert, Debevere, & Devlieghere, **2007**) may have been responsible for the reduction of the initial microbial populations. The cell disruption and cell wall permeation, as well as damage in the nuclear membrane and vacuoles, was observed after 2-3 pulses of 0.7 J/cm² (Takeshita et al., **2003**).

In terms of oenological parameters, the experimental wines produced with the treated and untreated grapes have similar profiles. Regarding ethanol volume (% v/v) there is no significant difference ($p < 0.05$) between the samples (**Figure 37**). The amount of acids did not show variations as well; lactic acid is low since there is no malolactic fermentation (MLF) while, on the other side, there is a volatile acidity, expressed as acetic acid concentration, rather high for the strain of *Saccharomyces cerevisiae* used in the experimental set up (**Figure 38**).

Loira et al. (**2012**) have reported values for acetic acid around 0.6 g/L for high fermentative power strains and higher values (> 1.75 g/L) for strains with lower fermentative power in previous analysis. The variation in this experiment might be linked to the volume of fermentation and the fermenter geometry and not strain related. The pH values and total acidity are also similar in all samples with no significant difference ($p < 0.05$) (**Figure 39**).

Figure 37 Ethanol content in experimental wines after PL treatment. Average and error ($n = 3$). Different letters denote a significant difference with 95% confidence level (LSD test).

Figure 38 Acetic acid and lactic acid concentration in experimental wines after PL treatment. Average and error ($n = 3$). Different letters in same column denote a significant difference with 95% confidence level (LSD test).

Figure 39 Malic acid, total acidity and pH values in wines after PL treatment. Average and error ($n = 3$). Different letters in same column denote a significant difference with 95% confidence level (LSD test).

No influence on the oenological parameters due to the usage of PL as antimicrobial treatment has been observed on the experimental wines produced.

3.2 Fermentative volatile compounds

3.2.1 Experiment 1

Volatile compounds were determined with GC-FID. The values shown in **Table 9** indicate the profile of volatiles observed in each of the experimental wines.

Table 9 Volatiles composition of finished wines. Mean $\pm$ STD ($n=3$).

Yeast	Strain	Higher Alcohols [mg/L]	Fruity Esters [mg/L]	Acetate Esters [mg/L]	Total Volatiles [mg/L]
<i>Schizosaccharomyces pombe</i>	938	1,190.5 $\pm$ 288.1 ^{ab}	12.6 $\pm$ 3.0 ^a	64.8 $\pm$ 15.8 ^a	1,377.6 $\pm$ 301.2 ^{ab}
<i>Saccharomycodes ludwigii</i>	979	805.6 $\pm$ 62.2 ^a	10.8 $\pm$ 0.8 ^a	433.3 $\pm$ 28.7 ^c	1,369.4 $\pm$ 81.0 ^{ab}
<i>Torulaspora delbrueckii</i>	1880	1,038.8 $\pm$ 50.4 ^{ab}	26.7 $\pm$ 5.1 ^b	121.3 $\pm$ 7.5 ^b	1,235.3 $\pm$ 48.1 ^a
<i>Saccharomyces cerevisiae</i>	7VA	1,413.3 $\pm$ 142.4 ^b	16.9 $\pm$ 1.7 ^a	79.9 $\pm$ 3.4 ^{ab}	1,730.3 $\pm$ 157.5 ^b

Different letters in the same column indicate significant differences between means ($p<0.05$)

The concentration of total higher alcohols for the species *Schizosaccharomyces pombe* (strain 938) is 190.6 ± 24.4 mg/L which is lower than 350 mg/L established as threshold to be considered aromatic defect in wines (Rapp & Mandery, **1986**; Rapp & Versini, **1995**); all the other yeast strains produced higher concentrations of total higher alcohols. Also, all the non-*Saccharomyces* fermentations produced fewer amounts of higher alcohols than *Saccharomyces*, so the use of non-conventional yeasts is a way to protect varietal aromas from grapes, because frequently are eclipsed by major fermentative volatiles such as fusel alcohols. The 2,3-butanediol has neutral aroma (Jackson, **2008**) but it contributes to sweeten and to soften the wine (Romano, Brandolini, Ansaloni, & Menziani, **1998**); the highest concentrations of this compound were obtained by the species *Saccharomyces cerevisiae* (strain 7VA) and *Schizosaccharomyces pombe* (strain 938).

Most of esters present in wines may contribute to the aroma profile of wines with fruity notes but the ethyl acetate that produces a characteristic aroma similar to solvent or glue (Peinado, Moreno, Medina, & Mauricio, **2004**). The amount of ethyl acetate produced by the yeast species *Saccharomycodes ludwigii* (strain 979) is 10 times higher to that produced by the species *Schizosaccharomyces pombe* (strain 938) and it reaches 425.2 ± 28.8 mg/L.

Considering all acetate esters that have an important effect on wine aroma by contributing fruity and floral characteristics (Boss et al., **2015**) the species *Torulaspora delbrueckii* (strain 1880) has produced the highest amount (23.1 ± 4.5 mg/L) followed by the species *Saccharomyces cerevisiae* (strain 7VA). Suárez-Lepe and Morata (**2012**) have stressed the fact that the yeast *Torulaspora delbrueckii* may enhance the aromatic profile in the same time that it reduces volatile acidity and lowers the production of volatile phenols.

The carbonyl volatile compounds identified in red wines are acetaldehyde, diacetyl and acetoin. Acetaldehyde is a precursor of more stable pigments such as pyranoanthocyanins like the vitisins (Morata, Benito, Loira, et al., **2012**; Oberholster et al., **2015**) and is mainly present in wine fermented with *Saccharomyces cerevisiae* (strain 7VA) followed by the fermentation carried out with *Schizosaccharomyces pombe* (strain 938). On the other hand, the acetoin and the diacetyl may become aromatic defects by giving undesirable sensory quality notes to the wine (Tilloy, Ortiz-Julien, & Dequin, **2014**) when reaching values higher than 10 mg/L or 2 mg/L, respectively (Benito et al., **2011**) due to its buttery aroma. In the wines evaluated, those fermented with *Saccharomycodes ludwigii* (strain 979) have produced higher amounts of both compounds while the yeast *Schizosaccharomyces pombe* (strain 938) has produced the lowest amount of diacetyl and the yeast *Torulaspora delbrueckii* (strain 1880) has produced the lowest concentration of acetoin.

According to the results obtained it can be seen that the yeast *Saccharomycodes ludwigii* (strain 979) has a tendency to produce compounds that may affect negatively the organoleptic profile of wines.

3.2.2 Experiment 2

Ethyl acetate is a compound responsible of solvent or glue aroma in wines when in high concentration (Peinado et al., **2004**); it can be seen that all pairs of fermentative yeasts having *Mp* had concentrations larger than 125 mg/L of ethyl acetate produced; the pair *Mp-Sp* had the largest maximum values with significant difference ($p < 0.05$) in all treatments with values as high as 280 mg/L for the treatment ProcyanidinC1 (**Table 10**). These values are considerably high in comparison to a single culture fermentation involving *Sc* producing approximately 4.7 mg/L or the sequential fermentation involving different strains of *Td* and

Sc with values between 6 mg/L and 30 mg/L according to research done by Loira et al. (2014).

Compounds such as acetaldehyde, acetoin and diacetyl are also shown in **Table 10**. Regarding the amount of acetaldehyde, it can be seen that the pair *Mp-Sp* had significant maximum values (above 30 mg/L) for the control and the treatments (+)-Catechin and ProcyanidinB2; the pair *Td-Sc* had the largest value for the treatment ProcyanidinC1 (>70 mg/L). The values for acetaldehyde concentration are in general larger for all the trials in the treatment ProcyanidinC1 than the other treatments. In terms of acetoin content, the pair *Mp-Sp* also had significant maximum values for the control and the treatments (+)-Catechin and ProcyanidinB2; the values reached are close to the threshold (10 mg/L) (Benito et al., 2011) and are similar to the results observed for the pair *Lt-Sp* in treatment ProcyanidinC1. Finally, the diacetyl was found to have significant maximum values only for the treatment ProcyanidinC1 for both trials having *Mp* while there is a significant minimum value for the trial *Td-Sp* for treatment ProcyanidinB2. The threshold for the diacetyl in wines is 2 mg/L according to Benito et al. (2011) and several trials are on the upper threshold level or even above it.

The concentration of total esters (**Table 11**) had same tendency to the ethyl acetate concentration. The trials where the *Mp* was used have significant maximum values. The pair *Mp-Sp* produced the largest concentration of esters (299.1 ± 7.8 mg/L) in treatment ProcyanidinC1. Other esters like ethyl lactate and 2-phenyl ethyl acetate were found to be statistically more abundant in trials with the yeasts *Lt-Sp*. The control and the treatment ProcyanidinB2 had larger concentrations of ethyl lactate (between 7.5 and 8.8 mg/L) while the treatment ProcyanidinC1 had larger amount of 2-phenyl ethyl acetate (23.3 ± 0.7 mg/L).

Even though some esters could induce odd aromas when overpassing the threshold levels, ethyl lactate produces coffee or strawberry and raspberry aromas (Vilanova, Genisheva, Masa, & Oliveira, 2010) and 2-phenylethyl acetate smells like roses, honey or apples (Duarte, Amorim, & Schwan, 2013). The overall all esters may contribute to fruity and flower aromas (Boss et al., 2015) and therefore any of the potential aroma defects were detected in these wines during the sensory evaluation.

Table 10 Concentration of volatiles from fermentative origin in finished wines [mg/L]. Mean $\pm$ STD (n=3).

Yeasts	Acetaldehyde	Diacetyl	Ethyl acetate	Acetoin	Ethyl lactate	2,3-butanediol	2-phenyl ethyl alcohol	2-phenyl ethyl acetate
<i>Control</i>								
Lt-Sc	14.0 $\pm$ 1.8 ^c	2.9 $\pm$ 0.8 ^a	44.8 $\pm$ 8.4 ^c	6.9 $\pm$ 0.2 ^b	6.9 $\pm$ 0.4 ^{ab}	501.8 $\pm$ 43.0 ^c	57.5 $\pm$ 4.5 ^a	5.4 $\pm$ 0.1 ^a
Mp-Sc	21.4 $\pm$ 1.3 ^b	2.9 $\pm$ 0.6 ^a	133.9 $\pm$ 18.1 ^b	7.3 $\pm$ 0.1 ^b	6.0 $\pm$ 0.0 ^{ab}	787.7 $\pm$ 33.9 ^a	60.5 $\pm$ 0.5 ^a	6.4 $\pm$ 0.2 ^a
Td-Sc	13.9 $\pm$ 0.6 ^c	3.3 $\pm$ 0.2 ^a	84.3 $\pm$ 4.5 ^{bc}	6.7 $\pm$ 0.1 ^b	6.0 $\pm$ 0.1 ^{ab}	556.6 $\pm$ 15.7 ^b	30.5 $\pm$ 1.8 ^b	5.3 $\pm$ 0.0 ^a
Lt-Sp	12.1 $\pm$ 0.7 ^c	2.2 $\pm$ 0.2 ^a	53.8 $\pm$ 4.3 ^c	6.9 $\pm$ 0.3 ^b	8.2 $\pm$ 0.5 ^a	510.0 $\pm$ 0.6 ^{bc}	45.0 $\pm$ 10.4 ^{ab}	5.2 $\pm$ 0.1 ^a
Mp-Sp	30.6 $\pm$ 2.4 ^a	3.1 $\pm$ 0.1 ^a	204.4 $\pm$ 64.9 ^a	10.5 $\pm$ 2.7 ^a	6.3 $\pm$ 0.1 ^{ab}	838.4 $\pm$ 21.0 ^a	59.3 $\pm$ 20.5 ^a	5.7 $\pm$ 0.3 ^a
Td-Sp	20.5 $\pm$ 3.2 ^b	2.7 $\pm$ 3.1 ^a	46.7 $\pm$ 8.8 ^c	7.8 $\pm$ 0.2 ^b	4.5 $\pm$ 3.9 ^b	552.9 $\pm$ 36.5 ^{bc}	35.3 $\pm$ 2.0 ^b	7.6 $\pm$ 3.9 ^a
<i>(+)-Catechin</i>								
Lt-Sc	15.3 $\pm$ 1.4 ^{bc}	2.2 $\pm$ 0.8 ^{ab}	49.7 $\pm$ 8.2 ^b	6.7 $\pm$ 0.0 ^b	6.6 $\pm$ 0.3 ^a	514.8 $\pm$ 15.5 ^d	51.0 $\pm$ 9.8 ^b	5.2 $\pm$ 0.1 ^{ab}
Mp-Sc	18.9 $\pm$ 1.1 ^b	2.0 $\pm$ 0.1 ^{ab}	138.0 $\pm$ 19.8 ^a	7.0 $\pm$ 0.4 ^b	6.2 $\pm$ 0.2 ^a	783.6 $\pm$ 43.7 ^b	61.6 $\pm$ 0.8 ^a	6.5 $\pm$ 0.1 ^{ab}
Td-Sc	12.5 $\pm$ 0.8 ^c	2.2 $\pm$ 0.1 ^{ab}	70.2 $\pm$ 13.9 ^b	6.8 $\pm$ 0.2 ^b	2.0 $\pm$ 3.5 ^b	565.1 $\pm$ 40.2 ^c	35.1 $\pm$ 4.5 ^d	3.5 $\pm$ 3.0 ^b
Lt-Sp	12.7 $\pm$ 0.6 ^c	1.5 $\pm$ 1.3 ^{ab}	44.4 $\pm$ 1.2 ^b	6.7 $\pm$ 0.1 ^b	7.8 $\pm$ 0.2 ^a	511.9 $\pm$ 8.4 ^d	41.1 $\pm$ 2.0 ^{cd}	5.2 $\pm$ 0.2 ^{ab}
Mp-Sp	30.8 $\pm$ 4.6 ^a	2.5 $\pm$ 0.3 ^a	136.0 $\pm$ 38.6 ^a	9.7 $\pm$ 2.7 ^a	5.9 $\pm$ 0.1 ^a	863.2 $\pm$ 18.0 ^a	47.1 $\pm$ 1.4 ^{bc}	5.7 $\pm$ 0.4 ^{ab}
Td-Sp	17.5 $\pm$ 5.7 ^{bc}	0.8 $\pm$ 1.4 ^b	47.1 $\pm$ 11.1 ^b	7.3 $\pm$ 0.4 ^b	6.4 $\pm$ 0.1 ^a	606.2 $\pm$ 8.1 ^c	35.8 $\pm$ 3.4 ^d	8.6 $\pm$ 5.8 ^a
<i>Procyanidin B2</i>								
Lt-Sc	15.0 $\pm$ 0.7 ^c	2.2 $\pm$ 0.2 ^{abc}	47.9 $\pm$ 1.8 ^d	6.7 $\pm$ 0.1 ^b	7.5 $\pm$ 0.5 ^{ab}	511.6 $\pm$ 22.5 ^c	39.2 $\pm$ 5.1 ^b	5.2 $\pm$ 0.1 ^c
Mp-Sc	20.9 $\pm$ 1.9 ^b	2.4 $\pm$ 0.2 ^{ab}	128.1 $\pm$ 8.5 ^b	7.1 $\pm$ 0.3 ^b	6.2 $\pm$ 0.1 ^{ab}	846.2 $\pm$ 34.0 ^a	56.8 $\pm$ 4.0 ^a	6.4 $\pm$ 0.1 ^a
Td-Sc	15.4 $\pm$ 1.2 ^c	2.5 $\pm$ 0.2 ^{ab}	62.9 $\pm$ 4.7 ^c	7.0 $\pm$ 0.0 ^b	2.0 $\pm$ 3.5 ^c	571.3 $\pm$ 20.4 ^b	32.6 $\pm$ 0.5 ^b	5.2 $\pm$ 0.0 ^c
Lt-Sp	15.3 $\pm$ 2.3 ^c	1.9 $\pm$ 0.2 ^{bc}	44.2 $\pm$ 2.3 ^d	6.8 $\pm$ 0.1 ^b	8.8 $\pm$ 1.4 ^a	560.1 $\pm$ 8.1 ^{bc}	35.9 $\pm$ 10.0 ^b	5.2 $\pm$ 0.1 ^c
Mp-Sp	33.9 $\pm$ 2.9 ^a	3.0 $\pm$ 0.2 ^a	166.3 $\pm$ 12.2 ^a	13.9 $\pm$ 6.8 ^a	6.1 $\pm$ 0.1 ^{ab}	878.9 $\pm$ 41.5 ^a	57.4 $\pm$ 11.7 ^a	5.7 $\pm$ 0.2 ^b
Td-Sp	25.7 $\pm$ 6.1 ^b	1.3 $\pm$ 1.2 ^c	43.0 $\pm$ 1.5 ^d	8.3 $\pm$ 0.6 ^b	4.4 $\pm$ 3.8 ^{bc}	563.3 $\pm$ 29.1 ^b	31.3 $\pm$ 2.6 ^b	5.6 $\pm$ 0.3 ^b
<i>Procyanidin C1</i>								
Lt-Sc	51.5 $\pm$ 10.2 ^b	2.3 $\pm$ 0.2 ^b	47.4 $\pm$ 3.6 ^{cd}	7.0 $\pm$ 0.1 ^b	6.8 $\pm$ 0.2 ^a	485.9 $\pm$ 29.5 ^d	62.1 $\pm$ 5.7 ^c	5.3 $\pm$ 0.1 ^c
Mp-Sc	28.4 $\pm$ 5.2 ^c	3.6 $\pm$ 0.7 ^a	195.5 $\pm$ 14.8 ^b	6.8 $\pm$ 0.2 ^b	4.3 $\pm$ 3.7 ^a	619.4 $\pm$ 11.1 ^b	76.2 $\pm$ 8.4 ^b	5.9 $\pm$ 0.2 ^b
Td-Sc	74.0 $\pm$ 12.0 ^a	2.3 $\pm$ 0.1 ^b	56.1 $\pm$ 1.7 ^c	7.4 $\pm$ 0.1 ^b	6.2 $\pm$ 0.2 ^a	535.0 $\pm$ 3.1 ^c	44.6 $\pm$ 1.5 ^d	5.2 $\pm$ 0.0 ^c
Lt-Sp	50.9 $\pm$ 12.6 ^b	2.5 $\pm$ 0.4 ^b	43.2 $\pm$ 1.7 ^d	9.4 $\pm$ 1.3 ^a	6.6 $\pm$ 0.4 ^a	488.2 $\pm$ 29.6 ^d	58.2 $\pm$ 5.2 ^c	23.3 $\pm$ 0.7 ^a
Mp-Sp	44.9 $\pm$ 3.4 ^b	3.3 $\pm$ 0.3 ^a	284.8 $\pm$ 7.7 ^a	7.4 $\pm$ 0.1 ^b	6.2 $\pm$ 0.1 ^a	734.0 $\pm$ 40.9 ^a	89.0 $\pm$ 2.4 ^a	5.3 $\pm$ 0.1 ^c
Td-Sp	36.7 $\pm$ 8.3 ^{bc}	2.1 $\pm$ 0.2 ^b	45.8 $\pm$ 3.9 ^{cd}	7.3 $\pm$ 0.7 ^b	6.3 $\pm$ 0.2 ^a	602.6 $\pm$ 10.7 ^b	42.7 $\pm$ 1.4 ^d	5.3 $\pm$ 0.1 ^c

Different letters in the same column indicate significant differences between means (p<0.05). Treatments are analysed separately.

Table 11 Concentration of esters, higher alcohols and total volatiles from fermentative origin in finished wines [mg/L]. Mean $\pm$ STD (n=3).

Yeasts	Esters	Higher alcohols*	Total Volatiles**
<i>Control</i>			
Lt-Sc	59.9 $\pm$ 8.8 ^c	401.1 $\pm$ 31.0 ^a	1,001.6 $\pm$ 71.7 ^b
Mp-Sc	157.0 $\pm$ 20.2 ^b	362.3 $\pm$ 4.4 ^{ab}	1,353.9 $\pm$ 55.0 ^a
Td-Sc	102.6 $\pm$ 5.4 ^c	314.2 $\pm$ 4.7 ^b	1,012.7 $\pm$ 15.4 ^b
Lt-Sp	67.2 $\pm$ 4.7 ^c	358.4 $\pm$ 34.4 ^{ab}	972.3 $\pm$ 32.1 ^b
Mp-Sp	223.2 $\pm$ 61.8 ^a	362.9 $\pm$ 89.5 ^{ab}	1,483.7 $\pm$ 171.4 ^a
Td-Sp	58.8 $\pm$ 8.9 ^c	303.6 $\pm$ 13.0 ^b	961.8 $\pm$ 56.8 ^b
<i>(+)-Catechin</i>			
Lt-Sc	63.9 $\pm$ 8.8 ^b	368.4 $\pm$ 37.6 ^a	986.2 $\pm$ 41.3 ^{bc}
Mp-Sc	159.7 $\pm$ 21.0 ^a	360.3 $\pm$ 8.5 ^a	1,347.0 $\pm$ 33.3 ^a
Td-Sc	80.3 $\pm$ 15.1 ^b	332.9 $\pm$ 26.2 ^{ab}	1,015.0 $\pm$ 65.9 ^b
Lt-Sp	57.5 $\pm$ 1.3 ^b	337.6 $\pm$ 8.5 ^{ab}	943.4 $\pm$ 9.6 ^c
Mp-Sp	153.2 $\pm$ 42.1 ^a	302.2 $\pm$ 12.9 ^b	1,376.0 $\pm$ 46.1 ^a
Td-Sp	62.8 $\pm$ 7.4 ^b	313.1 $\pm$ 4.8 ^b	1,023.8 $\pm$ 5.0 ^b
<i>Procyanidin B2</i>			
Lt-Sc	63.0 $\pm$ 2.5 ^{cd}	327.2 $\pm$ 31.9 ^{ab}	941.3 $\pm$ 7.3 ^d
Mp-Sc	147.6 $\pm$ 7.9 ^b	339.8 $\pm$ 4.3 ^{ab}	1,379.9 $\pm$ 39.6 ^b
Td-Sc	74.2 $\pm$ 3.1 ^c	337.0 $\pm$ 9.8 ^{ab}	1,023.2 $\pm$ 14.1 ^c
Lt-Sp	58.2 $\pm$ 3.7 ^d	300.4 $\pm$ 49.7 ^b	959.0 $\pm$ 38.0 ^d
Mp-Sp	184.8 $\pm$ 11.8 ^a	366.6 $\pm$ 47.3 ^a	1,497.0 $\pm$ 38.3 ^a
Td-Sp	55.7 $\pm$ 4.1 ^d	301.8 $\pm$ 5.0 ^b	972.6 $\pm$ 31.7 ^{cd}
<i>Procyanidin C1</i>			
Lt-Sc	61.7 $\pm$ 3.9 ^{cd}	475.6 $\pm$ 12.6 ^{ab}	1,099.5 $\pm$ 52.8 ^{cd}
Mp-Sc	213.5 $\pm$ 12.7 ^b	444.8 $\pm$ 48.4 ^{bc}	1,332.0 $\pm$ 58.1 ^b
Td-Sc	70.2 $\pm$ 1.8 ^c	425.0 $\pm$ 1.5 ^{cd}	1,129.8 $\pm$ 13.1 ^c
Lt-Sp	73.1 $\pm$ 1.7 ^c	402.5 $\pm$ 15.4 ^d	1,041.5 $\pm$ 54.3 ^d
Mp-Sp	299.1 $\pm$ 7.8 ^a	490.6 $\pm$ 7.4 ^a	1,595.0 $\pm$ 32.0 ^a
Td-Sp	57.4 $\pm$ 3.8 ^d	394.3 $\pm$ 10.1 ^d	1,116.6 $\pm$ 22.8 ^{cd}

Different letters in the same column indicate significant differences between means (p<0.05). Treatments are analysed separately.

*Esters account for the sum of: ethyl acetate, ethyl lactate, 2-phenylethyl acetate, isoamyl acetate and ethyl butyrate

**Higher alcohols account for the sum of: 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol, isobutanol, propanol, butanol, hexanol and 2-butanol.

Regarding higher alcohols, among the statistical maximum values for each treatment, the amount obtained in treatment ProcyanidinC1 for trial *Mp-Sp* reached 490.6 ± 7.4 mg/L, which also correlates to the large value obtained for 2-phenyl ethyl alcohol for this trial (85.0 ± 2.4 mg/L). The concentration of higher alcohols is close to the threshold 350 mg/L and it could be aromatic defect in wines (Rapp & Mandery, **1986**; Rapp & Versini, **1995**) in some cases. At last, the amount of 2,3-butanediol had statistical maximum values for trials having *Mp* as fermentative yeast while trials where *Lt* was used the concentration of this alcohol had minimum values in all four treatments. 2,3-butanediol may contribute to enhance organoleptic properties in trials with *Mp* since this compound has neutral aroma but sweetens and softens the wine (Jackson, **2008**; Romano et al., **1998**).

The concentration of total volatiles follows the trend observed for ethyl acetate, 2,3-butanediol and 2-phenyl ethyl alcohol where *Mp* was used as fermentative yeast; larger values were observed for this species with disregard of the yeast used for the sequential fermentation or the treatment. On the other hand, the use of *Td* in the control treatment lead to lower volatiles concentration values while, *Lt* produced less total volatiles in all three treatments where the flavan-3-oles were added. **Figure 40** shows the influence of the fermentative pair of yeasts in the production trend of esters, higher alcohols and total volatiles. Fermentations done with *Metschnikowia pulcherrima* (*Mp*) had a higher concentration of esters. It can also be seen that there is a trend in having higher concentration of all volatiles when using Procyanidin C1, shown as number 4 in the chart.

Figure 40 Content of esters, higher alcohols and total volatiles of the different fermentative pair of yeasts and the treatments. In this chart the numbers indicate the treatment: 1) control, 2) (+)-catechin, 3) procyanidin B2 and 4) procyanidin C1.

A PCA was done for the eleven volatile compounds plot in **Figure 41**. The distribution is explained by the first two components. PC1 is positively contributed by total volatiles, esters and ethyl acetate and, negatively contributed by ethyl lactate and 2-phenyl ethyl acetate. PC2 is positively contributed by acetaldehyde and higher alcohols and, negatively contributed by 2,3-butanediol. Four different clusters can be identified after PCA. Regarding PCA1 the fermentative non-*Saccharomyces* yeasts are highlighted and clusters are split into trials fermented with *Mp* with higher concentration of ethyl acetate and esters while trials fermented with *Td* and *Lt* are explained by the variables ethyl lactate and 2-phenyl ethyl acetate. Regarding PC2 the distribution highlights the treatment being the Control and ProcyanidinC1 –where polymeric pigments are scarce (ProcyanidinC1) or null (Control)– influenced by the acetaldehyde and higher alcohols concentration while treatments (+)-Catechin and ProcyanidinB2 are influenced by 2,3-butanediol.

Figure 41 Projection of the 72 wine trials in the first two components of the PCA. Identification of clusters formed: 1) Control and ProcyanidinC1 (yeasts *Lt* and *Td*); 2) Control and ProcyanidinC1 (yeast *Mp*); 3) (+)-Catechin and ProcyanidinB2 (yeasts *Lt* and *Td*); 4) (+)-Catechin and ProcyanidinB2 (yeast *Mp*).

3.2.3 Experiment 3

A summary of the fermentative volatiles is given in **Table 12** after having them grouped in 4 different categories; these categories are higher alcohols, carbonyl compounds, fruity esters and total volatiles. The sum of higher alcohols (except for 2,3-butanediol) are, in all trials, in concentrations below the threshold of 350 mg/L (Rapp & Mandery, **1986**; Rapp & Versini, **1995**), therefore their contribution to wines could not be considered as an aromatic defect; on the contrary, these compounds may enrich the aroma profile of the wines produced. The fermentative yeasts pair *Lt*-*Sc* has produced the highest concentration of total higher alcohols (between 227.7 ± 5.3 and 253.9 ± 8.3 mg/L) while fermentations done with *Sc* have reported the smallest concentrations (between 135.9 ± 4.4 and 179.8 ± 4.6 mg/L). Acetoin, diacetyl and ethyl acetate are grouped as carbonyl compounds; these three molecules in concentrations larger than their threshold can provide off-aromas to red wine. Ethyl acetate is a compound with fruity aroma that can also be related to solvent or glue when in high concentration

(Peinado et al., **2004**); acetoin with a threshold of 10 mg/L and diacetyl with a threshold in wines of 2 mg/L could provide buttery aromas (Benito et al., **2011**).

Regarding single molecules, in contrast to values of ethyl acetate larger than 125 mg/L obtained in sequential fermentations when using *Metschnikowia pulcherrima* (Escott et al., **2018**), the results obtained in these trials got values between 24.4 ± 0.1 mg/L and 49.4 ± 1.6 mg/L; the pair Lt-Sp obtained lower values while Lt-Sc got the highest. These values are, on the other hand, closer to the values observed by Loira et al. (**2014**) for fermentations involving *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* with values between 6 mg/L and 30 mg/L. In terms of diacetyl, the fermentative pair Lt-Sc had larger values but statistically different among them; the values ranged from 5.2 ± 0.3 mg/L to 11.3 ± 0.1 mg/L all of which are higher than the threshold and may be perceived by the consumer; according to Bartowsky and Henschke (**2004**), diacetyl can provide buttery aroma and flavour to a series of fermented foods and beverages but is also an intermediate in the production of acetoin and further production of 2,3-butanediol. The amount of acetoin produced is slightly higher, yet statistically significant, for the fermentative pair Lt-Sp with values between 7.5 ± 0.2 mg/L and 8.1 ± 0.3 mg/L, meaning all samples have produced concentrations of acetoin lower than the threshold. The values for these three compounds together are statistically higher for the fermentative pair Lt-Sc especially due to the larger amounts of ethyl acetate and diacetyl produced during fermentation.

The fruity esters are present in higher concentration in the trials fermented with the pair Lt-Sp followed by the pair Lt-Sc and finally, the trials with less ester were fermented with Sc. As observed with higher alcohols and carbonyl compounds, the fruity esters concentration seems to depend on the fermentative yeast for wine fermentation and not on the treatment followed. The largest values were obtained with two non-*Saccharomyces* yeasts yielding concentrations between 23.6 ± 0.7 mg/L and 27.2 ± 1.7 mg/L. Among the fruity esters evaluated, coffee or strawberry and raspberry aromas are produced by ethyl lactate (Vilanova et al., **2010**); roses, honey or apples may come from 2-phenylethyl acetate (Duarte et al., **2013**); and the contribution of the overall esters to wines, is expected to be fruity and flowery according to Boss et al. (**2015**).

Table 12 Volatiles with fermentative origin shown by family of compounds identified by GC-FID. The statistical analysis is given by parameter (columns) and the difference in letters indicates significant statistical difference (LSD method with 95% confidence level). Means $\pm$ STD ($n = 3$).

Treatment	Higher Alcohols ¹	Carbonyl compounds ²	Fruity Esters ³	Total volatiles
	mg/L	mg/L	mg/L	mg/L
<i>(+) - Catechin</i>				
Sc	135.9 $\pm$ 4.4 ^f	42.0 $\pm$ 1.7 ^c	8.7 $\pm$ 0.6 ^c	1316.6 $\pm$ 91.4 ^c
Lt-Sc	253.9 $\pm$ 8.3 ^a	59.8 $\pm$ 1.9 ^a	15.8 $\pm$ 0.4 ^b	1048.6 $\pm$ 11.2 ^d
Lt-Sp	196.1 $\pm$ 1.7 ^c	38.4 $\pm$ 0.6 ^{cd}	27.1 $\pm$ 1.2 ^a	933.5 $\pm$ 7.4 ^e
<i>PB2</i>				
Sc	154.9 $\pm$ 4.1 ^e	46.7 $\pm$ 0.8 ^b	8.4 $\pm$ 0.9 ^c	1615.7 $\pm$ 64.0 ^b
Lt-Sc	227.7 $\pm$ 5.3 ^b	62.2 $\pm$ 1.5 ^a	14.7 $\pm$ 0.4 ^b	1028.1 $\pm$ 9.1 ^{de}
Lt-Sp	203.0 $\pm$ 5.6 ^c	36.6 $\pm$ 1.5 ^d	23.6 $\pm$ 0.7 ^a	987.7 $\pm$ 7.4 ^{de}
<i>PC1</i>				
Sc	137.3 $\pm$ 1.0 ^f	36.8 $\pm$ 0.6 ^d	7.5 $\pm$ 0.6 ^c	1421.5 $\pm$ 53.5 ^c
Lt-Sc	233.7 $\pm$ 3.8 ^b	58.3 $\pm$ 1.0 ^a	14.0 $\pm$ 0.7 ^b	1047.9 $\pm$ 11.7 ^{de}
Lt-Sp	209.2 $\pm$ 4.8 ^c	36.5 $\pm$ 1.0 ^d	24.6 $\pm$ 0.5 ^a	981.7 $\pm$ 34.4 ^{de}
<i>PA2</i>				
Sc	179.8 $\pm$ 4.6 ^d	50.5 $\pm$ 0.8 ^b	8.8 $\pm$ 0.4 ^c	1746.0 $\pm$ 16.0 ^a
Lt-Sc	232.0 $\pm$ 7.6 ^b	58.4 $\pm$ 1.9 ^a	17.7 $\pm$ 1.6 ^b	1033.4 $\pm$ 18.3 ^{de}
Lt-Sp	206.8 $\pm$ 2.9 ^c	38.4 $\pm$ 1.8 ^{cd}	27.2 $\pm$ 1.7 ^a	1022.0 $\pm$ 15.1 ^{de}

¹Higher alcohols comprise the following compounds: 1-propanol, 2-buntanol, isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethyl alcohol;

²Carbonyl compounds comprise: diacetyl, acetoin, ethyl acetate; and

³Fruity esters comprise: isobutyl acetate, ethyl butyrate, ethyl lactate, isoamyl acetate, 2-phenylethyl acetate.

Most of the concentration of total volatiles accounts for 2,3-butanediol, therefore and besides the fact that the concentration of higher alcohols and carbonyl compounds is higher in trials fermented with Lt-Sc and that fruity esters are more abundant in trials fermented with Lt-Sp, total fermentative volatiles are in larger concentration in trials produced with Sc since this yeast has produced larger amounts of 2,3-butanediol (> 1000 mg/L). In these trials, Sc yeasts have reduced acetoin to 2,3-butanediol in larger extent than in the other fermentations as it was reported in fermentations performed with engineered *Saccharomyces cerevisiae* strains (Remize, Roustan, Sablayrolles, Barre, & Dequin, **1999**); this due to the fact that in the metabolic pathway from pyruvate involves the enzyme acetoin reductase which reduces acetoin to 2,3-butanediol (Celińska & Grajek, **2009**).

3.2.4 Experiment 4

No fermentations took place in experiment 4 since the evaluation was performed on wine model media. Therefore, no data for this experiment are available.

3.2.5 Experiment 5

Although there has been an effect in the degradation of pigments potentially due to oxidative reactions, the photodegradation do not seem to affect fermentative volatile compounds as it can be seen in **Table 13** for aromatic families' higher alcohols and esters.

Table 13 Volatile compounds from fermentative origin. Average and STD (n=3) Different letter denote a significant difference ($p<0.05$) (LSD test) for parameters in the same row.

	Control	5pPm	10pPm	5pPt	10pPt
Methanol	84.6±5.8 ^{bc}	81.7±2.9 ^c	93.4±2.7 ^{ab}	97.1±2.6 ^a	95.2±2.8 ^a
Higher alcohols	364.1±16.9 ^a	379.4±6.7 ^a	360.3±19.7 ^a	408.0±36.2 ^a	387.1±16.1 ^a
Esters	45.6±10.3 ^a	40.5±4.8 ^a	54.3±4.6 ^a	48.2±1.4 ^a	45.5±0.9 ^a
Total volatiles	2644.1±89.9 ^a	2653.7±199.8 ^a	2745.6±197.3 ^a	2927.5±170.1 ^a	2789.1±80.2 ^a

In the case of methanol, there is an increase in the concentration of this volatile compound as the PL energy increases. 10 pulses at medium energy (10pPm) have increased the concentration of methanol but, it was with higher energy pulses (5pPt and 10pPt) that this compound produced maximum concentrations. It is common to observe a proportional increase of methanol in red wines as the intensity of the maceration increases and, after the use of enzymes to promote the extraction of polyphenols (Jackson, **2008**). The difference in methanol's concentration is significant with 95% confidence level (LSD test) between the control and the high energy treatments.

3.3 Anthocyanins, pyranoanthocyanins and polymeric pigments determination

3.3.1 Experiment 1

Anthocyanins.

The concentration of non-acylated anthocyanins after fermentation varies in function of the yeast used for the fermentation of the must. The highest *concentration* of non-acylated anthocyanins are present in wine fermented with yeast *Schizosaccharomyces pombe* (strain 938) with 110 mg/L (**Figure 42**). It can be observed, that the concentration of monomeric anthocyanins in the experimental wines is related to the fermentative yeast used. The concentration of anthocyanins has been reduced between 55 % and 70 % with respect to the initial concentration in grape must.

Figure 42 Peaks of monomeric anthocyanins, also known as non-acylated anthocyanins, in a HPLC-DAD chromatogram. 1) delphinidin-3-O-glucoside, 2) cyanidin-3-O-glucoside, 3) petunidin-3-O-glucoside, 4) peonidin-3-O-glucoside and 5) malvidin-3-O-glucoside.

On the other hand, acylated anthocyanins, those conjugated with acetic acid, *p*-coumaric acid or caffeic acid through an ester formation at glucose position 6 (Morata et al., 2006) are present in lower concentration in finished wines. Caffeoyl derivatives are the least concentrated in all cases while *p*-coumaroyl derivatives are the most abundant among the acylated derivatives except for the fermentation done with *Saccharomyces cerevisiae* (strain 7VA). *p*-coumaroyl derivatives, specifically malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside is one of the main acylated derivatives of *Vitis vinifera* L. cv. Tempranillo (A. Morata et al., 2006) and its concentration after fermentation is higher for the non-*Saccharomyces* yeasts.

Pyranoanthocyanins

These pigments were found in all experimental fermentations while the polymeric pigments were found on those musts added either with (+)-catechin or with procyanidin B2. The vitisins A and B, formed by the condensation of anthocyanins with pyruvic acid and acetaldehyde, respectively (Morata et al., 2006), were identified by its molecular ions; vitisin A has a molecular ion $[M]^+$ with (m/z) 561 and aglycon ion with (m/z) 399 while vitisin B was identified with the molecular ion $[M]^+$ with (m/z) 517 and the aglycon ion with (m/z) 355 (Vergara, Mardones, Hermosín-Gutiérrez, & von Baer, 2010). The yeast *Schizosaccharomyces pombe* (strain 938) has produced the highest amount of vitisin A among all the yeasts evaluated, four times more than the others, and it has also produced a vitisin A type derivative with peonidin (Peonidin-3-*O*-glucoside pyruvic acid) (Figure 43).

Figure 43 Peaks from HPLC-DAD chromatogram corresponding to vitisin-type pigments in experimental wines. 1) peonidin-3-*O*-glucoside-pyruvic acid, 2) vitisin B, 3) vitisin A, 4) acetyl vitisin B

The molecular ion $[M]^+$ (m/z) 531 and the aglycon ion (m/z) 369 were used to identify the pigment Pn3G-pyruvic acid (He et al., **2012**). Vitisin B was found in higher proportion in the wine fermented with *Saccharomyces cerevisiae* (strain 7VA) and it corresponds with the high amount of acetaldehyde present in this wine after fermentation; acetaldehyde remains after pyruvic acid is used as substrate by yeasts when sugars become scarce during fermentation (Morata et al., **2007**) therefore the concentration of acetaldehyde is higher at the end of the fermentation.

Vinyl phenolic compounds were found in wines produced with yeasts from the species *Saccharomyces cerevisiae* (strain 7VA) and the sequential fermentation with *Torulaspora delbrueckii* (strain 1880). These compounds are formed due to enzymatic hydroxycinnamate decarboxylase activity of the species *Saccharomyces cerevisiae* (strain 7VA) and even though the species *Schizosaccharomyces pombe* may have this enzymatic activity (Morata, Benito, Loira, et al., **2012**) no vinyl phenols were found in the wine fermented with this yeast. The vinyl phenols identified are derivatives of malvidin-3-*O*-glucoside with 4-vinylphenol and 4-vinylguaiacol and the molecular ions $[M]^+$ were (m/z) 609 and (m/z) 639, respectively (He et al., **2012**).

Polymeric pigments

The polymeric pigments comprise the formation of adducts with flavanols. The formation of polymeric pigments is associated to wine aging (Pechamat, Zeng, Jourdes, Ghidossi, & Teissedre, **2014**) but in this investigation its production was enhanced by the addition of (+)-catechin and procyanidin B2 to fresh must. **Figure 44** shows the pigments identified in the wines produced with *Saccharomyces cerevisiae* (7VA) and added with either 80 mg/L (+)-catechin or 40 mg/L procyanidin B2 while **Figure 45** shows those identified in the experimental wines fermented with *Schizosaccharomyces pombe*. The dosages of added flavan-3-ols used may double the concentration of catechin and procyanidin B2 naturally found in the original must since Suriano, Alba, Tarricone and di Gennaro (**2015**) have reported up to 17.78 ± 0.74 mg/L of (+)-catechin and 45.87 ± 1.24 mg/L of procyanidin B2 in a Primitivo wine at racking; these values may increase if the grape is destemmed in lower extent.

Figure 44 Peaks of polymeric anthocyanins formed in fermentations of *Saccharomyces cerevisiae* with either catechin or procyanidin B2. 1) malvidin-3-*O*-glucoside-ethyl-(epi)catechin and 2) malvidin-3-*O*-glucoside-ethyl-procyanidin B2.

Figure 45 Peaks of polymeric anthocyanins formed in fermentations with *Schizosaccharomyces pombe* with catechin and procyanidin B2. 1) malvidin-3-*O*-glucoside-procyanidin B2, 2) peonidin-3-*O*-glucoside-catechin and 3) isomers of malvidin-3-*O*-glucoside-(epi)catechin

Total dimers formed with (+)-catechin are more abundant in the wine fermented with *Schizosaccharomyces pombe* (strain 938) (1.24 ± 0.10 mg/L) but it is the yeast *Saccharomyces ludwigii* (strain 979) the one that produced more variety of compounds. The ions used for the identification of these pigments were: Pt3G-(+)-catechin molecular ion $[M]^+$ (m/z) 767 and fragment ions 591, 303; M3G-(+)-catechin with molecular ion $[M]^+$ (m/z) 781 and fragment ions 619, 331; M3G-ethyl-(+)-catechin with molecular ion $[M]^+$ (m/z) 809 and fragment ions 657, 331 (He et al., **2012**; Monagas, Gómez-Cordovés, Bartolomé, Laureano, & Silva, **2003**).

In the case of the polymers formed with procyanidin B2 only one molecule was identified, the malvidin-3-*O*-glucoside-ethyl-procyanidin B2 (M3G-ethyl-procyanidinB2) which is slightly more abundant in wines fermented with *Schizosaccharomyces pombe* (strain 938) and followed by the fermentation with *Saccharomyces cerevisiae* (strain 7VA) ($>0,3$ mg/L). The M3G-ethyl-procyanidinB2 was identified with its molecular ion $[M]^+$ (m/z) 1097 and fragment ion (m/z) 519 (He et al., **2012**). A summary of all pigments found in the wines produced is shown as per main pigment groups in **Table 14**.

Table 14 Pigments present in wines and must expressed as [mg/L]. Pyranoanthocyanins involve vitisins and vinyl phenolic compounds while polymeric pigments include adducts formed with (+)-catechin and procyanidin B2. Mean $\pm$ STD (n=3).

Yeast	Strain	Anthocyanins [mg/L]	Pyranoanthocyanins [mg/L]	Polymeric pigments [mg/L]	Total [mg/L]
<i>Schizosaccharomyces pombe</i>	938	120.1 $\pm$ 6.7 ^b	5.2 $\pm$ 0.29 ^d	1.6 $\pm$ 0.08 ^d	126.9 $\pm$ 7.0 ^b
<i>Saccharomyces ludwigii</i>	979	82.5 $\pm$ 2.9 ^a	0.8 $\pm$ 0.04 ^b	1.3 $\pm$ 0.13 ^c	84.7 $\pm$ 2.8 ^a
<i>Torulaspora delbrueckii</i>	1880	92.3 $\pm$ 2.2 ^a	0.9 $\pm$ 0.12 ^b	1.0 $\pm$ 0.05 ^b	94.2 $\pm$ 2.3 ^a
<i>Saccharomyces cerevisiae</i>	7VA	82.7 $\pm$ 3.5 ^a	1.9 $\pm$ 0.02 ^c	1.5 $\pm$ 0.06 ^{cd}	85.9 $\pm$ 3.6 ^a
Must	-	264.5 $\pm$ 11.7 ^c	0.0 ^a	0.0 ^a	264.5 $\pm$ 11.7 ^c

Different letters in the same column indicate significant differences between means (p<0.05)

3.3.2 Experiment 2

Anthocyanins

The concentration of monomeric anthocyanins including the non-acylated, and the acetyl-, *p*-coumaroyl- and caffeoyl derivatives, vary in function of the treatment and the yeasts used in the sequential fermentations (see **Table 15**).

It can be seen that the treatment ProcyanidinC1 kept more non-acylated pigments over the fermentation comparing same yeast pairs between treatments. If we consider the pair *Lt-Sp*, this trial had the highest concentration of non-acylated anthocyanins at the end of the fermentation with 74.1 ± 0.7 mg/L with significant difference to all other results but considerably low compared to the initial 172.2 ± 1.3 mg/L found in the must. Regarding the rest of monomeric derivatives, it can be seen that the trials with highest amount of pigments are the pair *Td-Sc* for the acetyl derivatives in the treatment ProcyanidinC1 and the same pair for the *p*-coumaroyl and caffeoyl derivatives in the treatment ProcyanidinB2.

In terms of total anthocyanins, ProcyanidinC1 is consistently having the highest concentration among all treatments for each specific pair of yeasts. The single trials having the highest amounts of total anthocyanins with no significant difference were fermented with the yeasts *Lt-Sc*, and *Lt-Sp* (**Figure 46**).

Table 15 Concentration of anthocyanin pigments in wines and must expressed as [mg/L]. Mean $\pm$ STD (n=3).

Yeasts	Anthocyanins			
	Non Acylated ¹	Acetylglucosides ²	<i>p</i> -Coumaroylglucosides ³	Caffeoylglucosides ⁴
Must	172.2 $\pm$ 1.3 ^a	9.4 $\pm$ 0.1 ^a	12.6 $\pm$ 0.1 ^a	0.9 $\pm$ 0.0 ^a
Control				
<i>Lt-Sc</i>	62.6 $\pm$ 0.5 ^{def}	3.7 $\pm$ 0.1 ^{hij}	5.1 $\pm$ 0.0 ^{fgh}	0.7 $\pm$ 0.0 ^{bcd}
<i>Mp-Sc</i>	55.6 $\pm$ 0.5 ^{hij}	3.8 $\pm$ 0.0 ^{ghi}	5.1 $\pm$ 0.0 ^{fgh}	0.7 $\pm$ 0.0 ^{bcd}
<i>Td-Sc</i>	50.7 $\pm$ 0.1 ^{lm}	3.8 $\pm$ 0.0 ^{ghi}	5.1 $\pm$ 0.0 ^{fgh}	0.6 $\pm$ 0.0 ^{cdef}
<i>Lt-Sp</i>	59.8 $\pm$ 0.6 ^{fg}	4.2 $\pm$ 0.0 ^{def}	4.7 $\pm$ 0.0 ^{ikl}	0.6 $\pm$ 0.0 ^{hijk}
<i>Mp-Sp</i>	45.7 $\pm$ 0.5 ^{no}	3.3 $\pm$ 0.1 ^{kl}	5.1 $\pm$ 0.0 ^{fgh}	0.6 $\pm$ 0.0 ^{defgh}
<i>Td-Sp</i>	54.0 $\pm$ 0.4 ^{jk}	3.8 $\pm$ 0.1 ^{ghi}	4.3 $\pm$ 0.1 ^m	0.5 $\pm$ 0.0 ^{klm}
(+)-Catechin				
<i>Lt-Sc</i>	64.1 $\pm$ 2.1 ^{de}	4.0 $\pm$ 0.5 ^{efgh}	5.0 $\pm$ 0.2 ^{hi}	0.6 $\pm$ 0.0 ^{bcde}
<i>Mp-Sc</i>	54.0 $\pm$ 0.5 ^{jk}	3.6 $\pm$ 0.0 ^{ijk}	5.2 $\pm$ 0.1 ^{efg}	0.6 $\pm$ 0.0 ^{defg}
<i>Td-Sc</i>	51.7 $\pm$ 1.2 ^{kl}	3.4 $\pm$ 0.1 ^{kl}	5.3 $\pm$ 0.2 ^{def}	0.6 $\pm$ 0.0 ^{defg}
<i>Lt-Sp</i>	57.6 $\pm$ 0.5 ^{ghi}	3.9 $\pm$ 0.0 ^{efghi}	4.6 $\pm$ 0.1 ^l	0.5 $\pm$ 0.0 ^{ijklm}
<i>Mp-Sp</i>	44.4 $\pm$ 0.4 ^o	3.1 $\pm$ 0.1 ^l	5.0 $\pm$ 0.0 ^{gh}	0.6 $\pm$ 0.0 ^{ghij}
<i>Td-Sp</i>	55.0 $\pm$ 0.9 ^{ij}	3.9 $\pm$ 0.1 ^{fghi}	4.7 $\pm$ 0.2 ^{ijkl}	0.5 $\pm$ 0.0 ^{lm}
ProcyanidinB2				
<i>Lt-Sc</i>	63.1 $\pm$ 0.5 ^{de}	3.8 $\pm$ 0.0 ^{ghi}	5.5 $\pm$ 0.0 ^{bcd}	0.7 $\pm$ 0.0 ^{bc}
<i>Mp-Sc</i>	57.8 $\pm$ 1.2 ^{gh}	3.8 $\pm$ 0.0 ^{ghi}	5.7 $\pm$ 0.0 ^{bc}	0.7 $\pm$ 0.0 ^b
<i>Td-Sc</i>	57.4 $\pm$ 1.3 ^{ghi}	3.8 $\pm$ 0.0 ^{hij}	5.7 $\pm$ 0.0 ^b	0.7 $\pm$ 0.0 ^b
<i>Lt-Sp</i>	61.6 $\pm$ 1.3 ^{ef}	4.2 $\pm$ 0.0 ^{defg}	4.9 $\pm$ 0.0 ^{hij}	0.6 $\pm$ 0.0 ^{fghij}
<i>Mp-Sp</i>	48.1 $\pm$ 1.2 ^{mn}	3.3 $\pm$ 0.0 ^{kl}	5.2 $\pm$ 0.1 ^{fgh}	0.6 $\pm$ 0.0 ^{efghi}
<i>Td-Sp</i>	57.5 $\pm$ 0.4 ^{ghi}	3.6 $\pm$ 0.0 ^{ijk}	4.2 $\pm$ 0.0 ^m	0.5 $\pm$ 0.0 ^m
ProcyanidinC1				
<i>Lt-Sc</i>	71.8 $\pm$ 1.0 ^{bc}	4.4 $\pm$ 0.0 ^{cd}	4.7 $\pm$ 0.1 ^{ijkl}	0.6 $\pm$ 0.0 ^{ijkl}
<i>Mp-Sc</i>	62.0 $\pm$ 0.6 ^{ef}	4.3 $\pm$ 0.0 ^{def}	4.7 $\pm$ 0.0 ^{kl}	0.6 $\pm$ 0.0 ^{ijkl}
<i>Td-Sc</i>	64.9 $\pm$ 0.7 ^d	4.9 $\pm$ 0.0 ^b	4.9 $\pm$ 0.1 ^{hijk}	0.6 $\pm$ 0.0 ^{efghi}
<i>Lt-Sp</i>	74.1 $\pm$ 0.7 ^b	4.3 $\pm$ 0.0 ^{de}	3.9 $\pm$ 0.0 ⁿ	0.5 $\pm$ 0.0 ^m
<i>Mp-Sp</i>	51.2 $\pm$ 0.3 ^l	3.8 $\pm$ 0.0 ^{ghi}	5.5 $\pm$ 0.0 ^{cde}	0.7 $\pm$ 0.0 ^{bcd}
<i>Td-Sp</i>	69.7 $\pm$ 0.2 ^c	4.7 $\pm$ 0.0 ^{bc}	5.1 $\pm$ 0.1 ^{fgh}	0.6 $\pm$ 0.0 ^{bcde}

Different letters in the same column indicate significant differences between means (p<0.05).

¹Comprise the following pigments: delphinidin-3-glucoside, cyaniding-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside.

²Comprise the following pigments: delphinidin-3-(6"-acetylglucoside), cyanidin-3-(6"-acetylglucoside), petunidin-3-(6"-acetylglucoside), peonidin-3-(6"-acetylglucoside), malvidin-3-(6"-acetylglucoside).

³Comprise the following pigments: cyanidin-3-(6"-*p*coumaroylglucoside), malvidin-3-(6"-*p*-coumaroylglucoside)*cis*, petunidin-3-(6"-*p*coumaroylglucoside), malvidin-3-(6"-*p*coumaroylglucoside)*trans*.

⁴Comprise the following pigment: malvidin-3-(6"-caffeoylglucoside).

Figure 46 Plot to compare polymeric pigments and total pigments after fermentation. Clusters are defined by treatment; diamonds for control, squares for (+)-catechin, triangles for procyanidin B2 and circles for procyanidin C1. Pair of yeasts producing larger levels of total pigments are shown in italic black characters; yeasts forming the larger amounts of polymeric pigments are shown in underlined italic grey characters.

Pyranoanthocyanins

In terms of vitisins formation, the trials having higher concentration of these pyranoanthocyanins had fermented with *Lt-Sc* in treatment ProcyanidinC1 and with *Td-Sp* in treatment ProcyanidinB2 (both 1.3 ± 0.0 mg/L) followed by the same yeast pairs but in the treatments (+)-Catechin and Control respectively (both 1.1 ± 0.0 mg/L) (**Table 16**). The vitisins identified with the mass spectroscopy are malvidin-3-glucoside pyruvate (Vitisin A) and malvidin-3-glucoside acetaldehyde (Vitisin B) with molecular ion $[M]^+$ (m/z) 561 and fragment ion with (m/z) 399 and $[M]^+$ (m/z) 517 and fragment ion with (m/z) 355 (Vergara et al., 2010) respectively; malvidin-3-(6"-acetylglucoside) pyruvate and malvidin-3-(6"-acetylglucoside) acetaldehyde were identified with $[M]^+$ (m/z) 603 and fragment ion (m/z) 399 and $[M]^+$ (m/z) 559 and fragment ion (m/z) 355 (He et al., 2012) respectively. The Control as well as the treatment (+)-Catechin reported the presence of vitisin A and vitisin B; the treatment ProcyanidinB2 had vitisin A, vitisin B and malvidin-3-(6"-acetylglucoside)

pyruvate while the treatment ProcyanidinC1 had, besides vitisin A and vitisin B, malvidin-3-(6''-acetylglucoside) pyruvate in the trials fermented with *Lt-Sc* and malvidin-3-(6''-acetylglucoside) acetaldehyde in the trial fermented with *Td-Sc*.

The vinyl phenolic compounds identified were the malvidin-3-glucoside-4-vinylphenol and malvidin-3-glucoside-4-vinylguaiacol identified with the molecular ions $[M]^+$ (m/z) 609 and 639 respectively (He et al., **2012**). The pair *Mp-Sc* had the highest concentration of vinyl phenolics in all treatments and out of these ones the trial from the treatment ProcyanidinB2 is the one with the highest value ($0.15 \pm 0.0\text{mg/L}$). According to Suárez-Lepe and Morata (**2015**), non-*Saccharomyces* yeasts with hydroxycinnamate decarboxylase activity (HCDC) such as *Sp* and *Torulaspora* spp. among other species produced the decarboxylation of hydroxycinnamic acids into vinyl phenols. Despite these findings, there was no trace of vinyl phenolic compounds in the trials fermented with *Td-Sp* in any of the treatments and in the specific trials *Lt-Sc* in the control and *Lt-Sp* in the treatment ProcyanidinC1.

Table 16 Concentration of pyranoanthocyanins and polymeric pigments in wines and must expressed as [mg/L]. Polymeric pigments include adducts formed with flavanols. Mean $\pm$ STD (n=3).

Yeasts	Pyranoanthocyanins		Polymeric pigments ³	Total pigments
	Vitisins ¹	Vinylphenolics ²		
Must	0.00 $\pm$ 0.0 ^k	0.00 $\pm$ 0.00 ^k	0.00 $\pm$ 0.00 ^k	195.1 $\pm$ 1.4 ^a
Control				
<i>Lt-Sc</i>	0.89 $\pm$ 0.04 ^{efg}	0.00 $\pm$ 0.00 ^k	0.00 $\pm$ 0.00 ^k	72.9 $\pm$ 0.4 ^{def}
<i>Mp-Sc</i>	0.97 $\pm$ 0.01 ^d	0.12 $\pm$ 0.01 ^{bc}	0.00 $\pm$ 0.00 ^k	66.3 $\pm$ 0.6 ^{kl}
<i>Td-Sc</i>	0.89 $\pm$ 0.01 ^{efg}	0.08 $\pm$ 0.01 ^{ef}	0.00 $\pm$ 0.00 ^k	61.2 $\pm$ 0.1 ^{no}
<i>Lt-Sp</i>	1.09 $\pm$ 0.03 ^{bc}	0.10 $\pm$ 0.00 ^d	0.00 $\pm$ 0.00 ^k	70.5 $\pm$ 0.6 ^{fgh}
<i>Mp-Sp</i>	0.83 $\pm$ 0.00 ^{ghi}	0.12 $\pm$ 0.00 ^b	0.00 $\pm$ 0.00 ^k	55.6 $\pm$ 0.6 ^{pq}
<i>Td-Sp</i>	1.13 $\pm$ 0.01 ^b	0.00 $\pm$ 0.00 ^k	0.00 $\pm$ 0.00 ^k	63.8 $\pm$ 0.4 ^{lmn}
(+)-Catechin				
<i>Lt-Sc</i>	1.13 $\pm$ 0.03 ^b	0.05 $\pm$ 0.00 ^{hi}	0.64 $\pm$ 0.01 ^c	75.6 $\pm$ 2.3 ^{cd}
<i>Mp-Sc</i>	0.79 $\pm$ 0.02 ^{hi}	0.12 $\pm$ 0.00 ^b	0.46 $\pm$ 0.01 ^e	65.0 $\pm$ 0.6 ^{klm}
<i>Td-Sc</i>	0.84 $\pm$ 0.02 ^{fgh}	0.08 $\pm$ 0.00 ^e	0.44 $\pm$ 0.02 ^e	62.5 $\pm$ 1.2 ^{mn}
<i>Lt-Sp</i>	1.05 $\pm$ 0.03 ^c	0.07 $\pm$ 0.00 ^{fg}	0.81 $\pm$ 0.01 ^a	68.7 $\pm$ 0.6 ^{hij}
<i>Mp-Sp</i>	0.80 $\pm$ 0.03 ^{hi}	0.10 $\pm$ 0.01 ^d	0.52 $\pm$ 0.02 ^d	54.6 $\pm$ 0.3 ^r
<i>Td-Sp</i>	1.10 $\pm$ 0.02 ^{bc}	0.01 $\pm$ 0.00 ^k	0.74 $\pm$ 0.00 ^b	66.0 $\pm$ 1.2 ^{jkl}
Procyanidin B2				
<i>Lt-Sc</i>	0.90 $\pm$ 0.02 ^{ef}	0.05 $\pm$ 0.00 ^{hi}	0.09 $\pm$ 0.00 ^j	74.2 $\pm$ 0.5 ^{cde}
<i>Mp-Sc</i>	0.93 $\pm$ 0.03 ^{de}	0.15 $\pm$ 0.00 ^a	0.25 $\pm$ 0.00 ^h	69.3 $\pm$ 1.2 ^{ghi}
<i>Td-Sc</i>	0.98 $\pm$ 0.02 ^d	0.06 $\pm$ 0.00 ^{gh}	0.20 $\pm$ 0.00 ⁱ	68.8 $\pm$ 1.3 ^{hij}
<i>Lt-Sp</i>	1.06 $\pm$ 0.02 ^c	0.03 $\pm$ 0.00 ^j	0.28 $\pm$ 0.00 ⁱ	72.7 $\pm$ 1.4 ^{def}
<i>Mp-Sp</i>	0.83 $\pm$ 0.02 ^{ghi}	0.08 $\pm$ 0.00 ^e	0.22 $\pm$ 0.01 ⁱ	58.2 $\pm$ 1.2 ^{op}
<i>Td-Sp</i>	1.28 $\pm$ 0.02 ^a	0.00 $\pm$ 0.00 ^k	0.33 $\pm$ 0.00 ^f	67.5 $\pm$ 0.4 ^{ijk}
Procyanidin C1				
<i>Lt-Sc</i>	1.29 $\pm$ 0.02 ^a	0.03 $\pm$ 0.00 ^j	0.02 $\pm$ 0.00 ^k	82.9 $\pm$ 1.1 ^b
<i>Mp-Sc</i>	0.45 $\pm$ 0.01 ^j	0.11 $\pm$ 0.00 ^c	0.00 $\pm$ 0.00 ^k	72.1 $\pm$ 0.6 ^{efg}
<i>Td-Sc</i>	1.05 $\pm$ 0.01 ^c	0.09 $\pm$ 0.00 ^{de}	0.00 $\pm$ 0.00 ^k	76.5 $\pm$ 0.7 ^c
<i>Lt-Sp</i>	0.97 $\pm$ 0.01 ^d	0.00 $\pm$ 0.00 ^k	0.00 $\pm$ 0.00 ^k	83.8 $\pm$ 0.7 ^b
<i>Mp-Sp</i>	0.51 $\pm$ 0.00 ^j	0.04 $\pm$ 0.00 ⁱ	0.04 $\pm$ 0.01 ^k	62.0 $\pm$ 0.3 ^{mn}
<i>Td-Sp</i>	0.77 $\pm$ 0.00 ⁱ	0.00 $\pm$ 0.00 ^k	0.01 $\pm$ 0.00 ^k	81.0 $\pm$ 0.3 ^b

Different letters in the same column indicate significant differences between means (p<0.05).

¹Comprise the following pigments: malvidin-3-glucoside acetaldehyde (Vitisin B), malvidin-3-glucoside pyruvate (Vitisin A), malvidin-3-(6"-acetylglucoside) pyruvate, malvidin-3-(6"-acetylglucoside) acetaldehyde.

²Comprise the following pigments: malvidin-3-glucoside-4-vinylphenol, malvidin-3-glucoside-4-vinylguaiacol.

³Comprise the following pigment: malvidin-3-glucoside-ethyl-catechin.

Polymeric pigments

The intervention of acetaldehyde in the condensation mechanism for the formation of polymeric pigments with a pronounced effect in the presence of flavanols (Dallas, Ricardo-da-Silva, & Laureano, **1996**) is confirmed through the analysis of stable pigments especially in treatments (+)-Catechin and ProcyanidinB2 (**Figure 47**). The dimers formed with added (+)-Catechin were identified as malvidin-3-*O*-glucoside-ethyl-catechin enantiomers with molecular ion $[M]^+$ (m/z) 809 and fragment ions (m/z) 657, 331 (He et al., **2012**; Monagas et al., **2003**) and retention times of 10.5 min and 11.2 min. Both UV-Vis signals had a shoulder between wavelengths 450 nm and 460 nm that correlates with Morata et al. (**2016**) observations for ethyl linkages for indirect condensation of flavanols with anthocyanins.

The yeasts *Lt-Sp* yielded the largest concentration of dimeric polymers. In general, for treatment (+)-catechin, the trials where the yeast *Sp* was used for sequential fermentation had produced more polymeric pigments than its counterpart fermented with *Sc*.

Regarding the formation of polymers with procyanidin B2, the trials fermented with *Sp* as sequential fermentative yeast had larger concentration values of polymers. The compound identified in all these trials is malvidin-3-*O*-glucoside-ethyl-procyanidinB2 with molecular ion $[M]^+$ (m/z) 1097 and fragment ion (m/z) 519 (He et al., **2012**; Morata et al., **2016**). The shoulder observed in the UV-Vis signal for anthocyanin-flavanol ethyl linkage at 460 nm was not sharp for these polymeric compounds as it was for the (+)-catechin enantiomers. The trial *Td-Sp* registered the highest value of this polymeric pigment with 0.33 ± 0.0 mg/L.

The evidence of the formation of polymers by condensation of procyanidin C1 with anthocyanins was not possible to assess since the concentration may be scarce to distinguish single peaks in the chromatograms and the signal from the UV-Vis and the mass spectrometer had relatively high noise signal for such concentration level. Few peaks were indicated at retention time 10.6 min with (m/z) 1187 as potential non-identified oligomer condensate products. These products had a shoulder signal at wavelength 460 nm similar to that produced by an ethyl linkage previously observed in other anthocyanin-flavanol condensates.

Figure 47 Polymeric peaks identified with HPLC in trials A) Control, B) (+)-Catechin and C) Procyanidin B2. The peaks were identified as follows: peaks 1) and 2) malvidin-3-glucoside-ethyl-catechin enantiomers with molecular ion $[M]^+$ (m/z) 809 and fragment ions (m/z) 657, 331; peaks 3) and 4) malvidin-3-O-glucoside-ethyl-procyanidinB2 with molecular ion $[M]^+$ (m/z) 1097 and fragment ion (m/z) 519.

Nonetheless, statistically speaking, there is no significant difference in treatment ProcyanidinC1 with respect to the control with any polymeric pigments at all.

3.3.3 Experiment 3

The cell walls of the yeast strains may adsorb anthocyanins in different extents (Suárez-Lepe & Morata, **2012**) and some of them may even adsorb three times more than others (Morata et al., **2003**); this fact suggests that the reduction of pigments during fermentation may be conditioned to the fermentative strains selected. Such is the case observed in this experiment specially seen when using the fermentative strains Lt-Sp where a significant reduction in the amount of malvidin-3-*O*-glucoside and total pigments is observed (**Table 17**).

The use of Lt in the initial phase may lead to the first reduction in total pigments that is later accentuated in the sequential fermentation with Sp. The treatment CAT was the only one where monomeric malvidin derivatives, others than malvidin-3-*O*-glucoside, were found; the pigment identified in these trials is the malvidin-3-*O*-(*p*-coumaroyl)-glucoside with molecular ion $[M]^+$ (m/z) 639 and fragment ion (m/z) 331 (Dipalmo, Crupi, Pati, Clodoveo, & Di Luccia, **2016**).

Pyranoanthocyanins are shown in **Table 17** under the groups Vitisins and Vinylphenolics. Regarding vitisins content, the pigment malvidin-3-*O*-glucoside acetaldehyde (Vitisin B) with molecular ion $[M]^+$ (m/z) 517 and fragment ion with (m/z) 355 (Vergara et al., **2010**) was identified in all trials with no statistical difference among them; nonetheless the amount of vitisins seems smaller for sequential fermentation trials in treatment PA2. The malvidin-3-*O*-glucoside-4-vinylphenol with molecular ion $[M]^+$ (m/z) 609 according with He et al. (**2012**) was the only vinylphenolic pigment identified in the trials where Sc was used as pure culture and sequential fermentation; the concentration in the pure culture trials was between 0.25 and 0.35 mg/L while the concentration in the trials with the fermentative strains Lt-Sc was between 0.18 and 0.22 mg/L (**Table 17**).

Table 17 Summary of pigments found in experimental red wines. The statistical analysis is given by parameter (columns) and the difference in letters indicates significant statistical difference (LSD method with 95% confidence level). Means $\pm$ STD ($n = 3$).

Treatment	Malvidin ¹ mg/L	Vitisins mg/L	Vinylphenolics mg/L	Polymeric pigments ² mg/L
<i>(+)-Catechin</i>				
Sc	28.7 $\pm$ 1.8 ^b	0.1 $\pm$ 0.0 ^{abc}	0.3 $\pm$ 0.0 ^{ab}	1.0 $\pm$ 0.1 ^a
Lt-Sc	25.9 $\pm$ 0.8 ^c	0.1 $\pm$ 0.0 ^{bc}	0.2 $\pm$ 0.0 ^e	0.8 $\pm$ 0.0 ^b
Lt-Sp	14.0 $\pm$ 0.7 ^d	0.1 $\pm$ 0.0 ^{abc}	0.0 $\pm$ 0.0 ^f	0.6 $\pm$ 0.1 ^d
<i>PB 2</i>				
Sc	29.7 $\pm$ 0.3 ^{ab}	0.1 $\pm$ 0.0 ^{ab}	0.4 $\pm$ 0.1 ^a	0.7 $\pm$ 0.0 ^{cd}
Lt-Sc	26.7 $\pm$ 0.6 ^c	0.0 $\pm$ 0.0 ^c	0.2 $\pm$ 0.0 ^{cd}	0.6 $\pm$ 0.0 ^d
Lt-Sp	14.7 $\pm$ 1.3 ^d	0.1 $\pm$ 0.0 ^{abc}	0.0 $\pm$ 0.0 ^f	0.6 $\pm$ 0.0 ^d
<i>PC 1</i>				
Sc	30.8 $\pm$ 0.5 ^a	0.1 $\pm$ 0.0 ^a	0.2 $\pm$ 0.0 ^{bc}	0.7 $\pm$ 0.0 ^c
Lt-Sc	26.4 $\pm$ 0.3 ^c	0.1 $\pm$ 0.0 ^{abc}	0.2 $\pm$ 0.0 ^{de}	0.6 $\pm$ 0.1 ^{cd}
Lt-Sp	13.7 $\pm$ 0.6 ^d	0.0 $\pm$ 0.0 ^{bc}	0.0 $\pm$ 0.0 ^f	0.4 $\pm$ 0.0 ^e
<i>PA2</i>				
Sc	29.9 $\pm$ 0.5 ^{ab}	0.1 $\pm$ 0.0 ^{abc}	0.3 $\pm$ 0.1 ^a	0.6 $\pm$ 0.1 ^d
Lt-Sc	26.4 $\pm$ 0.3 ^c	0.0 $\pm$ 0.0 ^{bc}	0.2 $\pm$ 0.0 ^d	0.4 $\pm$ 0.0 ^e
Lt-Sp	14.3 $\pm$ 0.6 ^d	0.0 $\pm$ 0.0 ^{bc}	0.0 $\pm$ 0.0 ^f	0.4 $\pm$ 0.0 ^e

¹Comprise the content of malvidin-3-*O*-glucoside and its derivative malvidin-3-*O*-(6-*p*-coumaroylglucoside)

²Comprise the content of identified polymeric pigments and unidentified peaks described in **Table 18**.

The fermentative pair Lt-Sp did not produce vinylphenolics due to the lack of hydroxycinnamate decarboxylase activity; this enzymatic activity is associated to the evaluated Sc strain and it has resulted in the condensation of malvidin-3-*O*-glucoside and product from the decarboxylation of *p*-coumaric acid (Morata et al., **2006**). Although Suárez-Lepe and Morata (**2015**) have reported hydroxycinnamate decarboxylase activity (HCDC) in non-*Saccharomyces* spp., there is no production of vinylphenolic pigments in the trials with fermentative strains Lt-Sp in any of the various treatments evaluated.

Polymeric pigments were formed during fermentation in all treatments and with all fermentative strains (**Table 18**). In treatment CAT the isomers malvidin-3-*O*-glucoside-vinylcatechin and malvidin-3-*O*-glucoside-vinyl-epicatechin with molecular ion $[M]^+$ (m/z) 805 (Asenstorfer et al., **2001**) were found in similar amounts in trails fermented with Sc while in sequential fermentations with Lt-Sp the quantity of the epicatechin derivative was higher; the third oligomer found in this treatment was malvidin-3-*O*-(*p*-coumaroyl)-glucoside-8-ethyl-(epi)catechin with molecular ion $[M]^+$ (m/z) 955 and fragment ions with (m/z) 803 and 647 (He et al., **2012**), this structure correspond to a dimer arrangement flavanol – anthocyanin ethyl linked.

Treatment PB2 produced slightly larger amounts of polymeric pigments with statistical significance ($p < 0.05$) for the fermentative strains Lt-Sp; the polymeric pigments identified in this treatment are malvidin-3-*O*-glucoside-epicatechin with molecular ion $[M]^+$ (m/z) 781 and fragment ion (m/z) 619, malvidin-3-*O*-glucoside-4-ethyl-epicatechin with molecular ion $[M]^+$ (m/z) 809, malvidin-3-*O*-glucoside-4-vinyl-epicatechin with molecular ion $[M]^+$ (m/z) 805 and malvidin-3-*O*-glucoside-4-vinyl-diepicatechin with molecular ion $[M]^+$ (m/z) 931 (He et al., **2012**). The last three pigments correspond to a flavanyl-pyranoanthocyanin structure and in particular, the last pigment, may correspond to the condensation of one malvidin unit with a procyanidin B2 (dimer) to form a trimer. The trimer is more abundant in trials using strain Sc in pure culture fermentation. The maximum absorption wavelength of these polymeric pigments is between 530 nm and 540 nm denoting a bathochromic effect or a displacement of the wavelength towards blueish tonality.

In treatment PC1 three dimers and a trimer were identified: the dimers were the isomers malvidin-3-*O*-glucoside-8-ethyl-catechin and malvidin-3-*O*-glucoside-8-ethyl-epicatechin

with molecular ion $[M]^+$ (m/z) 805 (Asenstorfer et al., **2001**; He et al., **2012**) and molecular structure flavanol-anthocyanin ethyl linked and the non-acetaldehyde mediated malvidin-3-*O*-glucoside-(epi)catechin with molecular ion $[M]^+$ (m/z) 781 and fragment ion (m/z) 619 (He et al., **2012**); the trimer found, with a slightly higher concentration in trials with strains Lt-Sp, was the malvidin-3-*O*-glucoside-4-vinyl-diepicatechin with molecular ion $[M]^+$ (m/z) 931 (Dipalmo et al., **2016**).

There were no pigments identified from the direct condensation of procyanidin C1 (trimer) with malvidin-3-*O*-glucoside. The fourth treatment, PA2, produced the least concentration of oligomeric pigments, specially the fermentative strain Sc in pure culture fermentation with 0.09 ± 0.01 mg/L; the polymeric pigments found in this treatment are malvidin-3-*O*-glucoside-epicatechin with molecular ion $[M]^+$ (m/z) 781 and fragment ion (m/z) 619, the flavanol pyranoanthocyanin dimer malvidin-3-*O*-glucoside-4-vinyl-epicatechin with molecular ion $[M]^+$ (m/z) 805 and the also flavanol pyranoanthocyanin trimer malvidin-3-*O*-glucoside-4-vinyl-diepicatechin with molecular ion $[M]^+$ (m/z) 931 (Dipalmo et al., **2016**).

The direct condensation of dimers flavanol-anthocyanin happens at slower and at lower yielding rates (Eglinton et al., **2004**) than the polymeric pigments formed when acetaldehyde is present; in this way, Dallas, Ricardo-da-Silva, and Laureano (**1996**) have also documented the direct condensation of procyanidin B2 with malvidin-3-*O*-glucoside in the absence of acetaldehyde.

The condensation of most of the polymeric pigments identified in this experimental design was mediated by acetaldehyde. The oligomer formation is not restrictive to just having one moiety of anthocyanin and another moiety of flavanol; two flavanol units may also condensed in the presence of acetaldehyde and even the anthocyanin could be in the form of and hemiacetal structure. From these polymeric structures formed, the flavanol-ethyl-flavanol oligomers are less stable (Es-Safi & Cheynier, **2004**). The copigmentation equilibrium phenomena may increase the colour of wine displacing some of the colourless anthocyanins into coloured oligomer species (Darias-Martín, Carrillo, Díaz, & Boulton, **2001**) after the condensation of hemiacetal forms with flavanols favouring the protonation of the anthocyanin afterwards. Three peaks are labelled as unidentified since the molecular weight does not correlate to expected molecular structures. Unidentified peaks 1 and 3 occur in treatments CAT and PB2 while all three are present in treatments PC1 and PA2.

Table 18 Stable pigments identified with HPLC-DAD-ESI/MS with DAD signal at 525nm in experimental wines.

Compound	t _R (min)	[M] ⁺ <i>m/z</i>	Fragment <i>m/z</i>	λ ^{max} _{VIS} (nm)	Reference
Malvidin-3- <i>O</i> -glucoside-epicatechin	12.9	781	619	530	<i>He et al. 2012</i>
Unidentified Peak 1	13.4	679	-	514	
Malvidin-3- <i>O</i> -glucoside-4-vinyl-diepicatechin	13.9	931	-	533	<i>Dipalmo et al. 2016</i>
Malvidin-3- <i>O</i> -glucoside-ethyl-epicatechin	14.2	809	-	538	<i>He et al. 2012</i>
Unidentified Peak 2	14.3	995	782	538	
Malvidin-3- <i>O</i> -(6- <i>p</i> -coumaroylglucoside)-8-ethyl-(epi)catechin	14.5	954	852	520	<i>He et al. 2012</i>
Malvidin-3- <i>O</i> -glucoside-8-ethyl-epicatechin	14.6	809	643	542	<i>Dipalmo et al. 2016</i>
Malvidin-3- <i>O</i> -glucoside-4-vinyl-epicatechin	14.9	805	331	540	<i>Asenstorfer et al. 2001</i>
Malvidin-3- <i>O</i> -glucoside-8-ethyl-catechin	15.3	809	643	538	<i>He et al. 2012</i>
Diepicatechin-malvidin-3- <i>O</i> -glucoside	16.2	619	-	506	<i>He et al. 2012</i>
Unidentified Peak 3	17.3	829	691	512	

t_R) retention time; [M]⁺) molecular ion; λ) maximum absorption wavelength.

The contribution of the yeast strains and the different treatments to the evolution and stability of pigments can be seen in a principle component analysis (PCA) chart in **Figure 48**. Two principal components explain the 84.7% of the variability of the experimental design. Component 1 had a positive contribution from malvidin-3-*O*-glucoside, total pigments and vinylphenolics while Component 2 had positive contribution from polymeric pigments and vitisins.

Figure 48 Principal Component Analysis (PCA) for pigments from experimental trials. The clusters identified by rectangles correspond to each individual fermentation (block font). The numbers refer to each of the four treatments evaluated: 1) CAT; 2) PB2; 3) PC1 and 4) PA2.

In this way there are three different clusters observed with respect to Component 1 where those trials having more pigments are located to the right side of the chart and correspond to pure culture fermentation with Sc while trials having the least concentration are located to the left; this behaviour was observed by Escott et al. (2018) for other non-*Saccharomyces* spp. including *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*. Regarding Component 2, the contribution of the treatments to the pigment formation can be seen for each fermentative media; treatment CAT had produced the largest amount of polymeric pigments in all three clusters followed by treatment PB2 for trials with Lt-Sp and by treatment PC1 for trials Sc and Lt-Sc.

3.3.4 Experiment 4

The number of anthocyanins including the non-acylated, the acetyl-, *p*-coumaroyl- and caffeoyl- derivatives, decreased in all cases when aldehydes were added (**Figure 49** left); SYRN produced less pigments reduction while, on the contrary, PHPR yielded the lowest concentration of pigments after the evaluation. The DAD signal and the MS spectra from the HPLC-DAD-ESI/MS analysis allowed the identification of the peaks observed on **Figure 49** (right) at full scale although, in some cases, the concentration was rather low.

Figure 49 HPLC chromatogram at same scale (left) and at full scale (right).

The following anthocyanins were identified in all six samples: delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin-3-*O*-(6-*p*-coumaroyl)-glucoside, cyanidin-3-*O*-(6-*p*-coumaroyl)-glucoside, petunidin-3-*O*-(6-caffeoyl)-glucoside and malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside. Besides these peaks, other peaks with unidentified nature are also reported in samples PHAC, PHPR, SYRN and HMBZ (**Table 19**).

Table 19 Anthocyanins found in trials after four weeks. Retention times (t_R) and spectral features in LC-DAD-ESI/MS analysis are shown for all treatments.

Anthocyanin	t_R	$\lambda_{\max}$ (nm)	$[M]^+$ (m/z)	MS/MS (m/z)
Delphinidin-3- <i>O</i> -glucoside	7.43	524	465	303
Cyanidin-3- <i>O</i> -glucoside	8.67	518	449	287
Petunidin-3- <i>O</i> -glucoside	9.50	526	479	317
Peonidin-3- <i>O</i> -glucoside	10.48	518	463	301
Malvidin-3- <i>O</i> -glucoside	10.97	528	493	331
Delphinidin-3- <i>O</i> -(6-acetyl)-glucoside	12.45	530	507	303
Cyanidin-3- <i>O</i> -(6-acetyl)-glucoside	13.57	528	491	284
Petunidin-3- <i>O</i> -(6-acetyl)-glucoside	14.07	530	521	317
Peonidin-3- <i>O</i> -(6-acetyl)-glucoside	15.09	522	505	301
Malvidin-3- <i>O</i> -(6-acetyl)-glucoside	15.29	530	535	331
Delphinidin-3- <i>O</i> -(6- <i>p</i> -coumaroyl)-glucoside	15.51	532	611	303
Cyanidin-3- <i>O</i> -(6- <i>p</i> -coumaroyl)-glucoside	16.46	524	595	287
Petunidin-3- <i>O</i> -(6-caffeoyl)-glucoside	16.76	534	625	317
Malvidin-3- <i>O</i> -(6- <i>p</i> -coumaroyl)-glucoside	17.67	532	639	331
<i>PHAC</i>				
Unidentified	13.62	506	613	453
Unidentified	14.85	515	701	347
Unidentified	15.99	502	563	401
Peak 1 ¹	16.62	508	593	431
<i>PHPR</i>				
Unidentified	18.48	537	915	603
<i>SYRN</i>				
Unidentified	16.03	538	655	
<i>HMBZ</i>				
Peak 2 ²	13.33	542	1094	331
Unidentified	14.40	540	1189	657/521
Unidentified	17.43	542	899	563/317

Notes. CTRL groups monomeric anthocyanins and acyl derivatives found in all trials.

¹ Concentration accounts for 0.28 mg/L.

² Concentration accounts for 0.63 mg/L.

In the case of PHAC the unidentified peaks had maximum absorbance wavelengths between 502 and 515 nm, lower than those of monomeric anthocyanins but similar to the range of absorption seen for the vitisins (Araújo, Fernandes, de Freitas, & Oliveira, **2017**; Morata et al., **2006**). One of these peaks, labelled Peak 1 in **Table 19** had a molecular ion $[M]^+$ at (m/z) 593 and fragment ion with at (m/z) 431 (**Figure 50**) which may potentially correspond to the intramolecular interaction of a malvidin-3-*O*-glucoside moiety and the phenylacetaldehyde molecule; this peak has a λ_{max} of 508 nm and t_R of 16.62 min.

Figure 50 Mass spectrum with molecular ions identified for peak 1. Maximum absorbance from DAD-signal was observed at 508 nm (top-right)

Regarding trials PHPR, SYRN and HMBZ, the unidentified peaks found had higher λ_{max} , towards bluish tonality. The weight of the ions $[M]^+$ of these unidentified peaks resembles that of polymeric pigments as well as the shift of wavelengths as a result of a bathochromic effect (Mateus, Silva, Santos-Buelga, Rivas-Gonzalo, & De Freitas, **2002**).

A second peak, Peak 2 (**Table 19**), with $[M]^+$ at (m/z) 1094 and λ_{max} at 542 nm found in the trail HMBZ is similar to that reported for the formation of an oligomeric unit from the reaction of malvidin-3-*O*-glucoside and (+)-catechin in the presence of vanillin aldehyde

(Sousa et al., 2007); such pigment formed had $[M]^+$ at (m/z) 917 and $\lambda_{\max}$ 549 (purple colour); the vanillin aldehyde was found to form an aryl linkage. In the current case study, the reaction may have taken place between two anthocyanin units; the $[M]^+$ at (m/z) 1094 got fore Peak 2 would potentially correspond to a malvidin-3-*O*-glucoside unit and a cyaniding-3-*O*-glucoside molecule linked by one moiety of 4-hydroxy-3-methoxybenzaldehyde (HMBZ) and a fragment ion at (m/z) 331. The mass spectrum relative abundance and the DAD signal at 525 nm for this peak are shown in **Figure 51**. Nonetheless the molecular ions give information regarding the nature of the components, the techniques used do not allow the identification of molecular structures produced.

Figure 51 Mass spectrum with molecular ions identified for peak 2. Maximum absorbance from DAD-signal was observed at 542 nm (top-left)

3.3.5 Experiment 5

In this experiment, the following pigments were identified: delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside; Acetyl glucosides: delphinidin-3-*O*-(6"-acetylglucoside), cyanidin-3-*O*-(6"-acetylglucoside), petunidin-3-*O*-(6"-acetylglucoside), peonidin-3-*O*-(6"-acetylglucoside) and malvidin-3-*O*-(6"-acetylglucoside); Coumaroyl glucosides: delphinidin-3-*O*-(6"-*p*-coumaroylglucoside)

cyanidin-3- *O* -(6"-*p*-coumaroylglucoside), petunidin-3- *O* -(6"-*p*-coumaroylglucoside), peonidin-3- *O* -(6"-*p*-coumaroylglucoside), and malvidin-3- *O* -(6"-*p*-coumaroylglucoside); Vitisins: malvidin-3- *O* -glucoside acetaldehyde (vitisin B), malvidin-3- *O* -glucoside pyruvic acid (vitisin A), and malvidin-3- *O* -(6"-acetylglucoside) acetaldehyde (acetyl vitisin B); vinyl phenolics: malvidin-3-*O*-glucoside-4-vinylphenol and malvidin-3-*O*-glucoside-4-vinylguaiacol. All pigments were grouped in the five families shown in **Table 20**. The concentrations per family, even though are very similar, happen to have significant difference ($p<0.05$). There is an apparent lower total pigment concentration in treatments 5pPm and 10pPt which may be attributed to other factors such as the fermenter and the fermentation volume or pigment adsorption on yeast cell walls (Morata, Gómez-Cordovés, Colomo, & Suárez, **2005**). In this case, the population growth during tumultuous phase may affect colour in different extent.

Table 20 Pigment matter profile in experimental wines. Pigments are grouped by families. Average and STD ($n=3$). Different letters denote a significant difference with 95% confidence level (LSD test).

Pigment family	Control	5pPm	10pPm	5pPt	10pPt
Monomers	56.3±7.2 ^a	38.4±5.5 ^b	51.4±15.9 ^{ab}	41.4±6.8 ^b	39.1±1.2 ^b
Acetyl - glucosides	2.8±0.3 ^a	2.0±0.2 ^b	2.5±0.7 ^{ab}	2.1±0.4 ^b	2.1±0.1 ^b
Coumaroyl - glucosides	3.1±0.5 ^a	2.1±0.2 ^{ab}	3.0±1.1 ^{ab}	2.3±0.4 ^{ab}	2.0±0.1 ^b
Vitisins	7.1±0.6 ^a	6.7±0.8 ^a	7.1±1.2 ^a	7.2±0.4 ^a	6.9±0.1 ^a
Vinyl phenolics	0.04±0.0 ^a	0.03±0.0 ^b	0.05±0.0 ^b	0.05±0.0 ^{ab}	0.04±0.0 ^{ab}
Total pigments	73.4±8.0 ^a	53.0±6.9 ^b	68.2±19.3 ^{ab}	56.9±8.1 ^{ab}	53.8±1.2 ^b

Other explanation is that the pigments from the grape's skin went under photodegradation. PL treatments irradiate the sample with a fraction of UV-C light. The continuous irradiation of pigments with UV-C light has led to a reduction of anthocyanins between 8-16% (Pala & Toklucu, **2011**). An oxidative degradation has been also observed in grape processing when using e-beam irradiation (Morata, Bañuelos, et al., **2015**).

3.4 Colour assessment

3.4.1 Experiment 1

All wines present less colour intensity with respect to the original colour found in musts potentially due to the precipitation of anthocyanins during fermentation and to the adsorption of pigments by the yeast cell walls (Morata et al., **2006**). Among the wines produced, the wines fermented with *Saccharomyces ludwigii* (strain 979) had slightly higher colour intensity and tonality for the wines “control” and “procyanidinB2” since its yellow and blue fractions are higher while the red fraction is lower in comparison to the other wines.

On the other hand, the wines produced with species *Saccharomyces cerevisiae* (strain 7VA) tend to have lower colour intensity potentially associated to the formation of insoluble polymeric pigments (Figueiredo-González et al., **2014**) and lower tonality values than those fermented with non-*Saccharomyces* yeasts generally associated to oxidative processes. Variations in colour can also be seen in the wines fermented with (+)-catechin and procyanidin B2 since the red fraction increases when these flavan-3-ols are present during fermentation and the blue fraction decreases in the same samples (**Table 21**).

Table 21 Characterization of colour of finished wines with UV-Vis spectrophotometry. Mean $\pm$ STD (n=3).

Strain	Colour Intensity ¹	Hue ²	% Yellow ³	% Red ³	% Blue ³
Control					
Sp 938	0.47 $\pm$ 0.04 ^a	0.97 $\pm$ 0.02 ^a	45.9 $\pm$ 0.3 ^b	47.3 $\pm$ 1.2 ^a	6.8 $\pm$ 1.3 ^a
SI 979	0.51 $\pm$ 0.03 ^a	1.25 $\pm$ 0.01 ^a	50.9 $\pm$ 0.1 ^c	40.5 $\pm$ 0.5 ^a	8.6 $\pm$ 0.5 ^a
Td 1880	0.38 $\pm$ 0.04 ^a	1.11 $\pm$ 0.03 ^a	49.8 $\pm$ 0.3 ^c	44.8 $\pm$ 1.2 ^a	5.5 $\pm$ 1.3 ^a
Sc 7VA	0.25 $\pm$ 0.01 ^a	0.88 $\pm$ 0.01 ^a	46.9 $\pm$ 0.7 ^b	53.4 $\pm$ 0.8 ^a	0.3 $\pm$ 1.4 ^a
Must	1.06 $\pm$ 0.04 ^b	0.89 $\pm$ 0.01 ^a	41.7 $\pm$ 0.2 ^a	46.8 $\pm$ 0.4 ^a	11.5 $\pm$ 0.5 ^b
With (+) - Catechin addition					
Sp 938	0.38 $\pm$ 0.07 ^a	0.90 $\pm$ 0.07 ^a	45.3 $\pm$ 1.1 ^b	50.3 $\pm$ 2.8 ^a	4.4 $\pm$ 2.2 ^a
SI 979	0.22 $\pm$ 0.03 ^a	0.89 $\pm$ 0.01 ^a	48.6 $\pm$ 0.3 ^c	54.6 $\pm$ 0.5 ^a	3.2 $\pm$ 0.6 ^a
Td 1880	0.25 $\pm$ 0.02 ^a	0.94 $\pm$ 0.02 ^a	48.9 $\pm$ 0.4 ^c	51.9 $\pm$ 1.1 ^a	0.8 $\pm$ 1.3 ^a
Sc 7VA	0.26 $\pm$ 0.02 ^a	0.85 $\pm$ 0.00 ^a	45.6 $\pm$ 1.0 ^b	53.9 $\pm$ 1.0 ^a	0.5 $\pm$ 1.9 ^a
Must	1.12 $\pm$ 0.04 ^b	0.91 $\pm$ 0.01 ^a	41.7 $\pm$ 0.2 ^a	46.0 $\pm$ 0.4 ^a	12.3 $\pm$ 0.5 ^b
With Procyanidin B2 addition					
Sp 938	0.37 $\pm$ 0.06 ^a	0.87 $\pm$ 0.06 ^a	44.8 $\pm$ 0.7 ^b	51.5 $\pm$ 3.4 ^a	3.8 $\pm$ 3.0 ^a
SI 979	0.39 $\pm$ 0.06 ^a	1.15 $\pm$ 0.06 ^a	50.3 $\pm$ 0.7 ^c	43.9 $\pm$ 3.4 ^a	5.8 $\pm$ 3.0 ^a
Td 1880	0.28 $\pm$ 0.01 ^a	0.96 $\pm$ 0.00 ^a	48.6 $\pm$ 0.4 ^c	50.4 $\pm$ 0.4 ^a	1.0 $\pm$ 0.8 ^a
Sc 7VA	0.27 $\pm$ 0.00 ^a	0.86 $\pm$ 0.02 ^a	45.3 $\pm$ 0.5 ^b	52.7 $\pm$ 0.4 ^a	2.0 $\pm$ 0.2 ^a
Must	1.13 $\pm$ 0.04 ^b	0.89 $\pm$ 0.01 ^a	41.4 $\pm$ 0.2 ^a	46.3 $\pm$ 0.4 ^a	12.3 $\pm$ 0.5 ^b

¹ Σ of absorptions at $\lambda_{\max}$ 420nm, $\lambda_{\max}$ 520nm and $\lambda_{\max}$ 620nm

² Ratio $\lambda_{\max}$ 420nm / $\lambda_{\max}$ 520nm

³ Fraction of colour (yellow, red or blue) obtained with the formula % = $[(\lambda_{\max} \times 100) / \text{Colour Intensity}]$

Different letters in the same column indicate significant differences between means (p<0.05)

3.4.2 Experiment 2

The colour intensity as well as the hue were determined for all the sequential fermentations in all treatments and, as it can be seen in **Table 22** (section A), the fermentations done with added procyanidin B2 had higher colour intensity values with no significant difference among the trials within the same treatment; the trials in the control set had significant difference between the sequential fermentations done with *Sc* and those done with *Sp*, the trials fermented with *Sc* had lower colour intensity values.

On the other hand, there are no significant difference in hue values in the trials of all treatments except for the fermentations done with *Mp-Sp* where, in all treatments, had the highest values with significant differences in each treatment (**Table 22** – section B).

The values of the total polyphenol index (TPI) had the same profile than the colour intensity with the highest values obtained with the addition of procyanidin B2 (**Table 22** – section C).

Table 22 Colour and total polyphenol index (TPI) assessed in experimental wines with UV-Vis spectrophotometry. Mean $\pm$ STD (n=3).

Yeast \ Treatment	Control	(+)-Catechin	ProcyanidinB2	ProcyanidinC1
A				
Colour Intensity¹				
<i>Lt-Sc</i>	0.11 $\pm$ 0.0 ^b	0.18 $\pm$ 0.0 ^a	0.28 $\pm$ 0.0 ^a	0.15 $\pm$ 0.0 ^a
<i>Mp-Sc</i>	0.11 $\pm$ 0.0 ^b	0.17 $\pm$ 0.0 ^a	0.27 $\pm$ 0.1 ^a	0.16 $\pm$ 0.0 ^a
<i>Td-Sc</i>	0.10 $\pm$ 0.0 ^b	0.17 $\pm$ 0.0 ^a	0.26 $\pm$ 0.1 ^a	0.17 $\pm$ 0.0 ^a
<i>Lt-Sp</i>	0.14 $\pm$ 0.0 ^{ab}	0.21 $\pm$ 0.0 ^a	0.27 $\pm$ 0.0 ^a	0.17 $\pm$ 0.0 ^a
<i>Mp-Sp</i>	0.15 $\pm$ 0.0 ^a	0.20 $\pm$ 0.0 ^a	0.25 $\pm$ 0.0 ^a	0.16 $\pm$ 0.0 ^a
<i>Td-Sp</i>	0.16 $\pm$ 0.0 ^a	0.17 $\pm$ 0.0 ^a	0.28 $\pm$ 0.0 ^a	0.17 $\pm$ 0.0 ^a
B				
Hue²				
<i>Lt-Sc</i>	1.0 $\pm$ 0.0 ^c	0.8 $\pm$ 0.0 ^c	0.8 $\pm$ 0.0 ^d	0.8 $\pm$ 0.0 ^d
<i>Mp-Sc</i>	1.0 $\pm$ 0.0 ^{bc}	0.9 $\pm$ 0.0 ^{bc}	0.9 $\pm$ 0.0 ^{bc}	0.9 $\pm$ 0.0 ^c
<i>Td-Sc</i>	1.1 $\pm$ 0.0 ^{bc}	0.9 $\pm$ 0.0 ^{bc}	0.9 $\pm$ 0.0 ^{cd}	0.8 $\pm$ 0.0 ^d
<i>Lt-Sp</i>	1.2 $\pm$ 0.1 ^b	1.0 $\pm$ 0.0 ^{bc}	1.0 $\pm$ 0.1 ^{bc}	0.8 $\pm$ 0.0 ^{cd}
<i>Mp-Sp</i>	1.4 $\pm$ 0.0 ^a	1.2 $\pm$ 0.0 ^a	1.2 $\pm$ 0.0 ^a	1.2 $\pm$ 0.0 ^a
<i>Td-Sp</i>	1.2 $\pm$ 0.2 ^b	1.1 $\pm$ 0.2 ^b	1.0 $\pm$ 0.0 ^b	1.0 $\pm$ 0.0 ^b
C				
Total polyphenol index				
<i>Lt-Sc</i>	4.0 $\pm$ 0.7 ^a	12.4 $\pm$ 1.4 ^{ab}	23.6 $\pm$ 0.1 ^b	9.4 $\pm$ 1.0 ^{ab}
<i>Mp-Sc</i>	4.7 $\pm$ 0.7 ^a	13.5 $\pm$ 1.1 ^{ab}	25.4 $\pm$ 0.6 ^a	8.9 $\pm$ 0.9 ^{ab}
<i>Td-Sc</i>	3.2 $\pm$ 0.7 ^a	14.4 $\pm$ 0.6 ^a	24.6 $\pm$ 0.6 ^{ab}	7.0 $\pm$ 0.8 ^b
<i>Lt-Sp</i>	3.4 $\pm$ 0.4 ^a	9.5 $\pm$ 0.6 ^b	23.8 $\pm$ 0.5 ^b	11.2 $\pm$ 1.2 ^a
<i>Mp-Sp</i>	4.6 $\pm$ 0.9 ^a	13.5 $\pm$ 1.4 ^a	25.4 $\pm$ 0.5 ^a	10.4 $\pm$ 0.9 ^a
<i>Td-Sp</i>	3.4 $\pm$ 0.2 ^a	10.0 $\pm$ 0.1 ^b	24.7 $\pm$ 0.5 ^{ab}	7.2 $\pm$ 1.0 ^b

¹ Σ of absorption at $\lambda_{\max}$ 420nm. $\lambda_{\max}$ 520nm and $\lambda_{\max}$ 620nm

² Ratio $\lambda_{\max}$ 420nm / $\lambda_{\max}$ 520nm

Different letters in the same column indicate significant differences between means (p<0.05). Parameters (A, B and C) were treated separately.

3.4.3 Experiment 3

Spectrophotometry, in particular UV-Vis, has been used to determine colour parameters as well as polyphenols due to the ability of anthocyanins and flavanols to absorb at different wavelengths in function of their molecular structure. **Table 23** summarizes all parameters obtained from the absorption at wavelengths in the UV and the visible spectrum. It can be seen that the colour intensity and the hue have greater values for the trials fermented with the pair Lt-Sp in all treatments; there is also a slight tendency to increase values of these two parameters in the following order of fermentative yeasts $Sc < Lt-Sc < Lt-Sp$. The anthocyanins ionization degree (α in **Table 23**) is also higher for the fermentative pair Lt-Sp meaning that the fraction of pigments with flavylium form is higher thus the colour observed may be more intense.

On the other hand, the amount of pigments measured with this technique is inverse to the colour intensity, meaning that trials fermented with Sc have larger amount of pigments in the end of the fermentation, and, as observed with HPLC-DAD-ESI/MS, the concentration of malvidin-3-*O*-glucoside is larger in these trials followed by the trials where Lt-Sc were used. The trials with the least concentration of polymeric pigments and total pigments correspond to those fermented with Sp. The trend of these values differs from that observed with HPLC-DAD-ESI/MS making evident a larger measuring range of this technique.

Table 23 Results from UV-Vis determination of colour parameters and polyphenols. The statistical analysis is given by parameter (columns) and the difference in letters indicates significant statistical difference (LSD method with 95% confidence level). Means $\pm$ STD ($n = 3$).

Treatment	I AU	N	α %	TPI AU	ChA	PI %	PP AU	TP AU
<i>(+) - Catechin</i>								
Sc	0.78 ± 0.0^{bc}	1.59 ± 0.0^c	17.76 ± 0.8^{bc}	13.81 ± 0.1^b	0.13 ± 0.0^{bc}	12.77 ± 0.6^{de}	0.13 ± 0.0^{bc}	1.03 ± 0.1^a
Lt-Sc	0.80 ± 0.0^{ab}	1.65 ± 0.0^c	30.84 ± 1.8^a	13.41 ± 0.1^b	0.23 ± 0.0^a	23.12 ± 0.6^{ab}	0.15 ± 0.0^a	0.66 ± 0.0^d
Lt-Sp	0.82 ± 0.0^{ab}	2.25 ± 0.0^{ab}	23.53 ± 3.6^b	14.77 ± 0.1^a	0.25 ± 0.0^a	25.27 ± 2.2^a	0.15 ± 0.0^a	0.59 ± 0.0^d
<i>PB2</i>								
Sc	0.71 ± 0.1^d	1.59 ± 0.0^c	16.39 ± 0.2^c	13.70 ± 0.5^b	0.12 ± 0.0^c	11.80 ± 0.7^e	0.12 ± 0.0^c	1.02 ± 0.1^{ab}
Lt-Sc	0.81 ± 0.0^{ab}	1.64 ± 0.0^c	22.86 ± 2.2^{bc}	13.39 ± 0.0^b	0.19 ± 0.0^b	19.17 ± 1.1^{bc}	0.16 ± 0.0^a	0.82 ± 0.1^c
Lt-Sp	0.84 ± 0.0^a	2.17 ± 0.0^b	22.68 ± 4.2^{bc}	15.04 ± 0.2^a	0.25 ± 0.0^a	24.61 ± 1.3^a	0.16 ± 0.0^a	0.64 ± 0.1^d
<i>PC1</i>								
Sc	0.74 ± 0.0^{cd}	1.64 ± 0.0^c	18.49 ± 1.6^{bc}	13.61 ± 0.1^b	0.13 ± 0.0^{bc}	12.84 ± 0.6^{de}	0.12 ± 0.0^c	0.95 ± 0.1^b
Lt-Sc	0.80 ± 0.0^{ab}	1.65 ± 0.0^c	22.48 ± 1.2^{bc}	13.36 ± 0.1^b	0.18 ± 0.0^b	18.44 ± 0.4^c	0.15 ± 0.0^a	0.82 ± 0.0^c
Lt-Sp	0.84 ± 0.0^a	2.20 ± 0.1^{ab}	24.53 ± 2.9^{ab}	14.64 ± 0.3^a	0.27 ± 0.0^a	26.70 ± 2.7^a	0.16 ± 0.0^a	0.59 ± 0.0^d
<i>PA2</i>								
Sc	0.75 ± 0.0^{cd}	1.64 ± 0.0^c	20.60 ± 1.2^{bc}	13.81 ± 0.1^b	0.16 ± 0.0^{ab}	15.84 ± 0.7^{cd}	0.14 ± 0.0^b	0.85 ± 0.0^c
Lt-Sc	0.82 ± 0.0^{ab}	1.66 ± 0.0^c	23.22 ± 2.5^b	13.38 ± 0.2^b	0.19 ± 0.0^b	19.05 ± 1.9^c	0.16 ± 0.0^a	0.82 ± 0.1^c
Lt-Sp	0.84 ± 0.0^a	2.26 ± 0.0^a	21.77 ± 2.0^{bc}	14.86 ± 0.2^a	0.25 ± 0.0^a	25.31 ± 0.9^a	0.16 ± 0.0^a	0.62 ± 0.0^d

I) Colour intensity; N) Hue (N); α) anthocyanin ionization degree; TPI) total polyphenols index; ChA) chemical age; PI) polymerization index; PP) polymerized pigments; TP) total pigments.

Regarding ChA and PI (**Table 23**), the values are higher for the fermentative pair Lt-Sp as also observed for hue (N) mainly due to the fact that the ratio oligomeric pigments to total pigments is higher for these trials even though the total pigments is smaller as already observed by Chen et al. (2018). According to these authors, the amount of monomeric and total pigments decreased when using Sp as fermentative yeasts, but the fraction of oligomeric pigments increased. The fact of having more oligomeric pigments and therefore larger values for ChA suggests an increase in compounds with absorption at smaller wavelengths similar to those pigments found in aged wines. The values of the TPI are statistical different for the trials Lt-Sp with a slightly higher numerical difference from the other trials.

3.4.4 Experiment 4

The change in colour was monitored with instrumental colour measurements; the spectrophotometric technique recorded the variations in absorbance at different wavelengths (420nm – for yellow, 520nm – for red and 620nm – for blue). During the month that the experiment spanned, the percentage of these three colours remained steady for the control (**Figure 52**). On the other hand, variations in the colour composition have been observed when using different aldehydes.

The use of PHPR decreased the percentage of red while on the other hand blue and yellow increased over time. CINN and PHAC have the largest amount of yellow among all aldehydes and the lowest contribution from red; regarding blue fraction, CINN decreases over time while PHAC increases it. SYRN and HMBZ had the greater values for red percentage after the control with a slight decrease towards the end of the evaluation; SYRN has similar values for yellow percentage than the control while HMBZ has slightly higher values; regarding blue content both aldehydes have larger percentage of blue with a trend to increase it with SYRN and to decrease with HMBZ towards the end of the evaluation.

Figure 52 Colour variations due to the contribution of the five phenolic aldehydes to media wine model.

The measurement of fixed absorption wavelengths also allowed us to follow up the changes in in colour intensity (CI) and hue (N) shown in **Figure 53**. As it can be seen, the control has a steady CI and N during the experiment while the addition of phenolic aldehydes produces changes in these two parameters. The aldehydes PHPR, PHAC and SYRN increase CI over time; on the other hand, CINN and HMBZ had a decreasing trend. Nonetheless, CI values are higher than the control after 4 weeks for the trials with aldehydes as a result of the increased absorptions in yellow and blue fractions. The pronounced gain in yellow percentage by addition of CINN and PHAC (**Figure 52**) has resulted in an increase of N three times more than the control with a trend to having larger N values over time; SYRN and HMBZ had N values similar to the control with a slight increase over time. Having larger N values are generally related to wine ageing processes where red wine pigments may undergo co-pigmentation phenomena with phenolic compounds found in wine, yielding molecules with lower maximum absorbance wavelength (ca. 505 nm), towards brick-red hue (Mateus et al., 2002). Shifts in the maximum absorbance wavelength of a pigment is known as bathochromic

effect and if it happens towards higher wavelengths, it is named bluing effect in wines (Cavalcanti, Santos, & Meireles, **2011**).

Figure 53 Colour intensity (CI) and hue (N) for trials having the five different aldehydes against the control.

The values from the CIE $L^*a^*b^*$ coordinates allowed us to build up a colour palette chart (**Figure 54**) that shows the evolution of colour over the span of the experiment. The lightness (L^*) is larger for PHAC followed by PHPR after one week and is has similar values for PHPR, CINN and PHAC at the end of the evaluation; higher lightness values may be related to lower colour stability since lightness decrease in wine samples over time indicates higher colour stabilization (García-Estévez, Alcalde-Eon, Puente, & Escribano-Bailón, **2017**); these authors correlate the decrease in L^* with an addition of oenological tannins increasing the amount of pigments in the wine. The reduction of stability observed in the experimental trials, and therefore an increase in L^* values, might be explained by the fact that aldehydes in excess promote the precipitation of pigments in wine model solutions.

Figure 54 Colour palette for media wine solutions with aldehydes obtained with CIE Lab coordinates.

The CIE $L^*a^*b^*$ values for the control trial were considered as initial reference conditions for determining differences among samples after four weeks. Absolute colour difference (ΔE^*_{ab}), variations in lightness (ΔL^*), chroma (ΔC^*_{ab}) and hue (Δh^*_{ab}) are shown in **Table 24**. Positive values for ΔA_{520} are the effect of having fortified CI as a consequence of the addition of copigments and an increase in absorbance at 520 nm known as hyperchromic effect or fortified colour intensity (Rein, **2005**); this hyperchromic effect may be the result of shifting the anthocyanins molecules equilibrium towards the flavylium coloured form (Davies & Mazza, **1993**).

Table 24 Mean values of λA_{520} and CIE $L^*a^*b^*$ differences ΔE^*_{ab} , ΔL^* , ΔC^*_{ab} and Δh_{ab} . Average and SD ($n = 3$). Different letters denote a significant difference with 95% confidence level (LSD test).

	PHPR	CINN	PHAC	SYRN	HMBZ
ΔA_{520}	-0.8 ± 0.002^e	-0.8 ± 0.001^d	-0.6 ± 0.001^c	$+1.1 \pm 0.002^a$	$+0.3 \pm 0.001^b$
ΔE^*_{ab}	12.0 ± 0.01^d	13.9 ± 0.01^b	16.7 ± 0.02^a	13.0 ± 0.01^c	5.7 ± 0.18^e
ΔL^*	$+0.6 \pm 0.01^b$	$+3.0 \pm 0.03^a$	-0.5 ± 0.01^c	-9.1 ± 0.02^e	-4.9 ± 0.03^d
ΔC^*_{ab}	-8.4 ± 0.02^e	-7.0 ± 0.02^c	-8.4 ± 0.01^d	$+8.0 \pm 0.01^a$	$+2.8 \pm 0.01^b$
Δh_{ab}	2.3 ± 0.08^d	11.7 ± 0.01^b	14.4 ± 0.02^a	5.0 ± 0.03^c	1.4 ± 0.06^d

The smaller the ΔE^*_{ab} the less colour variation observed between samples; in this way HMBZ had the least colour variation from the control while PHAC had the greatest. The change in colour in all samples having aldehydes was perceived by the human eye and this can be explained analytically since the value for ΔE^*_{ab} was greater than 3 CIEL^{*}*a*^{*}*b*^{*} units, which was experimentally determined with red wines and a panel of observers with normal colour vision (Martínez, Melgosa, Pérez, Hita, & Negueruela, **2001**). In terms of lightness (ΔL^*), PHPR and CINN had positive variation due to an increase in L^* while the others had negative values for lower L^* values. The greater variation in hue (Δh^*_{ab}) was observed in CINN and PHAC. Another study observed a bathochromic effect at low pH values (1 to 3) for vanillic, syringic and coniferil aldehydes and also a hyperchromic effect at higher pH values (from 3 to 5); the molar ratio of pigment/copigment also showed to have an influence on the hyperchromic effect being the molar ratio 1:100 the one having greater effect (Zhang, He, Zhou, Liu, & Duan, **2016**).

In terms of chroma, lightness and hue, the relative contribution of each parameter to the colour change that the media wine models went through after 4 weeks, is compared in **Figure 55**.

Figure 55 Relative contribution of lightness (ΔL^* - blue), chroma (ΔC^*_{ab} – orange) and hue (Δh^*_{ab} – yellow) to total colour difference for each phenolic aldehyde.

From these data we can observe that the highest contribution for colour changing in PHPR was ΔC^*_{ab} while Δh_{ab} had larger contribution for CINN and PHAC and ΔL^* was greater for SYRN and HMBZ.

From the overall results of the colour evolution assessment, those obtained with SYRN and HMBZ are interesting since N is steadier with respect to the control but have, on the other hand, larger CI, positive chroma and a hyperchromic effect under this concrete pH value and aldehyde concentration. These two aldehydes, unlike the rest, have multiple functional groups besides the aldehyde group (**Figure 56**).

Figure 56 Chemical structure of the five phenolic aldehydes used in the experimental set up.

3.4.5 Experiment 5

In terms of colour, the fact that the control is having higher total anthocyanins is directly reflected in having greater colour intensity (CI) and larger hue (**Table 25**). There is, on the other hand, no significant difference ($p < 0.05$) between treatments in terms of CI. Hue is lower for treatments 5pPm, 5pPt and 10pPt.

Table 25 Colour parameters from UV-Vis determination. Average and STD ($n=3$). Different letters denote a significant difference with 95% confidence level (LSD test)

	Control	5pPm	10pPm	5pPt	10pPt
CI	1.12 ± 0.1^a	0.85 ± 0.2^b	0.91 ± 0.2^b	0.81 ± 0.1^b	0.85 ± 0.1^b
Hue	0.73 ± 0.0^a	0.63 ± 0.0^c	0.66 ± 0.1^b	0.63 ± 0.0^c	0.62 ± 0.0^c

3.5 Sensory evaluation

3.5.1 Experiment 1

No sensory analysis was performed in this first experiment since the volume of experimental trials was minimal. The fermentations were carried out in micro-fermenters.

3.5.2 Experiment 2

Most of the vinification trials did not have statistical differences in the descriptive analysis except for those attributes highlighted with letters “a” and “b” on the **Figure 57**. These attributes correspond to the aroma quality, the fruitiness and the overall impression. The trial *Td-Sc* in the control was rated with the highest score for the attributes aroma quality and fruitiness with statistical maximum values; the trial *Mp-Sc* for treatment ProcyanidinB2 was attributed the lowest score for aroma quality which, regarding volatile compounds analysis, it may correspond to the rather large amount of diacetyl, ethyl acetate and 2-phenyl ethyl alcohol (**Table 10**).

The overall impression of the trial *Td-Sp* obtained the highest score for the treatment ProcyanidinB2; this trial had some of the lowest concentrations of total esters measured including the low amount volatiles responsible of wine faults. Out of the results obtained, especially the impression got for the trial *Mp-Sc* differ from the experience obtained by Sadineni, Kondapalli and Obulam (2012) where both non-*Saccharomyces* yeasts were compared after fermenting mango wine and both obtained better scores whilst the wine fermented with pure *Sc* scored worse; in terms of preference González-Royo et al. (2015) showed that 6 out of 9 tasters identified single culture *Sc* fermentation from sequential fermentation with *Td* and 5 of these 6 tasters that noted the difference preferred the sequential fermentation wine.

Sun et al. (2014) found that non-*Saccharomyces* yeast reinforced sweet, green and fatty notes and improved fruity odour. The results shown in this work compared the effect of these three non-*Saccharomyces* yeasts in sequential fermentations establishing *Td* as the one improving

the attributes of red wines in general. Any negative effect attributed to procyanidins addition was detected in the sensory evaluation.

Figure 57 Spider net chart for taste panel results for wine sensory evaluation: 1) Control, 2) (+)-Catechin and 3) ProcyanidinB2. Series with letters indicate significant difference between means ($p < 0.05$).

3.5.3 Experiment 3

This experiment did not consider sensory analysis due to the fact that the fermentation was done on corrected must with the addition of malvidin-3-O-glucoside and flavanols. No varietal aromas neither the effect of the fermentative yeast was to be evaluated through sensory analysis.

3.5.4 Experiment 4

No fermentations took place in experiment 4 since the evaluation was performed on wine model media. Therefore, no data for this experiment are available.

3.5.5 Experiment 5

Parallel to the volatiles determination, the sensory analysis shows that there is no significant difference ($p < 0.05$) for any parameter from the wine tasting except for herbal notes in nose (**Figure 58**). The aroma quality and the overall impression perceived as similar in wines by the panel of tasters with no significant difference ($p < 0.05$) express the lack of detrimental effect of PL used as grapes' processing antimicrobial. Despite of these results, there seems to be a general reduction in varietal aromas on the samples treated with PL as it has been previously documented by the use of e-beam irradiation (Morata, Bañuelos, et al., **2015**).

Figure 58 Sensory analysis of wines after PL treatments. Different letters in one parameter denote a significant difference ($p < 0.05$) (LSD test).

There is, nonetheless, significant difference ($p < 0.05$) in the parameter herbs to nose. The control wine had more herbaceous aroma than the PL treated samples and it is interesting to determine whether the irradiation had an effect on compounds responsible of green notes. Green notes compounds are generally found in grapes not fully mature.

Although the difference in oxidation between the control vs. the treated samples, was no significant ($p < 0.05$), the tasters noticed a slightly lower oxidized notes in PL treated samples. This result may suggest that perhaps the treatment had a particular effect on the reduction of molecules responsible for giving oxidized aromas in wine.

4. GENERAL DISCUSSION

The main objective of this doctorate thesis was to evaluate the potential use of non-*Saccharomyces* yeasts to enhance the quality of red wines. Throughout the experimental set up followed, the selection of non-*Saccharomyces* yeasts able to promote the formation of stable pigments, pyranoanthocyanins and polymeric pigments, was performed. In the same time, these yeasts have shown to contribute to modifying the aroma profile of young red wines. Yet, the fact of having an enriched aroma profile does not warranty these wines will be better accepted by the consumers.

Through the experimentation performed in experiments 1 to 3, the selection of suitable non-*Saccharomyces* yeasts was narrowed until selecting a strain of *Lachancea thermotolerans* capable of modifying more sensory attributes during the first stage of the fermentation of a sequential fermentation with a higher fermentative power yeast such as the strain of *Schizosaccharomyces pombe*. The slightly increase on lactic acid production by *Lachancea thermotolerans* together with the reduction of malic acid as a consequence of the metabolism of *Schizosaccharomyces pombe* had created an interesting experimental wine which metabolites content would optimally contribute for red wines to maintain colour intensity and tonality. Nevertheless, one drawback observed for this fermentative pair of yeast strains was the reduction of pigments in solution probably associated with the interaction of these molecules with the microbial cell walls. On the other side, an increase in colour intensity together with a reduction of anthocyanins may also suggest an increase in the polymerization rate.

Experiments 4 enhanced the search of having alternative ways to microbiological pathways of promoting the colour stability through the formation of pigments with different structure over ageing phase and, experiment 5 confirmed to be a useful cold pasteurization technique that allows the winemaker to control the implantation of pure culture or mixed fermentations.

The overall conclusions from the contribution of the experiments previously presented in this thesis work, are:

- *Saccharomyces cerevisiae* strain preserves monomeric anthocyanins but reduces colour intensity
- Pure culture fermentation with *Schizosaccharomyces pombe* produces wines with high content of stable pyranoanthocyanins.

- Sequential fermentations with *Schizosaccharomyces pombe* increased the content of polymeric pigments.
- Addition of flavanols to the must accelerates the formation of polymeric pigments.
- (+)-Catechin is prone to forming more polymeric pigments followed by Procyanidin B2.
- Not all non-*Saccharomyces* yeasts increased the amount of stable pigments in the presence of these added flavanols (e.g. *Metschnikowia pulcherrima*).
- Sequential fermentations increase metabolites (acetaldehyde and pyruvic acid) for stable pigments formation.
- Acetaldehyde was confirmed to be key in polymeric pigments condensation.
- Non-*Saccharomyces* yeasts increase hue values and polymerization index.
- Non-*Saccharomyces* sequential fermentations enrich the complexity of the aroma profile in wines, potentially protecting varietal aromas by lowering higher alcohols.
- PL may reduce the use of sulphites in winemaking, thus increase colour stability in musts and wines
- PL can control microorganism in grapes favouring the use of non-*Saccharomyces* yeasts in sequential and mixed cultures of yeast-lactic acid bacteria

The contribution of non-*Saccharomyces* yeasts to improve red wine's quality has been confirmed although trials in larger volume (semi-industrial scale) may be required to assess their impact in a better way. There are room for the use of non-*Saccharomyces* yeasts in the red winemaking industry with the eventual modification of sensory attributes; this biotechnology will differentiate the product among competitors parallel to providing solutions for colour stability during fermentation and throughout the ageing process.

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PUBLICATIONS

Articles published in SCI Journals

- 1) **Escott, C.**, Morata, A., Loira, I., Tesfaye, W., & Suarez-Lepe, J. A. (2016). Characterization of polymeric pigments and pyranoanthocyanins formed in microfermentations of non-*Saccharomyces* yeasts. *Journal of Applied Microbiology*, 121(5). <https://doi.org/10.1111/jam.13255>
- 2) Bañuelos, M. A., Loira, I., **Escott, C.**, Del Fresno, J. M., Morata, A., Sanz, P. D., ... Suárez-Lepe, J. A. (2016). Grape Processing by High Hydrostatic Pressure: Effect on Use of Non-*Saccharomyces* in Must Fermentation. *Food and Bioprocess Technology*, 9(10). <https://doi.org/10.1007/s11947-016-1760-8>
- 3) Chen, K., **Escott, C.**, Loira, I., Del Fresno, J. M., Morata, A., Tesfaye, W., ... Suárez-Lepe, J. A. (2016). The effects of pre-fermentative addition of oenological tannins on wine components and sensorial qualities of red wine. *Molecules*, 21(11). <https://doi.org/10.3390/molecules21111445>
- 4) **Escott, C.**, Vaquero, C., del Fresno, J. M., Bañuelos, M. A., Loira, I., Han, S. yu, ... Suárez-Lepe, J. A. (2017). Pulsed Light Effect in Red Grape Quality and Fermentation. *Food and Bioprocess Technology*, 10(8), 1540–1547. <https://doi.org/10.1007/s11947-017-1921-4>
- 5) Del Fresno, J. M., Morata, A., Loira, I., Bañuelos, M. A., **Escott, C.**, Benito, S., ... Suárez-Lepe, J. A. (2017). Use of non-*Saccharomyces* in single-culture, mixed and sequential fermentation to improve red wine quality. *European Food Research and Technology*, 243(12), 2175–2185. <https://doi.org/10.1007/s00217-017-2920-4>
- 6) **Escott, C.**, Del Fresno, J. M., Loira, I., Morata, A., Tesfaye, W., González, M. D. C., & Suárez-Lepe, J. A. (2018). Formation of polymeric pigments in red wines through sequential fermentation of flavanol-enriched musts with non-*Saccharomyces* yeasts. *Food Chemistry*, 239. <https://doi.org/10.1016/j.foodchem.2017.07.037>
- 7) Chen, K., **Escott, C.**, Loira, I., del Fresno, J. M., Morata, A., Tesfaye, W., ... Benito, S. (2018). Use of non-*Saccharomyces* yeasts and oenological tannin in red winemaking: Influence on colour, aroma and sensorial properties of young wines. *Food Microbiology*, 69, 51–63. <https://doi.org/10.1016/j.fm.2017.07.018>

Chapters in Books

- 1) **Escott, C.**, Loira, I., Morata, A., Bañuelos, M. A., & Suárez-Lepe, J. A. (2017). Wine Spoilage Yeasts: Control Strategy. In *Yeast - Industrial Applications*. InTech. <https://doi.org/10.5772/intechopen.69942>

Participation in International Congresses

- 1) **Escott, C.**, Loira, I., & Morata, A. (2016). Characterization of polymeric pigments and pyranoanthocyanins produced with non-*Saccharomyces* yeasts. 10th Congress on Polyphenols Applications. Porto, Portugal. Poster.
- 2) Loira, I., Morata, A., **Escott, C.**, Tesfaye, W., González, M.C., & Suárez-Lepe, J.A. (2016). Effects of ultrasound treatment on the phenolic fraction of a red wine aged on lees. 10th Congress on Polyphenols Applications. Porto, Portugal. Poster.
- 3) **Escott, C.**, Loira, I., del Fresno, J.M., Morata, A., Tesfaye, W., & Suárez-Lepe, J.A., (2017). Polymeric pigments formed in sequential fermentation of red fresh must by adding flavan-3-ols. 40th World Congress of Vine and Wine. Sofia, Bulgaria. Poster.
- 4) Del Fresno, J.M., Morata, A., Loira, I., **Escott, C.**, Cuerda, Rafael, Calderón, F., & Suárez-Lepe, J.A. (2017). Acceleration of ageing on lees in red wines by application of ultrasounds. 40th World Congress of Vine and Wine. Sofia, Bulgaria, Poster.

Participation in National Congresses

- 1) **Escott, C.** (2016). Caracterización de pigmentos poliméricos y piranoantocianos en vinos elaborados con no-*Saccharomyces*. XXXI Reunión del Grupo de Trabajo de Experimentación en Viticultura y Enología. Madrid, Spain. Short communication.
- 2) Del Fresno, J.M., Morata, A., Loira, I., **Escott, C.**, Cuerda, R., & Suárez-Lepe, J.A. (2018). Evolución de parámetros enológicos durante el proceso de crianza sobre lías asistido con ultrasonidos. 9^o Congreso Internacional de Química. Murcia, Spain. Poster.

RESEARCH STAY ABROAD

- 1) Anthocyanin and tannin fraction characterization of experimental wines produced with non-*Saccharomyes* yeast in sequential fermentations. **(2017)**. Instituto Superior de Agronomia, Universidade de Lisboa. Lisbon, Portugal. 13 weeks. Supervisor: Jorge M. Ricardo da Silva.

- 2) Characterization of seeds and skin extracts of black gapes *V. vinifera* L. cv. Merlot with potential for ocular-disease treatment. **(2016)**. Centro de Investigación y Desarrollo de Proteínas (CIDPRO), Instituto Tecnológico de Monterrey, Monterrey, NL, México. 4 weeks. Supervisora: Bertha Alicia Barba Dávila.

R+D Project

This thesis work has been done as part of the project “Formación de pigmentos piranoantociánicos y poliméricos estables durante la fermentación con no-*Saccharomyces* y *Saccharomyces*” funded by Ministerio de Economía y Competitividad (MINECO) with the grant number AGL2013-40503-R