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Departamento de Biologia

JOANA PINTO FERNANDES

VINHO&SAÚDE: APLICAÇÕES BIOTECNOLÓGICAS PARA PROMOVER A SAÚDE DO CONSUMIDOR

WINE&HEALTH: BIOTECHNOLOGY TOOLS TO PROMOTE CONSUMERS' HEALTH

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Ana Catarina Gomes, Investigadora do Instituto de Investigação Interdisciplinar da Universidade de Coimbra, e co-orientação da Doutora Gabriela Moura, Professora do Departamento de Ciências Médicas da Universidade de Aveiro.

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Esta dissertação é dedicada aos meus pais e ao Samuel

o júri

presidente	Doutor António Manuel Melo de Sousa Pereira Professor Catedrático da Universidade de Aveiro
	Doutor Manuel António da Silva Santos Professor Associado da Universidade de Aveiro
	Doutora Creina Stockley The Australian Wine Research Institute, Australia
	Doutor Filipe Centeno Diretor de Inovação e de Produção da Proenol – Indústria Biotecnológica SA
orientadora	Doutora Ana Catarina Gomes Investigadora Principal, Centro de Neurociências e Biologia Celular da Universidade de Coimbra

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palavras-chave

Aditivos enológicos, antioxidantes, biodisponibilidade, cancro colo-rectal, colagem, estabilidade proteica, extratos proteicos de levedura, polifenóis, resveratrol, síndrome de Progeria.

resumo

O vinho é uma mistura complexa de uma vasta gama de metabolitos, os quais impactam a saúde humana de uma forma sinergística. A multiplicidade de possíveis combinações metabólicas depende de muitos fatores, desde a variedade de uva e terroir até aos processos de vinificação, envelhecimento e armazenamento. Muitos dos constituintes do vinho foram estudados nas últimas décadas, pelo que é atualmente aceite que, principalmente devido ao alto teor de antioxidantes, o consumo regular e moderado de vinho pode proteger contra certos tipos de cancro, doenças cardiovasculares e neuronais. Apesar dessas descobertas notáveis, algumas preocupações surgiram recentemente no setor em termos da segurança e saúde, devido a reações alérgicas de consumidores que são suscetíveis a certos aditivos enológicos de origens exógenas, frequentemente aplicados durante a vinificação. Por outro lado, é atualmente necessária uma pesquisa sobre os benefícios associados a moléculas específicas que existem no vinho e com que eficácia estas atingem órgãos-alvo em humanos, após a sua passagem através da extensa via digestiva.

De modo a superar as preocupações do consumidor relacionadas com a ampla aplicação de aditivos exógenos, estudámos novos agentes de colagem proteicos obtidos a partir de estirpes enológicas de Saccharomyces cerevisiae. Os resultados demonstraram que os extratos proteicos de levedura são especialmente eficazes na clarificação e estabilização de vinhos brancos, devido à sua superior capacidade de prevenir e tratar fenómenos de oxidação, quando comparadas com proteínas de origem animal. Por outro lado, investigámos os potenciais benefícios dos componentes antioxidantes do vinho, através do estudo de diferentes misturas de polifenóis e suas potenciais sinergias a partir de um modelo celular de envelhecimento inovador, baseado em células de Progeria. Os resultados obtidos indicaram que a matriz completa dos vinhos causa maior redução de stress oxidativo, em comparação com polifenóis individuais, como sendo o resveratrol. Além disso, através de um ensaio in vivo, explorámos a biodisponibilidade de metabolitos derivados de um polifenol do vinho em amostras de plasma, confirmando a existência de possíveis formas bioativas após 6 semanas de intervenção.

Em geral, ao combinar os três eixos i) o desenvolvimento de tratamentos de levedura alternativos para a colagem de vinho, que substituem aditivos exógenos; (ii) as novas descobertas sobre o potencial antioxidante de diferentes tipos de vinhos; e (iii) o estudo dos potenciais benefícios anticancerígenos dos constituintes bioativos do vinho para a saúde dos consumidores, esta tese abre novos horizontes sobre como um conhecimento mais generalizado sobre o vinho e a saúde podem contribuir para uma indústria vinícola mundial melhorada, mais segura e com maior conscientização sobre a saúde do consumidor.

keywords

Antioxidants, bioavailability, clarification, colorectal cancer, polyphenols, Progeria syndrome, oenological additives, protein stability, resveratrol, yeast protein extracts.

abstract

Wine is a complex mixture of a vast range of metabolites that synergistically impact human health. The vast array of possible metabolic combinations depends on many factors, from the grape variety and terroir influence to the winemaking processes, aging and storage. Many of these wine constituents have been studied over the last decades, so it is currently accepted that mostly due to a high antioxidant content, a regular and moderated consumption of wine may protect against certain types of cancer, cardiovascular and neuronal diseases. Despite these remarkable findings, some safety and health concerns have been recently raised among the sector due to allergic reactions of susceptible consumers to some oenological additives from exogenous sources that are frequently applied during winemaking. On the other hand, further research is necessary on the benefits of specific wine bioactive molecules and to what extend they reach the target organs in humans after their passage through an extensive digestive pathway.

In order to overcome the consumer's concerns on the extensive application of exogenous additives, we have studied novel proteinaceous fining agents obtained from oenological Saccharomyces cerevisiae strains. Results exposed that these yeast protein extracts are especially efficient for white wines clarification and stabilization, due to their superior capacity to prevent and cure oxidation phenomena when compared to animal fining proteins. On the other hand, we have investigated the potential benefits of wine antioxidant components by studying different polyphenolic mixtures and their potential synergies in an innovative aging cell model based in Progeria cells. Results demonstrated that the whole wine matrix increases oxidative stress reduction when compared to individual polyphenols, such as resveratrol. Further, through a human study we explored the bioavailability of polyphenolic metabolites in plasma samples and confirmed their existence in possible bioactive forms after 6 weeks of intervention.

Overall, by combining the three axis i) the development of alternative yeast-based treatments for wine fining that substitute exogenous additives; ii) the findings on the potential antioxidant of different types of wines; and iii) the study of the potential anti-carcinogenic benefits of wine bioactive constituents to consumers' health, this thesis sheds new lights on how an extended knowledge on Wine&Health may contribute for a better and safer global wine industry, with a raised awareness on the consumer's health.

Abbreviations

ABTS	2,2´-azinobis-(3-ethyl-benzothiazoline-6-sulphonate
AP	Alkaline phosphatase
BCA	bicinchoninic acid assay
BCIP	5-bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt
BCV	BioClarVino
BSA	Bovine serum albumin
BSE	Bovine Spongiform Encephalopathy
CA	Cellulose acetate
CE	Capillary electrophoresis
CHD	Coronary heart disease
CHT	Chitinase
COX	Cyclooxygenase
CVD	Cardiovascular diseases
CWs	Cell wall materials
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSB	Double-strand breaks
ECF	Enhanced chemifluorescence
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ENO	Enolase
ESI	Electrospray ionization
FA	Formic acid
FTIR	Fourier transform infrared spectroscopy
GSH	Glutathione
HGPS	Hutchinson Gilford Progeria Syndrome
HMDB	Human Metabolome Database
HPLC	High performance liquid chromatography
IDY	Inactive dry yeast
LAB	Lactic acid bacteria
LC	Liquid chromatography
LDL	Low density lipoprotein
mDP	Mean degree of polymerization
MF	Molecular feature
MFOI	Molecular feature of Interest
MS	Mass Spectrometry
MSn	Multi-stage mass spectrometry
MW	Molecular weight
NAC	N-acetyl cysteine
NBT	Nitro-blue tetrazolium chloride
NC	Nuclei count
NM	Nuclear matrix

NT	Not treated wine / Untreated wine
NY	Nylon
OIV	International Organization of Vine and Wine
PAGE	Polyacrylamide gel electrophoresis
PA	Proanthocyanidin
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PES	Polyethersulfone
pI	Isoelectric point
PP	Polypropylene
PR	Pathogenesis-related
PRP	Proline-rich proteins
PVDF	Polyvinylidene difluoride
PVPP	Polyvinylpolypyrrolidone
QTOF	Quadrupole time-of-flight
ROS	Reactive oxygen species
RSC	Room temperature
RT	Retention time
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SIRT1	Sirtuin1
SMCs	Smooth muscular cells
TA	Titratable acidity
TBHP	tert-Butyl hydroperoxide
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline and Tween 20
TLP	Thaumatin-like protein
TPI	Total polyphenolic index
TPC	Total phenolic content
UPLC	Ultra-high performance liquid chromatography
VA	Volatile acidity
VP	Vegetable protein
VVTL	*Vitis vinifera* Thaumatin-like
YA	Yeast autolysate
YE	Yeast extract

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PART II. BENEFITS OF WINE MODERATE CONSUMPTION ON CONSUMERS' HEALTH

Outline

I. Wine History and cultural impact

Wine is a fermented beverage originated from *Vitis vinifera* grapes, whose first evidences were found from the age of the Persian cultures (3000-4000 BC). In some countries such as Rome, Greece and Egypt, the winemaking has been expanded when people started to cultivate crops instead of exclusively hunting (Kennedy *et al*. 2006; Fehér *et al*. 2005). Importantly, at those ancient times, wine had several purposes, not only as a beverage, but also as a disinfection, sedation and purgative agent, or even as a treatment to asthma, constipation, dyspepsia and epilepsy (Fehér *et al*. 2005; Jackson 2008).

Afterwards, references indicate that wine production arrived in Africa and Southern Europe around 1500BC, followed by India and China (500BC). Later, Romans were recognized to introduce the use of wood barrels to store the wines (200-400BC). The first plantation of vines in countries like South America, Australia or California only occurred in the 1500-1700s, due to the influence of Spanish and Dutch settlers. At that point, more sophisticated tools and techniques were also implemented in Western Europe, especially for storage and aging purposes (eg. use of glass bottles and cork sealing), which contributed to the wine quality improvement and further expansion.

Despite a severe phylloxera louse infestation (late 1900s) that almost destroyed all vineyard cultures in Europe (Banerjee *et al*. 2010; Du *et al*. 2011), the winemaking tradition subsisted and more knowledge about the vine fragilities and resistance to diseases was acquired. In addition, the investigation of the climate and soil impact on different grapevine varieties contributed to improve the wine yield and quality.

Along with the rapid evolution of both viticulture and winemaking processes, wine became an important component on human's diet (Fehér *et al*. 2005). In fact, across the history of mankind, wine always played an important role as a food complement and as a unique symbol of social living, culture, art and history.

II. Grapevine biodiversity and terroir: impact on wine bioactive composition

Worldwide, about 1400 different varieties of grapevine are used to produce wine (Santos *et al*. 2014). Alongside the evolutionary history of each grapevine variety, a high level of genetic variations occurred, resulting in the existence of various grapevine clones. These sources of biodiversity are highly valuable within the sector, as they permit the creation of distinct wines all over the world. By other side, the environmental conditions from each specific region (*terroir)* differently impact on the final wine characteristics. In brief, the climate conditions, soil, microbial activity and winemaking practices play a crucial role on the wine final style and identity.

The complex interplay resultant from both grapevine biodiversity and influence of *terroir* allows for the production of unique wines, since these factors cause variations on the concentration of the wine constituents as water, sugars, alcohol, but also on the vast range of organic and inorganic components. Among these elements, wines hold complex mixtures of important bioactive compounds as resveratrol, quercetin and gallic acid. Their variable concentration might originate different impacts on consumers' health (Alén-Ruiz *et al*. 2009; Muselík *et al*. 2007; Singh *et al*. 2016). Indeed, the potential synergies between individual polyphenols or between polyphenols and other wine constituents are still not fully understood.

Taken together, this raises the hypothesis that different varieties and/or clones should have different health impacts, raising an opportunity to further explore high-value bioactive combinations from grape and wine. Over long term, this would broaden the application of grapevine varieties, thus contributing to their sustainable conservation.

III. Winemaking processes and oenological additives

Winemaking is based in a natural process known as fermentation, where endogenous yeast and enzymes transform grape juice into wine. Despite being considered a rather traditional process, the wine production has largely benefitted from industrial evolution. Indeed, the industrialization of the fermentation process allowed for a more accurate monitoring and control of the processes (e.g. pH, temperature dissolved oxygen). The large-scale implementation of innovative biotechnological tools has opened an opportunity to standardize procedures, thus minimizing the occurrence of negative deviations to the process that cause unexpected losses in both yield and quality. As a result, winemaking was extended to its premium levels all over the wold, since wines ensured higher levels of quality and became more commercially attractive.

From harvest to fermentation, clarification, maturation and bottling, several winemaking techniques were developed to produce unique combinations of flavours, sweetness, dryness, alcoholic strength (Jackson 2008). In particular, several types of oenological additives were created not only to improve the fermentation efficiency, but also to prevent and correct aroma, texture and colour defects. The primary types of additives used in the wine industry originally included different formulations of fermentation nutrients, enological tannins, inactive yeasts, and fining agents.

Of these, fining agents are essential to enable the removal of undesired particles and/or molecules suspended/diluted existing in wine before bottling. Traditional fining practices involved the addition of elementary substances into wine, such as egg whites, blood, milk and isinglass. Afterwards, some of these products were banned due to health concerns (eg. Blood after the outbreak of bovine spongiform encephalopathy, BSE). Others, were refined, resulting in the general use of improved formulations, mainly protein-based, such as albumins derived from egg whites, caseins derived from milk, pork gelatines or fish isinglasses. Although most of these protein formulations are still widely used in winemaking, recent concerns have been raised among the wine industry due to: (I) their allergenic potential to susceptible individuals; (ii) dietary restrictions of vegetarian and vegan consumers;

(iii) environmental issues. At present, their production and application in wine became more restricted, according to the recent health and safety legislations defined by the International Organisation of Vine and Wine (OIV) and the Codex Alimentarius International Code of Standards.

Altogether, the development and implementation of innovative techniques and additives in winemaking is continuously required in order to avoid safety concerns and to fulfill the emerging consumption diets and trends.

IV. Wine chemistry

Wine is a complex alcoholic beverage, composed by more than one thousand different chemical compounds. Major wine components are water (80-85%) and ethanol (around 12-14%), followed by a wide diversity of other complementary molecules including organic acids, sugars, phenolic and volatile compounds. The combination between these wine constituents determine its aroma, colour, taste, mouthfeel and stability characteristics.

Wine aroma is highly influenced by the presence of volatile molecules, such as alcohols, esters, terpenes and thiols. Depending on their origin, volatiles are classified as (i) primary/varietal aromas if directly extracted from the grapes and thus, variable according to each grapevine variety and specific *terroir*; (ii) secondary aromas if generated during the fermentation processes through the action of yeasts and bacteria; (iii) tertiary aromas if produced during maturation and aging stages, either in vessel, barrel or bottle. In addition, non-volatiles as polyphenols and organic acids contribute to the overall levels of bitterness and acidity. Indeed, major organic acids as tartaric, malic and citric are important for taste, but also prevent harmful bacteria growth during and after fermentation.

Otherwise, wine mouthfeel and colour characteristics are strongly related to polyphenolic components. In particular, tannins are specific polyphenols derived from the grape skin and seeds that have the ability to precipitate salivary proteins. Higher tannic contents, mostly present in red wines, increase wine complexity, bitterness and astringency, a dry and rough mouthfeel sensation. By other hand,

polyphenolic plant pigments, named anthocyanins, define its colour intensity and hue. During wine maturation and aging, monomeric anthocyanins tend to polymerize to form more complex derived pigments, such as pyranoanthocyanins, resulting in hue changes and colour stabilization.

Altogether, the key components - alcohol, acidity, tannin, sweetness and aroma - should be well balanced to guarantee a correct wine elegance degree and stability over time. From fermentation until aging stages, wine chemistry must be followed by sensory analysis and analytical techniques to control chemistry and microbiological features.

V. Health benefits of regular and moderate wine consumption

Recent studies have highlighted the potential health benefits of the moderate wine consumption in different illness conditions, such as various forms of cancer, diabetes, cardiovascular and neurological diseases (Berlett & Stadtman 1997; Lippi *et al*. 2010; Rodrigo *et al*. 2011; Yochum *et al*. 1999; Obrenovich *et al*. 2010; Cooper *et al*. 2004). Those effects are mainly attributed to a high polyphenolic wine content, especially present in red wines.

In particular, research has been focused on the health benefits of resveratrol, a well-known antioxidant proved to be a strongly antioxidant that prevents cellular DNA damage and induces cellular differentiation (Zamora-Ros *et al*. 2015). Despite the evidences from previous studies showing that some wine-derived polyphenols are efficiently absorbed into the human blood (Walle 2011; Vitaglione *et al*. 2005a), it is still unclear to what extent these molecules preserve their biological activity when reaching the targeted organs (Manach *et al*. 2005). In fact, bioavailability studies only identified residual concentrations of the free form of resveratrol in plasma, after intake of either red wine or resveratrol tablets (Vitaglione *et al*. 2005a; Rotches-Ribalta *et al*. 2012). Besides, other authors have identified other resveratrol derived metabolites in plasma that might also possess bioactive functions in the human body (Juan *et al*. 2010).

Considering this, a single analytical technique is insufficient to access the metabolome of the wines or plasma samples. Therefore, holistic approaches as metabolomics are being developed, by combining high resolution techniques, data processing and multivariate statistics (Roullier-Gall, Witting, *et al*. 2014; Cozzolino 2016). This strategy allows the identification of a wider range of compounds with potential interest to health research.

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Objectives

Objectives

Health concerns have been recently raised among the wine sector on the impact of wine consumption to consumers. New strategies are currently required to reduce the application of exogenous additives, which are potentially harmful to consumers and to environment. On the other hand, the benefic effects related to wine moderate consumption need to be further validated and explored as most of the current studies are contradictory, or not conclusive.

In this thesis we have tackle these concerns under different and yet complementary perspectives, that ranged from the oenological additives to the consumers' health. Therefore, this work is presented divided into two distinct parts:

PART I. Endogenous alternatives for wine fining

Wine might cause intolerances and allergies to susceptible individuals, due to the residual presence of specific fining agents applied during clarification and stabilisation stages of the winemaking process (Vassilopoulou *et al*. 2011). To overcome this concern, novel strategies and oenological products need to be developed as substitutes to the traditionally fining practices.

In Part I, the main objective is to develop novel endogenous alternatives for wine finning and clarification from oenological yeasts origin. To achieve this main objective, three specific objectives were defined:

I.a) to benchmark the impact of the currently used fining agents from varied origins, comparing with yeast protein extracts produced at laboratorial scale;

I.b) to select the most efficient yeast protein extracts and validate their efficiency;

I.c) to develop a specific tool to detect unstable proteins in white wines.

Accordingly, the general introduction of Part I is addressed in Chapter 1 and each of the specific objectives is presented in the following chapters.

PART II. Benefits of wine moderate consumers' health

Moderate wine consumption is recognized to bring health benefits to consumers due to the wine content in important bioactive molecules, such as powerful antioxidant molecules. However, research is needed to further explore which compounds might be potentially applied in other industries as nutraceutical, pharmaceutical and cosmetics. Indeed, it is also unclear to what extent those compounds are absorbed and metabolised in the human body and, therefore, keep their bioactive functions.
In the Part II of this work, the main objective is to explore the cellular effects of polyphenolic mixtures and wines, as well as their bioavailability in human plasma after moderate consumption of red wine. To achieve this, two specific objectives were defined:

II. a) to assess the cellular impact of different polyphenols, polyphenolic complex mixtures and wines by using a Progeria cell model;
II. b) to explore the bioavailability of resveratrol metabolites and other compounds in human plasma after a dietary intervention study in humans.

Accordingly, the general introduction of Part II is addressed in Chapter 5 and each of the specific objectives is presented in the following chapters.

PART I

ENDOGENOUS ALTERNATIVES FOR WINE FINING

Chapter 1

PART I - General Introduction and Objectives

1.1. Winemaking process

The winemaking process plays a crucial role in the shape of the wine characteristics and, consequently, in the achievement of the desired wine style. In brief, winemaking is composed by five main stages: (i) harvesting; (ii) production of grape juice; (iii) must fermentation; (iv) post-fermentation techniques; (v) aging and bottling (**Fig 1.1**).

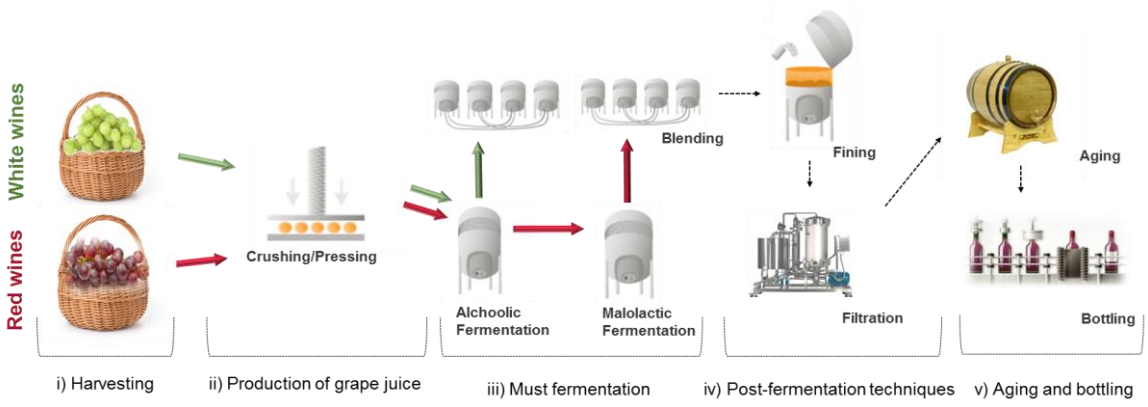

Fig. 1.1 - Scheme of winemaking steps for white and rosé wines.

1.1.1. Grape harvesting

During grape maturation, the accumulation and metabolism of a wide range of molecules occurs. Firstly, water, sugars and nitrogen compounds are transported via the plant phloem into the grape berries, where sucrose is hydrolysed to glucose and fructose and the synthesis of aroma precursors as flavonoids and volatile compounds starts.

Optimal ripeness is often determined by the correct biochemical ratio between sugar content, pH and varietal components such as aroma, flavour and colour compounds. For an accurate prediction, viticulturists and winemakers rely on the sensory analysis of the berries, combined with biochemical methodologies. In specific, standard methods are applied to measure potential alcohol content (°Brix), to verify the chemical stability (pH and total acidity) and to determine the total polyphenolic

content of the grapes. However, the most accurate harvest moment also varies according to each grape variety, climate and desired wine style.

The physical preservation and phytosanitary status of the grapes are also critical factors at this stage. When performed mechanically, harvest might cause damage of the grape skins, leading to excessive colour maceration that affects in particular, the production of white wines. Moreover, the skin damage leads to oxidation and accelerates fermentation phenomena that later compromise the aromatic quality of the wines. To avoid these, mechanical harvest is frequently performed overnight, since the lower temperatures reduce fermentation and contamination rates. The traditional alternative is the hand-picked harvest that assures a more accurate selection of the berries and less overall damage.

1.1.2. Production of grape juice

Once transported to the wineries, grapes are firstly sorted to guarantee the exclusion of unripe or rotten berries. Depending on the type of wine to be produced, different decisions are taken at the winery concerning downstream production processes, such as those related to destemming, crushing and pressing. During these steps, primary/varietal aroma compounds are released from the berries, including terpenes, C_{13}-norisoprenoids and C_6 compounds (Zalacain *et al*. 2007)

White wines are normally crushed and pressed in the presence of the grape stems and both of these steps are performed before the start of fermentation. During pressing, the white must is separated from the grape skins to avoid the extraction of an excessive amount of yellow pigments and/or undesired flavours. Contrarily, in red wine production, grapes are crushed right after sorting, but only pressed at a later stage or at end of primary fermentation. At that point, the fermented liquid is separated from the grape skins and other remaining solids. Herein, pressing is performed without including the grape stems, to avoid the extraction of excessive tannins, a type of polyphenols that are already abundant in the red grapes. It is also important to avoid the extraction of the 2-methoxy-3-isopropylpyrazine, which might add an undesirable vegetable aroma to wine. After the pressing step, the red juice is then left to macerate with skins during several days or even weeks to extract the

desired colour pigments, varietal aromas and polyphenols. Along this stage, dead yeast cells (lees) and other small particles precipitate to the bottom of the vessel, where a deposit is formed. Later, the wine is transferred to a clean vessel where additional fermentations or treatments might still be carried out.

To produce rosé wines, an intermediate process between white and red styles is applied. Red grapes are pressed straight after sorting and fermentation is firstly completed. Fermentation is then followed by a brief maceration period, to extract only a small portion of tannins and pigments that respectively confer its typical phenolic structure and pink tonality.

1.1.3. Wine fermentation

Grape juice is transferred to clean barrels or stainless steel tanks to start the fermentation process. At this step, winemakers might decide on a natural fermentation, performed by endogenous yeast strains naturally derived from the grapes skin and air. Indeed, the wine juice naturally holds a complex microbiota, where both *Saccharomyces* and non-*Saccharomyces* species co-exist (Fleet 1990). Conversely, fermentation might be forced to occur by the addition of specific *Saccharomyces* yeast strains that are commercially available, such as *S. cerevisiae or S. bayanus*. These cultured yeast strains are specifically selected due to a greater capacity to boost the whole process and, therefore, facilitate its forecast and efficiency.

S. cerevisiae is the most common yeast strain associated with wine fermentation. Although initially rare during the start of a natural fermentation, it gradually dominates until the end of the process. This yeast is responsible for the conversion of grape sugar into alcohol and CO_2, but also plays an important role in the enzymatic conversion of grape aroma precursors to wine secondary metabolites, such as esters, aldehydes, ketones and higher alcohols (Swiegers *et al*. 2005; Pretorius 2000; Falqué *et al*. 2002). During a typical fermentation completed by Saccharomyces cerevisiae, 95% of the sugar is converted to ethanol and carbon dioxide. Among the remaining sugar, 1% is converted to cellular material, and 4% to other products (Lambrechts & Pretorius 2000; Pretorius 2000).

Red wines commonly undergo a secondary fermentation, called malolactic fermentation, to convert the excess of malic acid into lactic acid, resulting in a softer - less coarse or tannic - and more complex taste on the palate. For that purpose, winemakers add lactic acid bacteria (LAB) to wine, such as *Oenococcus oeni*, which initiate decarboxylation, promoting the release of carbon dioxide and the de-acidification of the wine.

1.1.4. Post-fermentation processes

After the fermentation stages, wine is still turbid due to the presence of residues from grape skins and dead yeast cells. Therefore, different clarification and stabilization processes are applied to remove undesired components as yeast cell walls, oxidized pigments, colloids and excessive tannins.

The most common post-fermentation procedures are racking, filtration and fining. All of these techniques allow for the separation of the clarified wine from its sediment, gradually formed at the bottom of the vats.

Racking

The simplest and most traditional post-fermentation technique is racking and consists in the separation of the wine from its sediment into a clean container by the force of gravity. This technique is applied at various winemaking stages and it is most commonly used to clarify impurities in wine musts (e.g. dust, cell walls, soil, bacteria and fungi).

Despite being technically simple and cheap, this process implicates extensive settling periods that delay the overall production process and increases the risk of wine losses by its dilution with the sediment left behind. Moreover, the filling of the tanks has to be closely supervised, as excessive disruptions to wine resting might promote wine oxidation (Christaki & Tzia 2002) and/or aroma deterioration.

Filtration

Filtration is another common technique for wine clarification and stabilization, usually applied just before bottling. Several materials are accepted in winemaking filtration as diatomaceous earth precoat, cellulose sheets, synthetics polymer membranes, or more recently, inorganic and organic membranes. The most frequent membranes pore size are 0.1 and 0.22 μm for white wines and 0.2 μm for red wines (Urkiaga *et al*. 2002). The smaller the micron rating, the more particles the filter removes (Wilson 2015). Nevertheless, the use of smaller pore size membranes can introduce undesirable flavour and colour changes in the treated wines (Ferrando *et al*. 1998). In particular, a reduction of important wine components has been previously registered, specifically in tannins (4.8%), anthocyanins (2.4%) and polyphenols (10%) (Arriagada-Carrazana *et al*. 2005). Contrarily, if the membrane pore size is too large, undesired compounds might pass through the filtration membranes, compromising the clarification quality and both aromatic and phenolic profiles of the wine.

Fining

Considering the disadvantages of racking and filtration, fining is considered as the only available solution to achieve a more selective and efficient wine clarification. Fining consists in the addition of oenological compounds - fining agents, to the wine promoting flocculation and sedimentation of its undesired components. Fining is used with different purposes, such as: *(i)* to accelerate wine clarification; *(ii)* to remove targeted molecules or particles in excess; *(iii)* to increase stability and limpidity over time; *(iv)* to improve the organoleptic quality; *(v)* to facilitate the filtration process after fining; or *(vi)* to enhance the compactness of the resultant lees/sediment (Harbertson 2008; a. Braga *et al*. 2007; Castellari *et al*. 2001; Iturmendi *et al*. 2010).

The common application of a wide-range of exogenous fining agents, derived from different origins and sometimes at excessive dosages, has raised health concerns among both winemakers and consumers. Indeed, research is required to improve this technique and develop alternative fining agents that minimize health concerns

to consumers and that comply with the recent European Regulations [European Regulation (EU) No 579/2012].

1.1.5. Wine aging and bottling

During the last stage of wine production, wines might be retained inside oak barrels to age during few months to several years. Important volatile and non-volatile constituents from the wood are incorporated into the wine matrix, contributing to the improvement of the wine flavours and quality. Specifically, the oxidizing and reducing conditions, enzymatic reactions and oak contact regulate the formation of tertiary metabolites, including aldehydes, acids, oak lactones and volatile phenols. Oak barrels also allow for a controlled oxidation of the wine, which is important for the colour stabilization and the reduction of astringency in red wines. At the end of the winemaking process, bottles are filled and closed using natural corks or screw caps.

The final wine is a complex mix of metabolites, extracted and produced during the different stages of winemaking. The multiplicity of factors associated to grape varieties, environmental conditions, viticulture practices, yeast strains and winemaking methodologies directly influences the final wine chemical composition, which explains the existence of distinctive wine styles all over the word (Atanassov *et al*. 2009).

1.2. Applications of wine fining

1.2.1. Fining agents

A wide-range of additives, named ‘fining agents’, is used to interact with the undesirable particles/macromolecules dissolved or in suspension within the wine and remove them (**Fig. 1.2**). Existing fining agents are powder minerals and solid materials (e.g. bentonite clays), synthetic polymers (e.g. polyvinylpolypyrrolidone -

PVPP), animal proteins (e.g. casein, gelatine, ovalbumin and isinglass), or even vegetable proteins (e.g. wheat, rice, maize and potato proteins) (A. Braga *et al*. 2007; Cosme *et al*. 2009; Weber *et al*. 2007; Gambuti *et al*. 2012; Tschiersch *et al*. 2008). According to each fining purpose, wine type or market, the most suitable fining agents are selected by the winemakers (Tschiersch *et al*. 2008). Different combinations of fining agents are available on the market and they are formulated either as a single compound or as a mixture (A. Braga *et al*. 2007; Tschiersch *et al*. 2010; Cosme *et al*. 2012; Vincenzi *et al*. 2013; Fernandes *et al*. 2015).

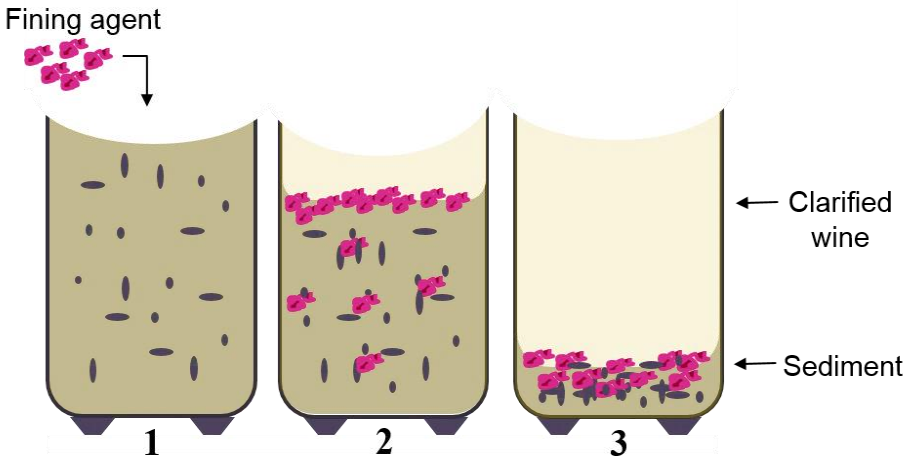

Fig. 1.2 - Scheme representing the fining process in a winery vat. 1) Addition of the fining agent in an untreated wine; 2) Formation of aggregates between fining agent and unstable compounds; 3) Complete sedimentation of the aggregates. Formation of sediment at the vat's bottom.

The physical and chemical properties of each fining agent are determined by its composition. In specific, their features, such as chemical or molecular nature, density, isoelectric point (pI) and molecular weight directly impact on the overall effect and efficacy of the fining process (Cosme *et al*. 2007).

Fining agents are presented in powder or liquid formulations and manufacture instructions must be rigorously followed before their application into wine. While liquid formulations are frequently ready-to-use products, some preparative steps are necessary before usage of the powder formulations, such as the resuspension with

distillate water or wine and a good homogenization. In general, the usage conditions and application limits should follow the regulations described in the *International Oenological Codex* (OIV 2015).

1.2.2. Preliminary fining trials – laboratorial scale

Preliminary fining trials are usually performed by winemakers in order to test for the proper quantities and fining conditions before applying these treatments at industrial scale. Fining trials consist in laboratorial-scale experiments that attempt to reproduce distinct fining conditions, using small glass tubes/flasks, vertically set in a laboratory bench. Small volumes of wine are used to perform these laboratorial experiments (usually 50 – 500 mL per assay) and thus, a higher number of replicates might be tested in parallel. Also, a wide range of wines, fining agents, proportions (in the case of fining mixtures) and concentrations can be further tested.

Overall, preliminary fining trials allow an accessible prediciton and selection of the most efficient fining agents and dosages to be later applied at the wineries (Tschiersch *et al*. 2008). Moreover, this procedure avoids the application of a surplus of the fining agent that usually leads to an over-fining effect. In that case, wine becomes highly turbid and it might acquire undesired off-flavours directly derived from the applied fining agent. Besides, the excess of treatment might cause removal of varietal aroma characteristics, loss of wine structure and colour destabilisation.

1.2.3. Fining applications considering wine types

Fining is differentialy applied, according to the wine type and winemaking purpose. For this reason, it is important to highlight the main applications of this technique in white, rosé and red wines.

1.2.3.1. White and rosé wines

Both white and rosé wines have comparable physicochemical properties as a low or residual concentration of tannins, colour pigmens and the existence of unstable proteins. Therefore, the fining process is similarly applied in both wine types to prevent oxidation reactions and haze formation after bottling. (Andrade *et al*. 2001; Monteiro *et al*. 2001; Cosme *et al*. 2008).

In these wines, oxidation is frequent as wine polyphenols (eg. catechins, proanthocyanidins, hydroxycinnamic acids and their derivatives) tend to be rapidly oxidize, compromising their colour, taste and aroma (Bonilla *et al*. 2001; Li *et al*. 2008; Gómez *et al*. 1995; Spagna *et al*. 2000). Although this reaction is slow at the pH of wine, the presence of metals, such as iron or copper, may accelerate oxidation (Oszmianski *et al*. 1996). Contrarily, redox-active constituents as SO_2 and ascorbic acid might reduce these reactions (Sioumis *et al*. 2006).

Due the complexity and difficulty to forecast these reactions, it is important for the winemakers to rapidly prevent oxidation by applying fining agents, namely:

- Animal proteins - Casein, potassium caseinate and swim bladder isinglass. These proteins were shown to interact with flavenol monomers and low molecular weight procyanidins, removing the oxidized species in suspension (Cosme *et al*. 2008). In particular, pigments are efficiently removed after the addition of casein and potassium caseinate, decreasing browning and preventing oxidation reactions (Cosme *et al*. 2008).
- Synthetic fining agents – Polyvinylpolypyrrolidone (PVPP). This agent has the capacity to adsorb browned pigments and phenols when added to white wine. In fact, previous studies showed that wines treated with PVPP contain lower anthocyanin levels and colour density, becoming more stable during storage (Castillo-Sánchez *et al*. 2008; Castillo-Sánchez *et al*. 2006).

The haze formation after bottling is a visual defect, which is often accompanied by the production of off characters. It is caused by the presence of specific grape proteins that remain until the end of fermentation, such as chitinases and thaumatin-like proteins (Dufrechou *et al*. 2012). Haze-forming proteins are generally removed by the application of mineral clays, namely bentonites, which interact

electrostatically with unstable proteins, promoting their flocculation (Ribéreau-Gayon *et al*. 2006). Importantly, bentonite treatments are not selective enough for these proteins and might cause a decrease of the quality in final wines. In fact, previous studies highlighted several problems concerning the usage of bentonite such as (i) losses of wine volume (1-3%), (ii) environmental costs of bentonite disposal, (iii) wine colour alteration and (iv) aroma losses (Falconer *et al*. 2010; Sauvage *et al*. 2010).

Moreover, several studies demonstrated that haze-forming proteins also interact with other wine components, such as phenolic compounds, polysaccharides and metal ions (e.g. sulfate cations), all of which play important roles in protein stability and aggregation (Hsu *et al*. 1987; E.J. Waters *et al*. 1995; Falconer *et al*. 2010). Chemical and physical factors such as ethanol concentration, wine pH, protein molecular weight and protein pI also play a critical role in the haze phenomena (Waters *et al*. 2005). Indeed, each wine requires adapted fining treatments not only according to its biochemical characteristics and existence of specific proteins and other interfering molecules, but also according to the desired wine style as defined by the winemaker.

1.2.3.2. Red wines

Red wines are rich in phenolic compounds, mainly derived from the grape skin and seeds. Depending on their chemical structure and composition, phenolic compounds play a role on different red wine properties. In specific, the astringency is caused by the presence of polymeric flavan-3-ols or tannins; the bitterness conferred by the catechins; and the colour resulting from its composition in anthocyanins (Waterhouse 2002). Overall, the oenological practices and the storage period are the main factors influencing the colour and phenolic compounds in wines (Gómez-Plaza *et al*. 2000).

Astringency

Astringency is a complex mouth sensation of friction and dryness, perceived after the intake of young red wines, where the tannic content is still high (McRae & Kennedy 2011). Tannins are polymeric flavan-3-ols (proanthocyanidins) that interact with proteins through hydrogen bonds, formed between the phenolic hydroxyl groups and the peptide bonds; or through and hydrophobic interactions (**Fig. 1.3**) (Vincenzi *et al*. 2013). Proline-rich proteins (PRP) - are the most prone to bind tannins, as the proline residues fold, confering a characteristic three dimentional structure to the protein that results in a 'string-like' arrangement (Granato *et al*. 2010; Marangon *et al*. 2010).

Indeed, such PRPs are present in human saliva and connective tissue (Harbertson 2008). Therefore, the formation of insoluble tannin–salivary protein aggregates decreases the oral lubrication, simultaneously increasing the overall friction perception during and after the wine tasting – the astringency sensation. Due to genetic differences, the amounts of PRPs in human saliva is highly variable between individuals. Accordingly, different persons possess different rates of protein-tannin aggregation after red wine intake and consequently, different levels of perception and tolerance to astringency. For this reason, it is important to reduce excessive tannins through the application of fining agents.

Again, winemakers commonly use animal origin proteins, as egg albumins, caseins and gelatines. All of those proteins are positively charged at the acidic wine pH, so they can efficiently flocculate the negatively charged tannins (McRae & Kennedy 2011).

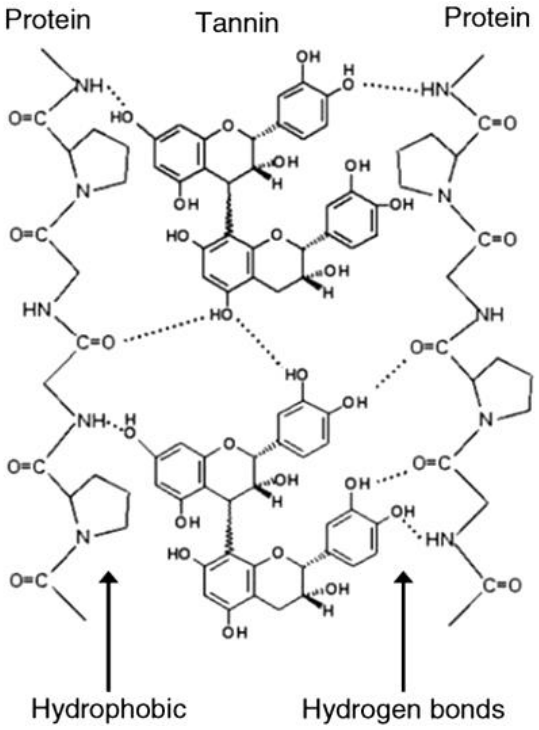

Fig. 1.3 - Protein-tannin interaction (Santos-Buelga, Celestino Freitas 2008).

Colour

Colour is a crucial characteristic in red wines and is determined by the presence of anthocyanin molecules with coloured forms at the wine pH (Rodrigues *et al*. 2012). Along the aging stage, the anthocyanin units tend to condensate and form long and stable polymeric chains. Fining agents, such as gelatine derivatives with lower molecular weight (with polydispersion below 43.0 kDa) are recognized to efficiently decrease colour intensity in young red wines, without significantly affecting the colour hue (a. Braga *et al*. 2007). Conversely, isinglasses were previously described to promote a softer reduction of colour density and hue when compared to caseins or gelatines (Bonerz *et al*. 2004).

The referred animal fining agents are commonly obtained through chemical hydrolysis, so the final products are composed of protein fractions with dissimilar molecular weights. The molecular weight directly impacts on the protein ability to

bind different categories of tannins and thus, to promote different effects on red wine. For instance, lower molecular weight gelatines (liquid form) are described to better reduce the polymeric tannin content, whereas the higher molecular weight gelatines (solid form) easily attach the oligomeric components (Cosme *et al*. 2007).

Besides, this interaction is strongly influenced by the tannic composition of each wine. Previous studies demonstrated that tannins with higher degree of polymerization (dP) and higher esterification with gallic acid units are more prone to stablish tannin-protein interactions (McRae & Kennedy 2011). According to this, wines with different tannic contents require the application of different and suitable gelatine-based products.

1.3. Exogenous vs. Endogenous fining agents

1.3.1. Exogenous fining agents: Health and environmental concerns

Animal fining agents

Currently, exogenous clarification and stabilization agents such as animal origin proteins raise increasing health and environmental concerns on wine consumers. According to the European Food Safety Authority (EFSA), wines fined with albumin, casein, caseinate or milk-based products may trigger adverse reactions in susceptible individuals. In particular, the incidence of allergic responses to albumin is around 0.3% in adults among the general population (The EFSA Journal 2005). Importantly, in recent years, consumers have become more and more informed and aware about the usage of these type of proteins in winemaking, and consequently there was an increase on its relevance within the wine sector.

Indeed, a previous study identified residual amounts of dried albumin in treated wine samples using the enzyme-linked immunosorbent assay (ELISA) procedure, confirming that adverse allergic reactions could not be excluded in sensitive individuals (Weber *et al*. 2007). However, Weber's results are not consensual, as they were not reproduced in other labs, who have not identified casein, isinglasses

nor egg proteins in latter studies, by using similar laboratorial methods as ELISA or Polymerase Chain Reaction (PCR) kits (Deckwart *et al*. 2014; Vassilopoulou *et al*. 2011). Despite this, Vassilopoulou *et al*. did report a residual allergenic response using the skin prick and basophil activation tests (Vassilopoulou *et al*. 2011), which proofs the presence of the allergens within the tested wines. Indeed, analytical methods with higher sensitivity as Liquid chromatography-mass spectrometry, LC-MS, did confirmed the presence of residual albumin in red wines, previously treated with only 5 g/hL of different egg-derived fining agents.

Altoghether, the existing studies on this topic are contradictory as different dosages and practices are being used in different countries, hindering the transferability of the results, and limiting the extent of their conclusions. Further investigation is therefore necessary to evaluate the presence of possible allergenic residues in fined wines. As result of the current concerns, European legislation has stablished new regulations in order to increase the awareness of producers and consumers about the use of these products. Accordingly, and since 2012, the presence of residual milk and/or egg based-products in final wines must now be declared on the label [European Regulation (EU) No 579/2012].

Mineral and vegetable fining agents

Alternatively, wine treatments with mineral and vegetable fining agents have been investigated over the last decades. Regarding mineral agents, it has been demonstrated that the use of bentonite clays (the mostly used) adversely affect the organoleptic properties of wines, causing aroma loss and colour alteration (Dufrechou *et al*. 2012). Moreover, its disposal raises significant environmental concerns such as oxygen deficiency (anoxia) in the soils, destruction of the soil absorption system and clogging of drainage systems (Fernando N Salazar *et al*. 2006; EPA (victoria) 2004).

Likewise, gluten and other vegetable derivatives were tested as potential substitutes to animal fining agents. Indeed, a first study has shown that the application of these compounds, in a dosage of 6 and 18 g/hL, promoted an efficient clarification of

Burgundy wines, as final turbidity was in the range of the values obtained with gelatine or egg protein treatments (Marchal *et al*. 2002). Nevertheless, gluten has also raised safety concerns to consumers susceptible with coeliac disease or gluten allergies (Simonato *et al*. 2011; Cattaneo *et al*. 2003; Restani *et al*. 2002). Although a preliminary study concluded that gluten residues were harmless to celiac individuals, no conclusions were taken concerning other allergy forms (Restani *et al*. 2002). Posteriorly, Cattaneo *et al.* (2003) and his co-authors proposed the use of gluten coupled with bentonite adjuvants in order to facilitate the removal of this immunoreactive protein from the white wines. More recently, mass spectrometry based methods, with lower detection levels, assured the presence of different forms of gluten proteins in red wine after fining with both partially hydrolyzed and nonhydrolyzed glutens in a dosage down to 1 g/hL (Simonato *et al*. 2011).

Alternativelly, recent comparative studies tested the effects of some novel fining agents from other vegetable origins, including:

- Rice proteins: Previous reports exposed lower effects on red wine colour, but efficient reduction of bitterness and astringency (Tschiersch *et al*. 2010).
- Corn zeins: The effect of corn proteins seems to be comparable to gelatine as regards to turbidity reduction, phenolic compounds removal and colour preservation of red wines (Simonato *et al*. 2009).
- Pea proteins: Results indicate that although this could be a potential alternative for potassium caseinate, it is less effective in reducing browning potential in white wines. (Cosme *et al*. 2012).
- Potato proteins: Later, the impact of potato protein (patatin) also appeared to reduce grape phenolics, brown pigments and also turbidity in white musts. The described effect was comparable or/and superior than registered by applying potassium caseinate (Gambuti *et al*. 2016).

1.3.2. Novel endogenous alternatives for wine fining

Yeast-based products

The intense use of the exogenous fining proteins has put aside the fact that both grape and wine hold a natural microbial population with a hidden potential as regards to possible biotechnology applications. Thousands of different yeast proteins, from the extensive myriad of yeast species that naturally colonize grape skins, are present throughout the entire fermentative process and embed a natural potential for fining and stabilizing the produced wines (Jolly *et al*. 2014). Therefore, it is crucial to acquire more knowledge about this fungal community and to stablish a link to the final wine properties and quality (Bokulich *et al*. 2011; Bokulich *et al*. 2014).

Indeed, endogenous yeasts play a central role for the Oeno-biotechnology stream, concerning the development of a wide range of yeast derivative products, with potential for winemaking applications. Currently, yeast derived-products applied to wine might include Inactivated Dry Yeast (IDY), Yeast Autolysate (YA), Yeast Extract (YE) or Yeast Mannoproteins (YM) (Petruzzi *et al*. 2015; González-Royo *et al*. 2013; Juega *et al*. 2012; Pozo-Bayón *et al*. 2009).

For example, inactive dry yeasts or their respective fractions might be used in enological processes to flocculate pigments or to enrich the sensory characteristics, the colour and the colloidal stability of wines (Mekoue Nguela & Vernhet 2015). Some authors also propose a possible contribution of these formulations to the enrichment of the wine antioxidant potential (Ángeles Pozo-Bayón *et al*. 2009; Pozo-Bayón *et al*. 2009; Jose *et al*. 2014). Recently, an innovative study also studied the possible use of IDY in the vineyard, through its directly application on the grapes. Results have shown benefits to the production and preservation of the final wine aroma, specifically with an increase of the wine sweetness and roundness (Šuklje *et al*. 2015).

Dead yeast cells (or lees) are also commonly used (Delgado de la Torre *et al*. 2015; Mazauric & Salmon 2006; Del Barrio-Galán *et al*. 2011). Lees consist in the sediment formed at the bottom of the wine vats resulting from fermentative or clarification steps. In general, red wine is left to age over lees in order to acquire

more complex aromas, reduce its astringency and increase colour stability (Delgado de la Torre *et al*. 2015; Bonilla *et al*. 2001).

Similarly, yeast mannoproteins - glycoproteins extracted from the yeast cell walls, which are composed from 15 up to 90% of mannose groups per weight - are commonly applied to stabilization processes (Guadalupe *et al*. 2010; Rodrigues *et al*. 2012; Juega *et al*. 2012). Previous studies tested the impact of these mannoproteins in red wines, but no positive correlation was found between their application and colour stabilization (Rodrigues *et al*. 2012; Guadalupe *et al*. 2010). Contrarily, it has been observed an important decrease of astringency and increase of sweetness and roundness, which suggests formation of tannin-mannoprotein aggregates, followed by precipitation (Guadalupe & Ayestarán 2008; Rodrigues *et al*. 2012).

Grape derived fining agents

The recent use of grape seed proteins was compared against exogenous fining proteins, and a positive reduction in red wine astringency was observed. Neverthless, gelatine had the greatest effects in most cases (Vincenzi *et al*. 2013). Similarly, Cell Walls (CW) from different varieties of grape pulp were recently tested. Pomace CWs from *Monastrell* grape presented a great adsorption capacity for high molecular weight proanthocyanidin (condensed tannin) and thus, to reduce red wine astringency (Bautista-Ortín *et al*. 2015). Nevertheless, further investigation is needed to confirm the fining potential of these vegetable origin treatments, obtained from grape seeds and pulp.

Overall, these evidences indicate that there is a clear opportunity for the oenology industry to explore and to develop potential alternative fining agents from endogenous origins, such as yeast, grape or must. Contrary to the current practices of adding exogenous compounds, these substitutes seem to be safer for consumers' health and more aligned with the current dietary trends.

The unmet need of these type of endogenous fining agents was in fact the trigger of this thesis, as it was driven by a challenge posed by the industrial partner of this work, *Proenol* – a Biotechnology SME willing to produce this type of alternatives.

1.2. Objectives

Main objective

In the Part I of this thesis, the main objective was to select efficient biological fining agents, naturally obtained from oenological yeast strains, which were previously isolated from natural wine fermentations.

To achieve this, the following specific objectives were defined:

a) to benchmark the impact of a wide range of exogenous fining formulations from animal, mineral and vegetable origins in different types of wines and compare these effects with novel alternatives produced at laboratorial scale, the yeast protein extracts;

b) to select the most efficient yeast protein extracts and validate their specific efficiency to stabilize and clarify white wines, when compared to the traditional fining agents;

c) to develop a specific immunological tool to target unstable proteins in white wines as chitinases and thaumatin-like proteins, which cause haze after bottling.

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Chapter 2

A Comprehensive Evaluation of Fifty-Five Fining Agents: Comparison with an Endogenous Alternative

A Comprehensive Evaluation of Fifty-Five Fining Agents: Comparison with an Endogenous Alternative

This Chapter aims at fulfilling the specific objective of this thesis “to benchmark the impact of a wide range of exogenous fining formulations from animal, mineral and vegetable origins in different types of wines and compare these effects with novel alternatives produced at laboratorial scale, the yeast protein extracts”, whose results were submitted in “Journal of Agricultural and Food Chemistry”, an international reviewed journal, from the ACS Publications.

Abstract

A large range of fining substances is commonly used in the winemaking industry to improve the organoleptic quality and long-term stability of wine. This study overviews the impact of exogenous commercially-available fining agents on three different types of wine. Altogether, fifty-five reference fining agents were grouped by their respective sources (caseins, albumins, gelatines, glutens, isinglass and bentonites). In parallel, four oenological yeast protein extracts (YPEs) were developed at laboratory scale and tested as a possible alternative to these exogenous products. YPEs had a similar effect on the chromatic characteristics of white and rosé wines, when compared with isinglass or bentonites. In red wines, YPEs promoted a reduction of colour intensity and polyphenol content, a comparable impact to that obtained using gelatines. Altogether, this study is regarded as the first benchmark on fining agents, and also indicates that endogenous yeast-based treatments could be a promising alternative to the commonly used fining agents.

Keywords: Allergenic, benchmarking, chromatic analysis, clarification, endogenous, fining agents, oenological yeasts.

2.1. Introduction

A variety of undesired particles and/or macromolecules remain in the wine after fermentation and their natural settlement is normally both time-consuming and unsuccessful. In contrast, the permitted fining technique of adding specific substances to wine efficiently gathers and flocculates the unstable undesirable material.

A widespread range of fining formulations is available on the market and various types of treatments can be added, at different dosages, alone or in combination. The decision of which fining agent(s) to apply, normally depends on the clarification or stabilization objective, desired style of the wine and destination market. In particular, fining is used to increase the limpidity, to correct the final colour and to guarantee an efficient stabilization of white and rosé wines (Neill 2002). It is mainly applied in red wines, however, to reduce excessive bitterness and astringency (Castillo-Sánchez *et al*. 2008; Iturmendi *et al*. 2010).

Animal proteins, such as gelatin or egg albumin are commonly used to interact with negatively charged species through the formation of hydrogen bonds (e.g. removal of aggressive tannins from young red wines) (Chagas *et al*. 2012). Apart from those, caseins and casein-derived products, prevent wine oxidation and bitterness due to their recognized ability to interact with very oxidized phenols (Braga *et al*. 2007). Other products such as isinglass - a collagen preparation from fish bladder – are similarly applied to clarify the yellow color of white wines, since they also remove phenolic compounds that are susceptible to oxidation (e.g. catechins, proanthocyanidins and hydroxycinnamic acids) (Cosme *et al*. 2008). The efficiency of the proteinaceous fining agents might be connected to their amino acid composition, isoelectric point, superficial charge density and molecular weight (Cosme *et al*. 2008). As an alternatively to proteinaceous fining agents, mineral substances such as bentonites are applied in white and rosé winemaking to remove specific unstable proteins that remain in wine after fermentation and cause haze problems after bottling (Pocock *et al*. 2011).

Although efficient, research studies suggest that consumers are increasingly concerned as well as informed about environmental and health problems involved

with the application of exogenous additives to wine. Therefore, a more sustainable and healthier need has been raised in the winemaking sector and novel environmentally-friendly fining agents are recently starting to be developed, such as vegetable proteins from gluten, pea, potato, grape seed or rice (Gambuti *et al*. 2012, Vincenzi *et al*. 2013). In addition, the application of yeast-based compounds has also been explored as possible alternative to the application of pork-derived gelatins in red wines (Iturmendi *et al*. 2010).

Exogenous fining additives of animal or even vegetable protein origin may cause adverse reactions in susceptible individuals (Vassilopoulou *et al*. 2011, Deckwart *et al*. 2014) and according to the European Food Safety Authority (EFSA), it is still difficult to stablish a clinical threshold to specific fining agents such as ovalbumin or egg white (European Food Safety Authority (EFSA) 2011). In contrast, endogenous alternatives such as yeast protein extracts (YPEs) are not required to be labelled as a potential allergen as are egg or milk derived products and their application in winemaking has already been approved by the International Organization of Vine and Wine, OIV (OIV-OENO 417-2011).

Herein, we have carried out an exhaustive analysis of the impact of different fining agents on the very same wine matrix, in which is, to the best of our knowledge, the first benchmark on several reference fining formulations, derived from wide-ranging sources. Further, we have tested YPE as potential fining alternatives in different wine types, and compared it against the most commonly used fining agents.

2.2. Material and Methods

2.2.1. Wines

Three young (Portuguese blends) white, rosé and red wines were provided by Proenol (Canelas, Portugal). At the end of their fermentations, a total of 50 L from each wine was stored in a bag-in-box, filled to the top to avoid oxidation. All wines were transported to the laboratory at room temperature and then stored at 14 ± 2ºC. Wines were very turbid and unstable, so sulphur dioxide was rectified to 40 mg/L of free SO_2, using potassium metabisulfite in powder form.

2.2.2. Reference fining formulations

Overall, samples of 55 different fining agents were acquired to their respective manufacturers. Treatments were categorized by sources: bentonites, isinglass, caseins, vegetable proteins, gelatins, albumins and mixtures (formulations with compounds derived from two or more distinct sources). As recommended by the suppliers, 29 fining agents were applied in both white and rosé winemaking and the other 26 were applied in red winemaking (**Table 2.1**). All samples were received as powder formulations, excluding the isinglasses and 12 samples of gelatines which were provided in liquid solutions. More detailed information about each individual fining formulation is provided as Table S2.1 (***Annex II***).

Table 2.1 - Fining formulations used in this study and respective application dosages.

White / rosé wines				**Red wine**			
Category	Code	Num. of samples	Dosages (g/hL)	Category	Code	Num. of samples	Dosages (g/hL)
Bentonites	B	8	5 - 120*	Gelatines	G	18	5 - 120*
Isinglasses	I	9	0,5 - 120*	Albumins	A	4	5 - 12*
Caseins	C	2	20/100	Isinglasses	I	1	5/100
Veg. Prot	V	1	10/30	Veg. Prot	V	2	5 - 30*
Mixtures	M	9	5 - 120*	Mixtures	M	1	5/10
TOTAL		29		**TOTAL**		26	

*Values diverge within the dosage range presented

2.2.3. Yeast protein extracts

Four different yeast strains were selected from a diverse collection of oenological yeasts, which were previously isolated from spontaneous wine fermentations. The respective YPEs were produced at laboratorial scale through a confidential methodology and further obtained in optimized liquid solutions. All the YPEs were tested in the three wines.

2.2.4. Characterization of the protein fining agents

Protein samples were prepared in *Laemmli* Sample Buffer 5X (pH 6.8) - containing 10% SDS, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2 M Tris-HCl and 0.05% bromophenol blue. Polyacrylamide resolving gels (12,5%) were used and the gel was afterwards stained using Coomassie Blue R250 reagent. Gel electrophoresis was performed on a Bio-Rad Protean II apparatus. Protein content was accessed using BCA Assay kit from Pierce.

2.2.5. Fining experiments

Glass tubes of 250 mL total volume were set vertically and filled with 200 mL of the tested wine type (white, rosé or red). Trials were performed in triplicates, during 48 hours at a temperature of 20ºC ± 2ºC. Overall, 576 individual fining assays were performed: (((29+4) white wine + (29+4) rosé wine + (26+4) red wine) x 2 dosages x 3 replicates). Due to the large number of assays, each wine was tested in a different round.

After fining, the clarified wines were transferred to clean, glass bottles, using a large-volume pipette to avoid disturbing the lees formed at the bottom of the tubes. In parallel, the samples were all filtered through qualitative filter paper (Q100, from Millipore) with pore size of 11 µm. For reference products, minimum (min) and maximum (max) dosages were defined according to suppliers. Optimal dosages of YPEs were defined after results obtained in previous trials extensively performed at laboratorial scale: 10 g/hL and 20 g/hL for white and rosé wines; 30 g/hL and 40 g/hL for red wine.

2.2.6. Fining efficiency

After 24 hours of fining – middle point of the process — two independent people separately observed each fining test and registered a qualitative evaluation in a scale of 0-5 about the following parameters: size of the particles, comparative limpidity and sedimentation rate.

After application of the fining agents in white wines, 1 mL samples were carefully collected from each wine surface (~1 cm to the top). Turbidity was then evaluated along time, from 5 min after fining until 48 h after fining (10 time points), by recording the samples absorbance at 540 nm in 1-cm path length quartz cells, using a Cary 100 BIO UV-Visible spectrophotometer (Batista *et al*., 2009). Values were then used to compare the sedimentation profile during the first 24 hours after fining using the different fining agents.

2.2.7. Conventional oenological parameters

Free and total SO_2, density, alcohol content, titratable acidity (TA), volatile acidity (VA), malic acid, tartaric acid, and pH were controlled using a FOSS Wine-Scan (FT-120) infrared Fourier-transform spectrometer. The phenolic content was expressed as Total Phenolic Index at 280 nm (TPI), according to the OIV method n. ENO/SCMAV/04/298 (2006).

2.2.8. Chromatic characterization

The absorption spectrum of each wine was recorded using a Cary 100 BIO UV-Visible spectrophotometer and the Cary WinUV Scan software. Absorbance was scanned over the range of 280-750 nm, using quartz cells of 1-cm path length. The chromatic coordinates of C* (chroma or 'saturation'), L* ('brilliance' for white/rose wines; 'clarity' for red wine), a* (redness), and b* (yellowness) were calculated using the CIELab system in the MSCV® software (Pérez-Magariño & González-Sanjosé 2003).

2.2.9. Statistical analysis

Statistical analysis was performed in the *GraphPad Prism* software, version 5.0. Standard deviation calculations, linear regression and One-way ANOVA analysis were performed. Differences of $p<0.05$ were considered significant. Principal Component Analysis was performed in the *Primer* software, version 6.1.16 (Clarke and Gorley, 2006).

2.3. Results and Discussion

2.3.1. Characterization of the fining agents

The common molecular weight (MW) profile of each protein fining agent category is presented in **Fig. 2.1**. For the gelatines and mixtures formulations, more than one characteristic protein pattern was observed. All isinglasses exposed two major individual bands, detected above 94 kDa (~100 and ~140 kDa), which might correspond to collagen components (β and α chains) (Pati *et al*. 2010; Chandra, M.V. Shamasundar 2015). A minor band (72 kDa), probably corresponded to elastin and lower MW bands (~40-45 kDa and <17 kDa) might correspond to gelatine degradation (Chandra, M.V. Shamasundar 2015).

No evidence of parvalbumin (~13 kDa), a fish allergen, were detected (Pati *et al*. 2010). Casein samples presented two intense bands at MW of ~30 and ~35 kDa, respectively, corresponding to β and α caseins (phosphoproteins). A weaker signal was observed at 75 kDa, probably derived from milk fat globule membrane (Murgiano *et al*. 2009). Lower MW bands might correspond to residual milk proteins such as β-lactoglobulin (~17 kDa). In contrast, liquid gelatines showed a low MW polydispersion (below 15 kDa), indicating substantial hydrolysis of these products (Braga *et al*. 2007). However, this pattern was not verified for all the gelatine powders, which presented a higher MW polydispersion, situated between ~30-250 kDa.

No differences were identified within the group of albumins. For these formulations, intense protein bands were identified within the range of 35-50 kDa, probably corresponding to ovalbumin. An intense band was also detected at ~74 kDa, possibly associated to ovotransferrin (Tolin *et al*. 2012). Concerning vegetable proteins, all samples presented a wide range of individual protein bands, a profile that correlates to typical wheat gluten extracts (Marchal *et al*. 2002).

The protein profile of mixtures shown different correspondences to protein fining agents, such as caseins (4 samples), wheat glutens (3 samples), gelatines (2 samples) and isinglass (1 sample). These indications confirm the high heterogeneity among this Category.

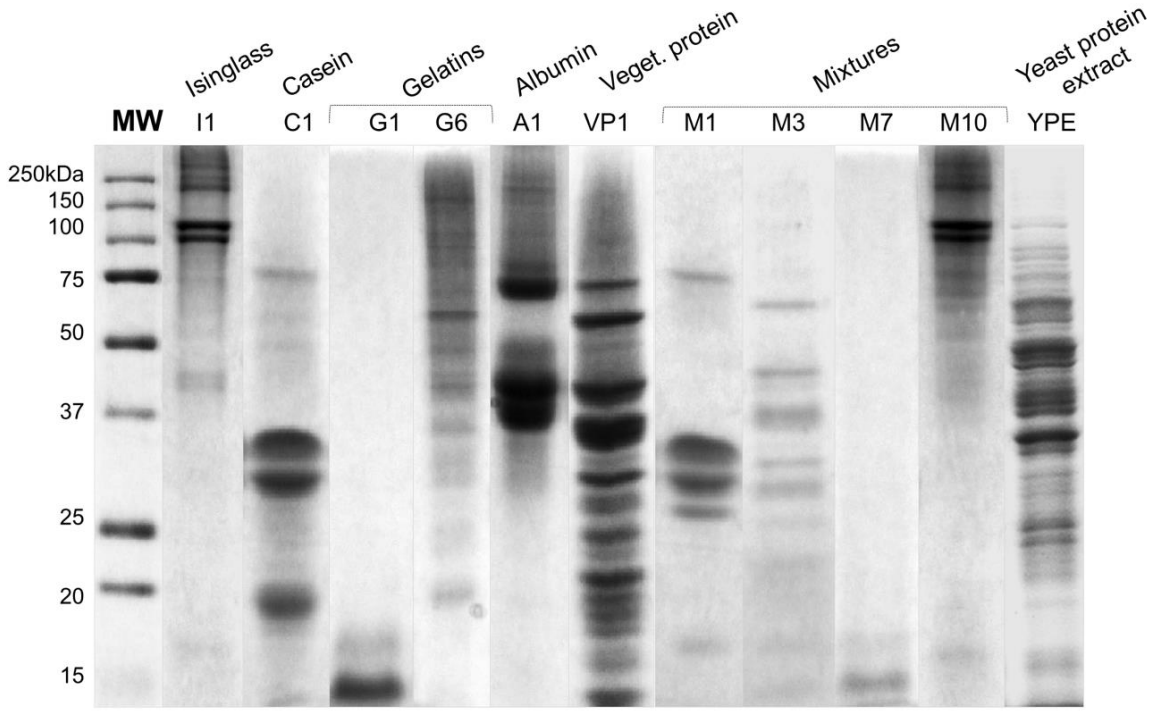

Fig. 2.1 - SDS-PAGE profile of the protein fining agents. Proteins were separated on a 12.5% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250. MW: molecular weight marker, I: isinglass, C: casein, G: gelatines, A: albumin (egg white), VP: vegetable protein (gluten), M: mixtures (formulations containing compounds derived from two or more distinct sources), YPE: yeast protein extract. The numbers following the referred letters, correspond to specific samples of reference fining agents (specified in Table S2.1 – *Annex II*). Gelatines G1 and G6, respectively correspond to samples provided in liquid and powder forms.

The electrophoretic profile of the YPEs revealed several individual protein bands (~10-100 kDa). In general, proteins exposed a MW from ~30-60 kDa and were well-defined (without smear appearance). Concerning protein composition, YPEs presented protein content (w/w%) above 50%, as required in the OIV regulation concerning the use of yeast protein extracts (OIV-Oeno 494-2012) (**Table 2.2**).

The mixture category presented the lower protein content, while albumins presented the highest protein content. More detailed information about each specific fining agent is presented as supporting information in in Table S2.1 (common fining agents) and S2.2 (YPEs) (***Annex II***).

Table 2.2 - Fining agents used in this study and respective protein content.

Category of fining agents	Protein content (w/w%) Mean ± SD
Isinglasses (n=10)	24,89 ± 11,85
Caseins (n=2)	56,90 ± 4,33
Gelatines (n=18)	30,50 ±12,02
Albumins (n=4)	67,49 ± 5,82
Vegetable (n=3)	56,01 ± 5,06
Mixtures (n=10)	16,92 ± 15,4
Yeast protein extracts (n=4)	54,20 ± 3,32

2.3.2. Fining efficiency

Visual qualitative evaluation - after 24 hours fining

After 24 hours of fining, a qualitative evaluation was performed by two independent observers on each experiment (including all triplicates). Since the colour of the red wine samples was excessively opaque, the visual evaluation was only performed for white and rosé wines. Particle size, sedimentation rate and comparative limpidity were evaluated in a scale from 0-5.

In both wines, positive correlations were found between the evaluated parameters (**Fig. 2.2**). Indeed, a higher sedimentation rate and an improved comparative limpidity were found to be consistently linked to the formation of larger particles/clusters. In fact, the Stokes Law mathematically describes that settling velocity depends on the squared radius of the particle (r), density of the liquid (d_l) and density of the particle (dp) (Ribéreau-Gayon *et al*. 2006), showing that the higher the ability of each fining complex/protein to bind the undesired particles/macromolecules, the greater the size of the aggregates and sedimentation rate (**Equation 2.1**).

Equation 2.1. Stokes Law:

$$v = \frac{2(d_l - d_p)r^2 g}{9\mu}$$

This qualitative evaluation revealed that after 24 hours fining, the application of bentonites, caseins and YPEs promoted the formation of the largest particles, along with faster sedimentation processes. Indeed, YPEs promoted the formation of medium-large particles and efficient precipitation in both white and rosé wines.

Considering results obtained for the common fining agents, previous suggestions were confirmed. Casein formulations are widely recognized to completely precipitate at wine pH (low over-fining probability), so the settling phenomenon was already expected to occur at a high rate by application of the maximum dosage. In addition, bentonites are also known to rapidly adsorb proteins or other positively charged particles in white and rosé wines and rapidly settle, creating a 'flaky' deposit, especially if no interfering colloidal material is present (Ribéreau-Gayon *et al*. 2006). Also, as expected, the application of the dissimilar mixtures resulted in a high heterogeneity in terms of the particles size. The application of isinglass and vegetable proteins (glutens) mostly produced low-medium size particles and, accordingly, the settling of the aggregates and limpidity was much lower. Previous fining studies actually reported that isinglass produces very light aggregates that settle slowly at the vats bottom (Ribéreau-Gayon *et al*. 2006). Apart from these, it is also known that other factors such as wine composition, particles charge, superficial density and protective colloids might play a role on the fining efficiency of each specific treatment (McRae & Kennedy 2011).

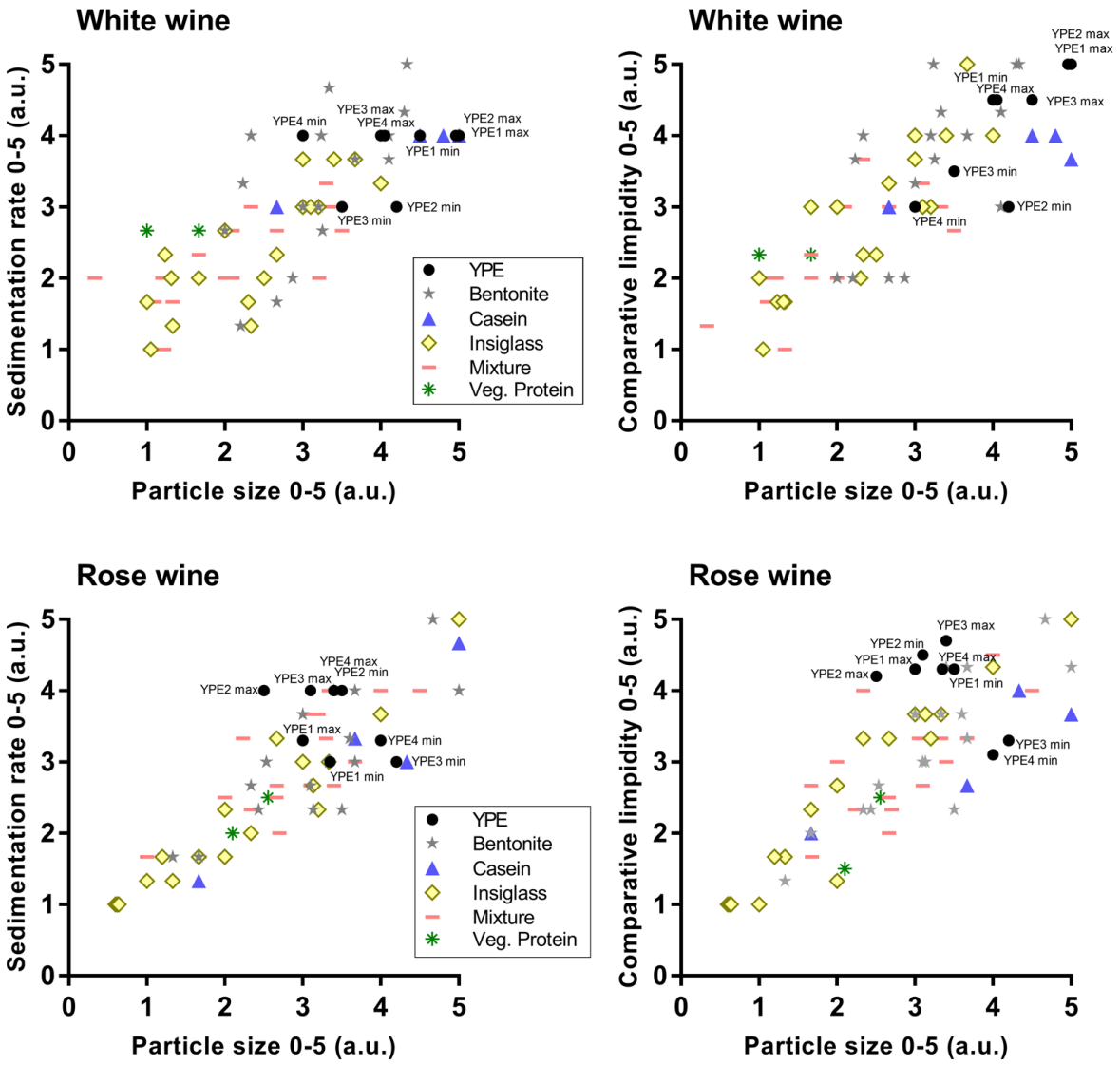

Fig. 2.2 - Visual evaluation of the fining trials performed in white and rosé wines. Each point represents an average evaluation of each fining assay (in triplicates). Two dosages were considered per treatment, but that distinction is only detailed herein for the YPEs. Values are given in arbitrary units (a.u.).

Sedimentation profile – during 48 hours fining

In the white wine trials, turbidity was recorded during the 48 hours after fining by measuring absorbance at 540 nm (**Fig. 2.3**). Results validated the previous qualitative comparison of fining efficacy, showing that YPEs are capable to efficiently reduce the turbidity levels along time, with a sedimentation rate similar to caseins or bentonites. In fact, it was observed that after 5 min of the fining

intervention, wines with isinglasses, mixtures and vegetable proteins, were still clearly more turbid than the others. Moreover, data also revealed that turbidity values recorded after 24 h fining, were already considerably low when YPEs were applied, confirming that less time is required to achieve an effective clarification treatment.

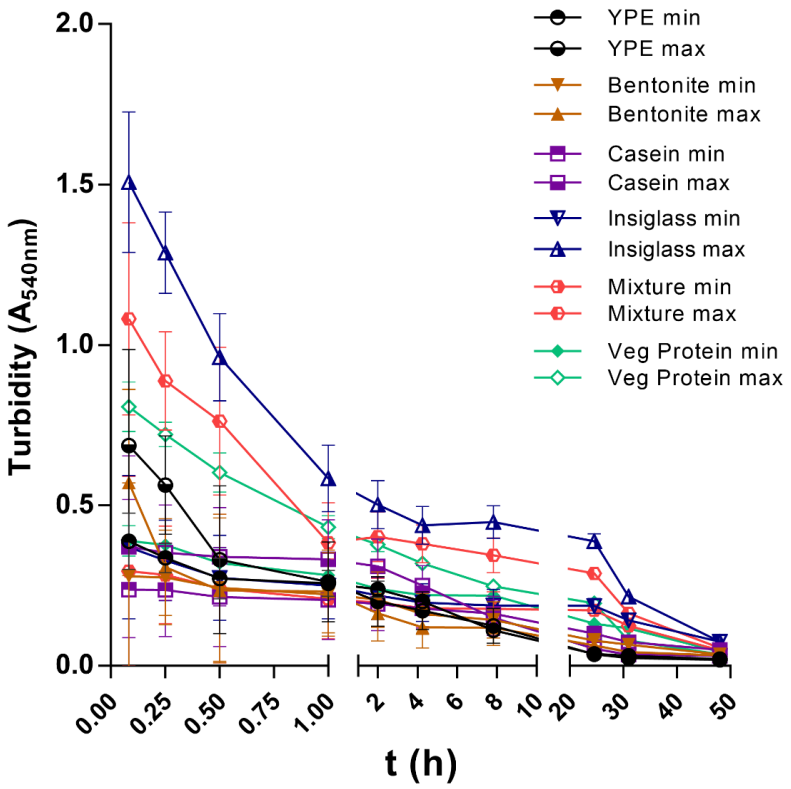

Fig. 2.3 – Sedimentation profile of the fining trials performed in the white wine. Values were recorder in 10 different time points, from 5 min to 48 h of fining. Mean and standard deviation values were calculated per category and dosage (min/max). Turbidity was evaluated by measuring the absorbance at λ = 540nm.

Lees thickness

After 48 hours, the clarified wines were carefully transferred to clean glass bottles. Results of the lees thickness revealed that some bentonites and gelatines produced the largest sediments in comparison with the other fining categories, in white/rose wines and red wines, respectively (15-23 mm) (**Fig. 2.4**). Also, the application of maximum dosages of bentonites was found to produced fluid and unstable deposits. Instead, minimum dosages produced more compact and steady sediments. The

application of casein and albumin induced the formation of medium-size lees (10-16 mm and 12-15 mm, respectively) and the aspect of the sediments was found to be slightly flexible, but still very homogeneous. Although the application of isinglasses could promote the formation of medium size lees (10-18 mm), more dispersed sediments were visualized at the bottom of the tubes.

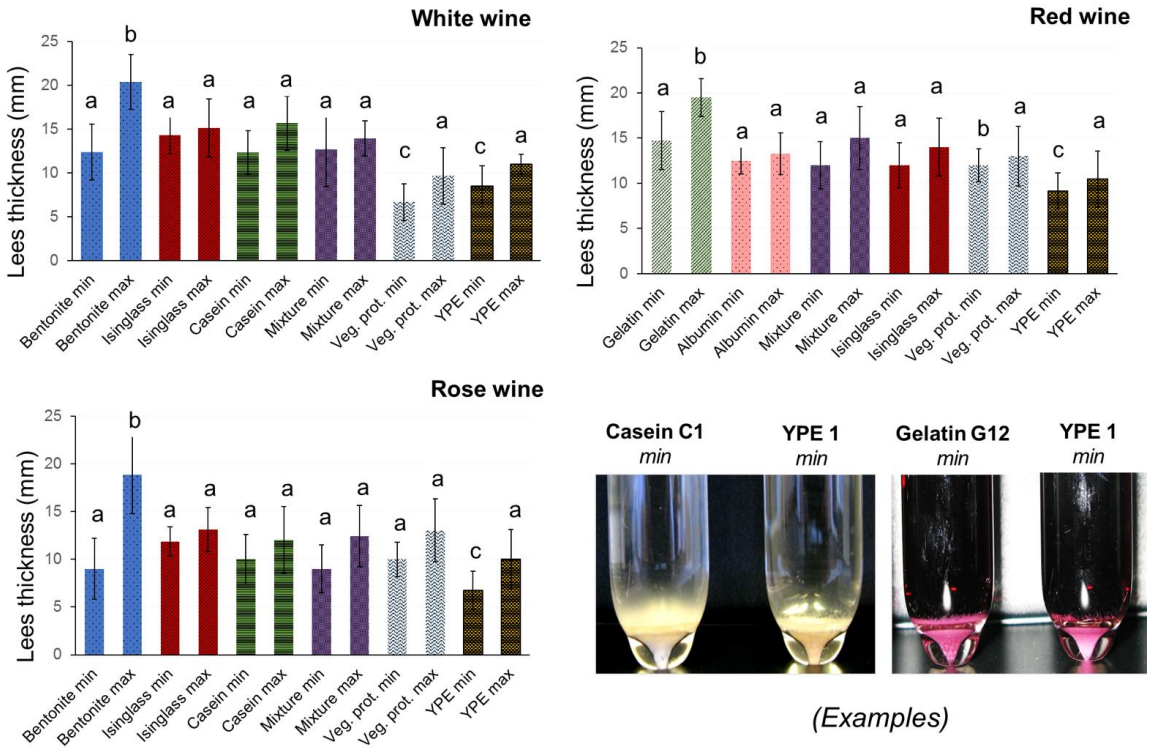

Fig. 2.4 - Lees thickness for each type of wine. Mean and standard deviation values were calculated per Category and dosage (min/max). Different letters correspond to significant differences (p<0.05).

Overall, the application of vegetable proteins (wheat glutens) and YPEs promoted the smallest lees (4-16 mm and 4-14 mm, respectively), which also had a superior level of firmness. This evidence was confirmed in the three types of wine. Previous studies also mentioned that yeast-based treatments produced smaller lees when compared with reference substances (Iturmendi *et al*. 2010; Fernandes *et al*. 2015). This finding might also increase the industrial interest of using YPEs as potential

alternatives to the remaining additives, due to the possibility to reduce wine losses during the racking stage.

2.3.3. Chromatic analysis

Results obtained through the chromatic analysis of white wine samples revealed that the application of some vegetable proteins and mixtures generated the less brilliant and the most saturated treated samples. Contrarily, wines treated with casein revealed the highest levels of brilliance (L*>96) and minor distinction was found between the groups of YPEs, bentonites, mixtures and isinglasses (**Fig. 2.5 Fig. 2.5A1**).As regards to colour impact, the application of casein formulations also promoted the highest decrease of the yellow colour component, which is in line with previous studies (Castillo-Sánchez *et al*. 2008). By other side, the impact of the YPEs is closer to the one obtained by applying bentonites or isinglasses (**Fig. 2.5 A2**). Similarly, the clarification effect of YPEs was verified in the rosé wine (**Fig. 2.5 B1-B2**). Isinglasses exposed the lowest potential to improve brilliance and reduce the wine saturation. Contrarily, the maximum dosages of caseins, confirmed its capacity to improve the final brilliance index (L*). In addition, YPEs revealed an important capacity to reduce the yellow intensity when compared with reference fining agents as bentonites, isinglasses or mixtures, and that was mostly marked with the application of YPE1 and YPE2.

Considering the red wines, the effect of the YPEs was also found to be similar to some common isinglasses, mixtures or caseins treatments. Gelatines did not considerably improve the clarity of this untreated wine and some products from this category promoted an undesired increase on saturation (circled in **Fig. 2.5C1**). Besides this fact, all treatments applied in red wine promoted a reduction in the red colour component. Indeed, the addition of YPEs promoted the most accentuated general colour reduction. As revealed for the brilliance analysis, colour measurements showed that wine samples treated with albumins, isinglasses and mixtures had similar effects (**Fig. 2.5C2**).

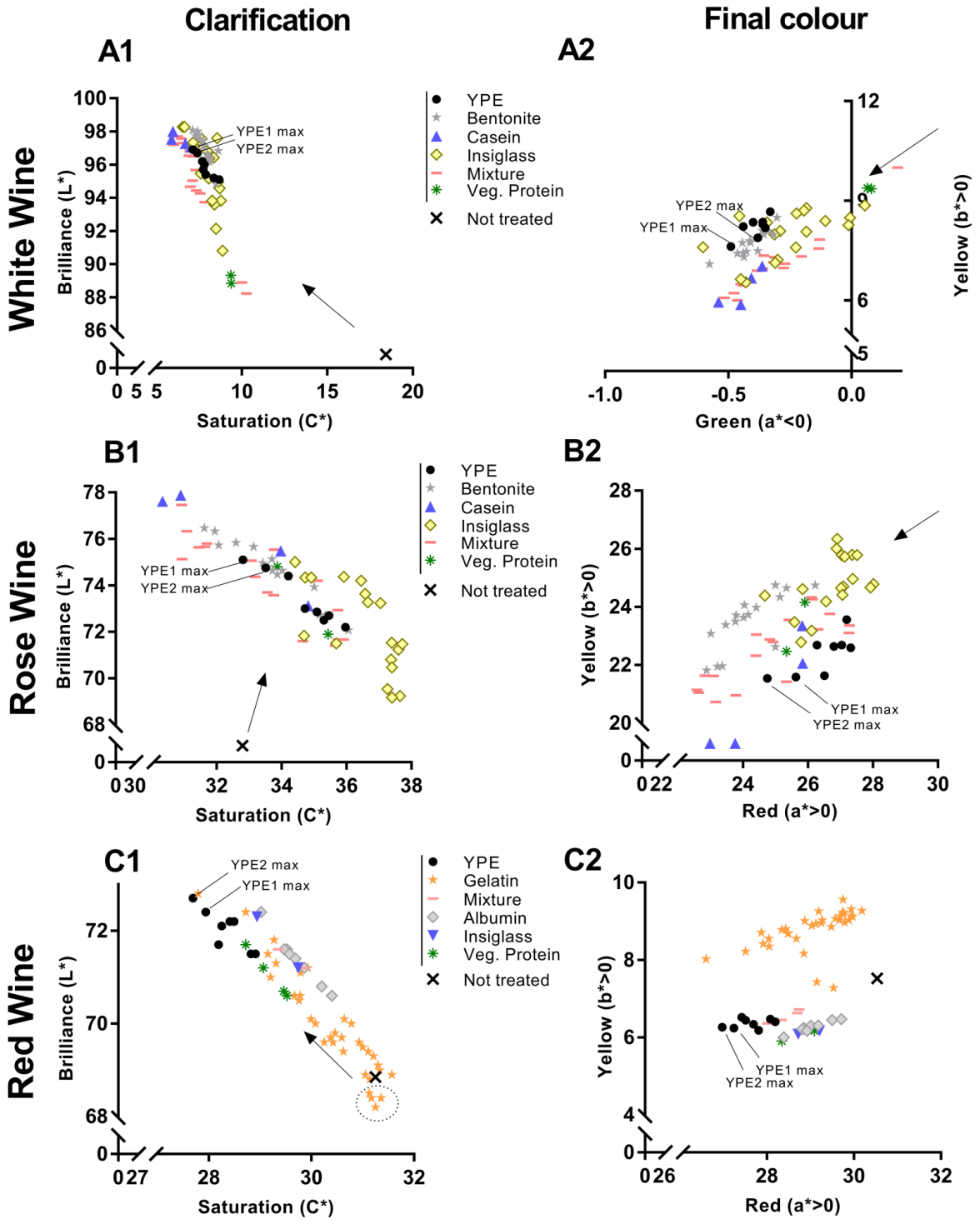

Fig. 2.5 - **Colour analysis in white, rosé and red wines.** Brilliance and Saturation, (L* vs. C*) and Colour components green (a*<0), red (a*>0) and yellow (b*>0). Experiments were performed in: **A)** White wine; **B)** rosé; **C)** Red wines and include the results for the respective untreated wines. Arrows indicate the general fining tendency; Samples treated with YPEs are highlighted (in black).

2.3.4. Other oenological effects

Conventional oenological parameters

The conventional enological parameters were controlled using Fourier transform infrared spectroscopy. In general, the principal component analysis revealed that the treatment using YPEs in the three types of wine tested in this study, did not induce negative or unexpected impacts, when compared with the reference substances (**Fig 2.6**).

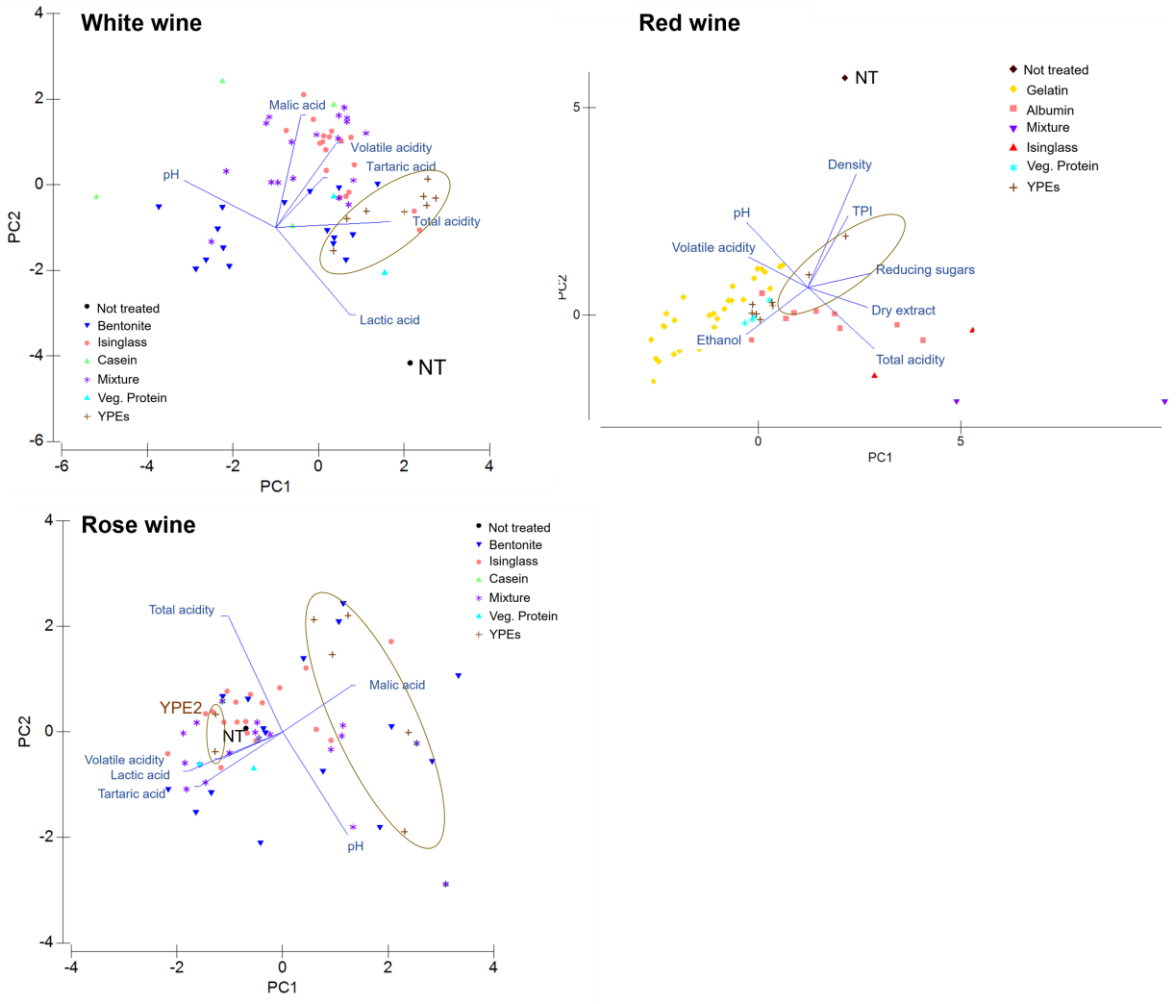

Fig. 2.6 - Principal component analysis (PCA) of the impact of different fining treatments on important oenological features in white, rosé and red wines. The two principal components, PCA1 and PCA2, respectively explained 49% and 25% (white wine); 42% and 31% (rose wine); 62% and 14% (red wine). The variance was plotted considering all treatments tested (in two different dosages). Arrows indicate the contribution of each conventional oenological parameter. The different groups are indicated by different colours; YPEs are circled in brown; NT: Not treated wine.

As regards to white wines, results showed that all treatments reduced the levels of lactic acid in the untreated wine, but no considerable variance was caused on total acidity or other types of organic acids. Similarly, no important grouping was verified for the rosé wine, but it is visible that YPE2 had a different effect, when compared to the remaining YPEs, which caused a higher deviation from the untreated wine. Since it has distinct characteristics, different features were evaluated on the red wine and results showed that the effect of YPEs was located in between to what in could be obtained by applying all the other treatments.

Polyphenolic reduction

Total polyphenolic index (TPI) was measured in all the samples of untreated and treated red wine to understand their capacity to reduce the respective polyphenolic content, which is normally associated to high levels of astringency in young red wines. Results revealed that all treatments could reduce the phenolic content of the untreated wine (**Fig. 2.7**) and in average, the application of the YPEs caused superior levels reduction (12.2-15.6%).

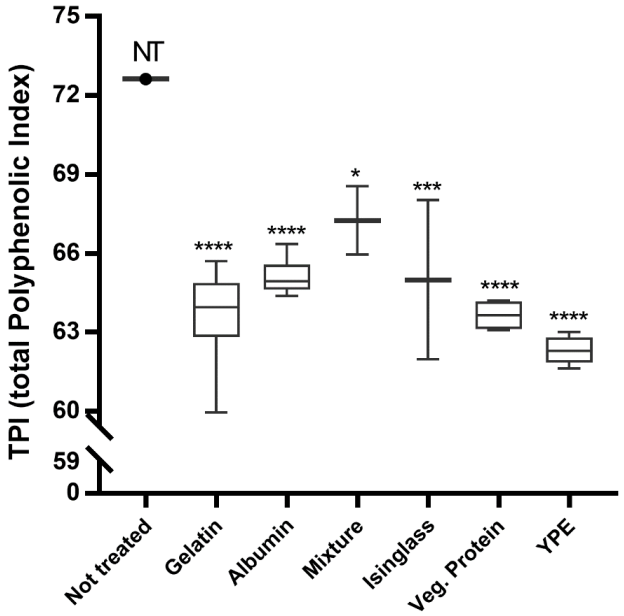

Fig. 2.7 - Total phenolic index (TPI) in red wine before and after different fining treatments. Mean and standard deviation values were calculated for each fining category and include results of both minimum and maximum dosages. Asterisk indicates a significant difference from Untreated wine and values were calculated using One-way ANOVA test. One asterisk (p<0.05); Three asterisks (p<0.0006); Four asterisks (p<0.0001).

Nevertheless, when comparing between categories (through a multi-comparison One-way ANOVA test), the effects caused by this alternative fining treatment were not significantly different from the one caused by gelatines, isinglasses or vegetable protein (p>0.05), an indication that corroborates with other fining study where yeast extracts were tested against gelatines (María Remedios *et al*. 2010). Contrarily, the decrease on TPI caused by YPEs was significantly higher, when compared to both mixtures (p<0.0007) and albumins (p<0.003).

2.4. Conclusions

Overall, this work has provided the first overview of the fining potential of a large range of currently available fining agents in comparison with potential new fining alternatives, namely the oenological yeast protein extracts (YPEs). After a first assessment on the fining potential of YPEs, results revealed that these novel materials were capable of efficiently improving the final colour of white and rosé wines in comparison with the reference formulations. Moreover, benefits were also observed regarding the sedimentation rate of the general fining process and the compactness of the resulting lees. Red wine samples treated with YPEs also presented improved levels of colour saturation and a reduced polyphenolic content, which was comparable with gelatine treatments. Besides the positive effects, it is also important to note that lower concentrations of these extracts (10- 40 g/hL) were sufficient to promote similar or superior effects when compared with higher dosages of the reference products. In particular, among all the tested extracts, YPE1 and 2 provided the most interesting results for all wines.

In future work, fining assays should be performed at a larger scale, along with more detailed post-treatment analysis as regards to each wine type, in order to confirm the obtained results. It is also necessary to support these preliminary results by testing different wine matrices in replicate laboratory trials. Contrarily to exogenous additives such as allergenic proteins, YPEs could be marketed as natural fining alternatives for wine clarification and/or stabilization.

Author Contributions

R.N. developed the yeast protein extracts, J.F. and F.C. performed the experiments; J.F, M.F.T. and A.C.G. designed the experiments; J.F. and A.C.G. wrote the paper.

Acknowledgments

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Chapter 3

Unveiling the potential of novel yeast protein extracts in white wines clarification and stabilization

Unveiling the potential of novel yeast protein extracts in white wines clarification and stabilization

This Chapter aims at fulfilling the specific objective of this thesis “to select the most efficient yeast protein extracts and validate their specific efficiency to stabilize and clarify white wines, when compared to the traditional fining agents”, whose results were published in *Frontiers in Chemistry*, an international reviewed journal, from the Elsevier Group (doi: 10.3389/fchem.2015.00020).

Abstract

Fining agents derived from animal and mineral sources are widely used to clarify and stabilize white wines. Nevertheless, health and environmental problems are being raised, concerning the allergenic and environmental impact of some of those fining products.

In this study, our aim is to validate the potential of yeast protein extracts, obtained from an alternative and safe source, naturally present in wine: the oenological yeasts. Three untreated white wines were used in this work in order to evaluate the impact of these novel yeast protein extracts (YPEs) in terms of the wine clarification and stabilization improvement. Two separated fining trials were thus conducted at laboratory scale and the yeast alternatives were compared with reference fining agents, obtained from mineral, animal and vegetable origins. Our results indicate that yeast protein extracts were capable to promote (i) brilliance/colour improvement, (ii) turbidity reduction (76-89% comparing with the untreated wines) and (iii) production of compact and homogeneous lees (44% smaller volume than obtained with bentonite). Additionally, after submitting wines to natural and forced oxidations, YPE treatments revealed (iv) different forms of colloidal stabilization, by presenting comparable or superior effects when particularly compared to casein. Overall, this study reveals that yeast protein extracts represent a promising alternative for white wine fining, since they are resultant from a natural and more sustainable origin, at present not regarded as potential allergenic, according to the Regulation (EC) No. 1169/2011.

Keywords: Browning potential, clarification, fining agents, oxidation, protein content, white wine, yeast protein extracts.

3.1. Introduction

The wine industry is one of the most competitive sectors all over the world. Accordingly, innovative oenological products and techniques constantly need to be optimized in order to produce high quality wines that are able to fulfill the demanding consumers. Particularly, white wines should present high levels of brilliance, a non-oxidized and pleasant final colour and a balanced organoleptic profile. In order to promote efficient clarification and stabilization processes, fining is still one of the most traditionally used techniques and therefore, a wide range of diverse fining agent formulations have been developed during the last decades (Braga *et al*., 2007; Cosme *et al*., 2007; Marangon *et al*., 2012; Lucchetta *et al*., 2013). Currently, fining products might be composed by animal, mineral and vegetable particles or macromolecules (Iturmendi *et al*., 2010; Gambuti *et al*., 2012) or even by mixed formulations that combine a miscellaneous of distinct compounds, trying to simultaneously promote parallel effects on wines.

Animal fining agents as casein and caseinates are well characterized to efficiently interact with problematic oxidizable compounds, due to their affinity to specific flavanol monomers and low molecular weight procyanidins (Cosme *et al*., 2008). By removing these undesired compounds that remain in the wine matter after fermentation, they are able to prevent the browning phenomenon after bottling. Despite their efficiency, some animal origin products as casein were shown to cause allergenic concerns on the consumers' health (Patzl-Fischerleitner and Eder, 2012; Schumann *et al*., 2013; Deckwart *et al*., 2014) and therefore, recent European legislations have already been adopted stating that if egg or milk derived proteins are detected on final wines, they must be declared on the respective bottle label [regulation (EU) No 579/2012]. On the other side, bentonite (montmorillonite mineral) is a non-protein fining agent that reduces the haze phenomenon in white through electrostatic adsorption to unstable proteins positively charged at wine pH (Blade and Boulton, 1988; Achaerandio *et al*., 2001; Sauvage *et al*., 2010; Pocock *et al*., 2011). These specific grape proteins remain in the wine until the end of fermentation, due to their highly resistance to proteases and to the low wine pH, but can aggregate into light-dispersing particles under elevated temperatures during

wine storage or transportation (Waters *et al*., 1992; Pocock *et al*., 2000; Le Bourse *et al*., 2011; Marangon *et al*., 2011). Although it is one of the most used fining agents, bentonite is already described to reduce essential aromatic compounds and consequently to compromise the wine final quality (Armada and Falque, 2007). Other problems involved with bentonite fining include long settling times, the associated manual handling requirements and the environmental costs for disposal of its waste (Hsu and Heatherbell, 1987; Marangon *et al*., 2012; Lucchetta *et al*., 2013).

In order to overcome the health and environmental concerns related with the use of the animal and mineral origin products, biological and more environmental-friendly alternatives should be further investigated (Marchal *et al*., 2002; Iturmendi *et al*., 2010; Patzl-Fischerleitner and Eder, 2012; Schumann *et al*., 2013). In fact, the wine sector has already made some efforts trying to find an efficient alternative to animal and mineral origin products. The application of particular vegetable fining alternatives extracted from wheat, pea and potato origin was already authorized by the International Organization of Vine and Wine (OIV) [Resolution OIV-OENO 495-2013]. More recently, proteins extracted from potato and grape seed actually showed some promising results in reducing harshness and astringency in red wines (Gambuti *et al*., 2012; Vincenzi *et al*., 2013). Regarding white wines, no efficient fining alternatives were still found to simultaneous clarification and stabilization, without necessity to add enological adjuvants during the fining process (Marchal *et al*., 2002; Cosme *et al*, 2012; Gambuti *et al*., 2012).

Alternatively, former studies explored novel fining treatments based in the application of yeast derivatives (e.g. cell wall components or yeast proteins), which have resulted in wine improvements such as a turbidity decrease, an astringency reduction and a stabilization potential (Dupin *et al*., 2000a; Bonilla *et al*., 2001; Caridi, 2006; Iturmendi *et al*., 2010). In fact, yeast plays a primary role in the winemaking process and thus, after fermentation, its autolysis naturally leads to a release of diverse cell compounds as enzymes, mannoproteins, fatty acids, nucleotides, peptides and free amino acids (Zhang *et al*., 2011). Nevertheless, Saccharomyces cerevisiae extracts are known to contain some particular proteins that are potentially involved in allergic responses (Lindberg *et al*., 1992)

(Kortekangas-Savolainen *et al*., 1993; Nermes *et al*., 1995; Savolainen *et al*., 1998; Nittner-Marszalska *et al*., 2001), namely the glycolytic enzymes, Enolase 1 (ENO1) and Enolase 2 (ENO2) that are involved in carbohydrate metabolism and therefore, intrinsic to the wine fermentation process (Varela *et al*., 2005; Kornblatt *et al*., 2013), contrary to all the exogenous products that are commonly applied and already described to raise both health and environmental problems.

In this work, our aim is to evaluate the potential of two alternative yeast protein extracts (YPEs), derived from a wine native source - oenological yeasts - as potential alternatives to problematic reference fining agents commonly used in white wines. For that purpose, the impact of the YPEs was measured in terms of different oenological parameters as turbidity, chromatic characteristics, lees volume, protein content, browning/oxidation prevention and curative potential after wine oxidation. The production of these two previously selected YPEs is currently being implemented at industrial scale to efficiently confirm their potential as innovative and natural fining agents for application in white wines, already authorized by the European Union [regulation (EU) No 144/2013].

3.2. Material and Methods

3.2.1. Wines and fining agents

Three young white wines (2013) were intentionally selected from three distinct Portuguese regions - Dão, Lisbon and Algarve - in order to cover different wine patterns and characteristics.

Those wines were received 1 week after wine alcoholic fermentation was completed and therefore, they were still turbid and unstable. Until used, they were stored at 14 ± 2ºC and the sulphur dioxide was rectified to avoid their premature evolution. Four fining products were used as reference for the fining experiments and prepared according to the supplier recommendations. These enological products were composed by different elements, respectively: Bentonite, Casein, Polyvinylpolypyrrolidone - PVPP and Vegetable protein derived from hydrolyzed

wheat gluten. Wines and fining agents were kindly provided by Proenol (Canelas, Portugal).

3.2.2. Yeast Protein Extracts (YPEs)

According to our preliminary fining trials, two specific yeast strains were selected from a diverse collection of oenological yeasts, all isolated from spontaneous wine fermentations. The corresponding protein extracts, namely BCV1 and BCV5, were produced through a confidential methodology developed at laboratory scale. Those protein yeast extracts were obtained in optimized liquid solutions with the final protein concentration of 50g/L.

3.2.3. Fining experiments

After alcoholic fermentation

After receiving the three untreated wines, the reference fining agents (x4) and the yeast protein extracts (x2) were simultaneously tested. Fining assays were conducted in triplicates, during 48 hours, using glass bottles of 275 ml of total volume. Just before the fining trials, each bottle was filled in with 250 ml of wine and the experiments were conducted at a controlled temperature of 22ºC ± 2ºC. For the application of the reference fining agents, the dosages (minimum and maximum) were defined according to the supplier recommendations. In the case of the two YPE, the fining dosages were determined per the previously fining experiments performed at laboratory scale. The tested dosages are presented in **Table 3.1**. Fining substances were left to flocculate and sediment to the bottom of the bottles during 48 hours. Clarified wine samples (supernatants) were then gently pipetted and filtered through a qualitative paper filter, pore size 11 µm (Whatman™ Grade 1) to new, clean bottles. Wine samples were posteriorly analyzed by the following wine analytical methodologies.

Table 3.1 - Fining agents and respective dosages tested.

Fining products	Minimum dosage (g/hL)	Maximum dosage (g/hL)
Bentonite	10	60
PVPP	10	80
Casein	20	100
Vegetable protein	20	60
BCV1	10	20
BCV5	10	20

After oxidation during 5 months

A volume of 5 L from each untreated wine barrel was purposely reserved in the large wine vessels (20 L) and stored at room temperature during 5 months. During this period, wines had natural contact with oxygen and no additional rectification with sulphur dioxide was performed. After 5 months, a second experiment of fining trials was performed, on the resultant highly oxidized and turbid wines. Again, the fining trials were conducted during 48 hours, but this time in glass bottles of 550 ml of total volume. Each glass bottle was filled in with 500ml of wine and the assays performed in duplicates, in this case, only testing the effect of YPEs (BCV1 and BCV5) and casein. After 48 hours fining, wine samples were processed according to the methodology previously described (section 2.3.1).

Protein fining agents characterization

As described by Vincenzi *et al*. (2005), a 10% stock solution of sodium-dodecyl sulphate (SDS) was prepared and then added to wine to achieve final concentrations of 0.1%. Samples were gently mixed during 2 min and then heated in a boiling water bath for 5 min. Potassium chloride (KCl) (2 M) was added to each sample to attain a final concentration of 200 mM. Samples were set to incubate for 1 hour and protein pellets were recovered by centrifugation at 14,000 g for 15 min at 4°C (Vincenzi *et al*., 2005). Pellets were further dissolved in 1x phosphate buffered saline, pH 7.4 (PBS buffer). Protein content (%w/w) was further accessed using BCA Assay kit from Pierce. Further, the protein molecular weight profile of

each protein fining agent was acquired by SDS-PAGE electrophoresis in concordance with the OIV resolution [Resolution OIV-OENO 452-2012]. Gel electrophoresis was performed on a Bio-Rad Protean II apparatus with power supply set at 100V/gel for the stacking gel and 150V/gel for the resolving gel. Protein samples were equally prepared in *Laemmli* Sample Buffer (5X) and boiled at 95ºC during 5 min. 12.5% polyacrylamide resolving gels were used to process the samples and the gel was afterward stained using Coomassie Blue R250 reagent. The isoelectric point of the protein fining agents and YPE was measured using the Stabino Charge Titration System, measured in triplicates.

3.2.4. Wine Analysis

Conventional oenological parameters

Free and total SO_2, density, alcohol content, titratable acidity (TA), volatile acidity (VA), malic acid, tartaric acid, pH, and glycerol content were controlled using a FOSS Wine-Scan (FT-120) infrared Fourier-transform spectrometer and a WineScan software Version 2.2.1 (FOSS, Hillerod, Denmark). The phenolic content was expressed as Total Phenolic Index at 280 nm (TPI) according to the OIV method n. ENO/SCMAV/04/298 (2006). According to Batista *et al*. (2009), final turbidity was measured at 540 nm in 1-cm path length quartz cells (Batista *et al*., 2009).

Lees volume

Lee volume was acquired by directly measuring the thickness of the sediment in the glass bottles after the fining trials were completed. Results were expressed as percentage of the initial volume of wine (%v/v).

Chromatic characteristics

The absorption spectrum of each wine was recorded through a spectrophotometry methodology in a Cary 100 BIO UV-Visible spectrophotometer, using the Cary WinUV Scan software. Absorbance was scanned for each sample over the range of 280-750 nm, using quartz cells of 1-cm path length. The absorbance values were recorded at 10 nm intervals. Following, the chromatic coordinates of C* (chroma or ‘saturation’), L* (lightness or ‘brilliance’), a* (redness), and b* (yellowness) were

calculated using the CIELab system in the MSCV® software, from Grupo de Colour de La Rioja, Logroño, Spain) (Perez-Magarino and Jose, 2002).

Wine unstable proteins

Protein content present in wine samples was measured by BCA kit methodology (Bio-Rad) using bovine serum albumin as the standard. The protein molecular weight pattern of each wine sample was assessed by SDS-PAGE electrophoresis, performed after protein precipitation with the KDS-method, according to Vicenzi *et al*, 2005 (Vincenzi *et al*., 2005).

Heat instability

Heat instability was accessed by the heat test methodology described in other studies (Fusi *et al*., 2010; Benucci *et al*., 2014) and also recommended by OIV (OIV-Oeno 494-2012) with small modifications. In brief, wine samples of 10 ml volume were set in test tubes and closed using screw caps. The tubes were heated at 80ºC in a water bath for 30 minutes and then allowed to cold to room temperature during 2 hours. The increase in turbidity was detected by spectrophotometry (Cary 100 BIO UV-Visible spectrophotometer and Cary WinUV software) at 540 nm in 1 ml quartz cuvettes and expressed as ΔA_{540nm}. All measurements were performed in triplicate.

Browning prevention effect: Forced and Natural oxidation

Browning prevention was tested in two separated assays. In the first test, browning potential was measured by submitting the wine samples to oxidize during 3 days. Two different tubes were prepared for each wine sample: In tube A (control), 15 ml of each wine sample was introduced into a 15 ml falcon tubes and carefully nitrogen-sparged during 2 minutes. In tube B, 10 ml of each wine sample was introduced into a 15 ml falcon tubes and 3% (w/v) hydrogen peroxide (Merck, Millipore) was added, without nitrogen addition (Cosme *et al*., 2008). All the tubes were left to incubate at room temperature during 3 days. In a separated experiment, the natural oxidation of the wines was also tested. This test was performed by simply leaving the wines treated with YPEs and casein in totally filled glass bottles of 200 ml volume during

5 and 9 months. For both tests, the browning variation was accessed by measuring the optical density variation at 420 nm (ΔA_{420nm}).

Curative test

For this experiment, the three base wines were stored to naturally oxidize during 5 months. After that period, the oxidized wines were treated with YPEs and casein in 500 ml fining assays. After fining, wine samples were analyzed by CIELab methodology and the achievements compared with the first fining trials, performed after alcoholic fermentation.

3.2.5. Statistical analysis

Statistical analysis was performed in Primer software, version 6.1.16 (Clarke and Gorley, 2006). Standard deviation values calculations and One-way ANOVA tests were performed. Differences of $p<0.05$ were considered significant.

3.3. Results and Discussion

3.3.1. Protein fining agents

The physicochemical characteristics of the protein fining agents used in this study are presented in **Table 3.2**. Both YPEs (BCV1 and BCV5) presented isoelectric point values (pI=4.4 and 4.5) close to casein (pI=4.6).

Table 3.2 - Fining agents used with the respective physicochemical characteristics

Fining products	*Isoelectric point (mean ± SD)*	*Protein content (% w/w)*
Bentonite	-	-
PVPP	-	-
Casein	4,6 ± 0,1	65,3
Vegetable protein	5,2 ± 0,0	19,5
BCV1	4,5 ± 0,2	62,4
BCV5	4,4 ± 0,1	60,1

In terms of protein composition, YPEs presented protein contents (%w/w) above 50% as required in the OIV regulation regarding yeast protein extracts fining (OIV-Oeno 494-2012). The low protein content presented by the vegetable protein fining agent, might indicate that this product probably contains other fining compounds on its formulation. In addition, SDS-PAGE analysis shows that 50% of the total yeast protein is located above 15 kDa of molecular weight, which is also in accordance with the OIV demand (**Fig. 3.1**). The distribution of the protein bands was observed between the molecular weight range of 10-150 kDa and the electrophoretic profile revealed low signs of protein degradation. Proteins detected at 48 kDa might correspond enolases (ENO1 and ENO2).

Casein presented intense individual bands located at 18, 30 and 34 kDa, probably respectively corresponding to the whey protein β-lactalbumin, α_s-Casein subunit and β-Casein subunit (Gambuti *et al*., 2012; Fleminger *et al*., 2013). Regarding the vegetable protein (VP) from gluten origin, the protein bands varied from 10-70 kDa of molecular weight. This protein profile corresponds, as expected, to hydrolyzed wheat proteins and the different bands correspond to distinct glutenin subunits (Chinuki *et al*., 2013).

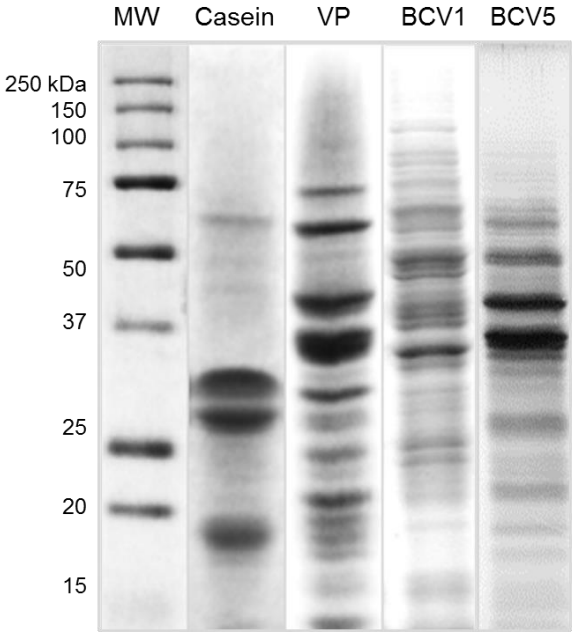

Fig. 3.1 - Protein molecular weight profile. Protein samples of the fining agents used in this study by Comassie-stained SDS-PAGE. (VP: Vegetable protein).

3.3.2. Clarification potential

Final turbidity

After the fining trial performed in 250 ml glass bottles, the impact of the YPEs and reference fining agents was first compared. No significant differences were detected regarding the conventional oenological parameters accessed by infrared Fourier-transform spectrometer. Contrarily, YPEs revealed a superior capacity to reduce turbidity, comparing with the efficiency of all the reference fining agents tested in this study (**Fig. 3.2**). Indeed, both extracts shown ability to clarify the most turbid wines as Wine 1 (reduction of 89%) and Wine 2 (76% reduction), but also to reduce the turbidity of the least turbid wine (79% reduction). In agreement to this, previous studies concerning the application of reference fining agents from animal, mineral and vegetable origins show comparable or inferior percentages of turbidity reduction when compared with the effect of our YPEs (Bonilla *et al*., 2001; Marchal *et al*., 2002; Braga *et al*., 2007; Sauvage *et al*., 2010; Lucchetta *et al*., 2013).

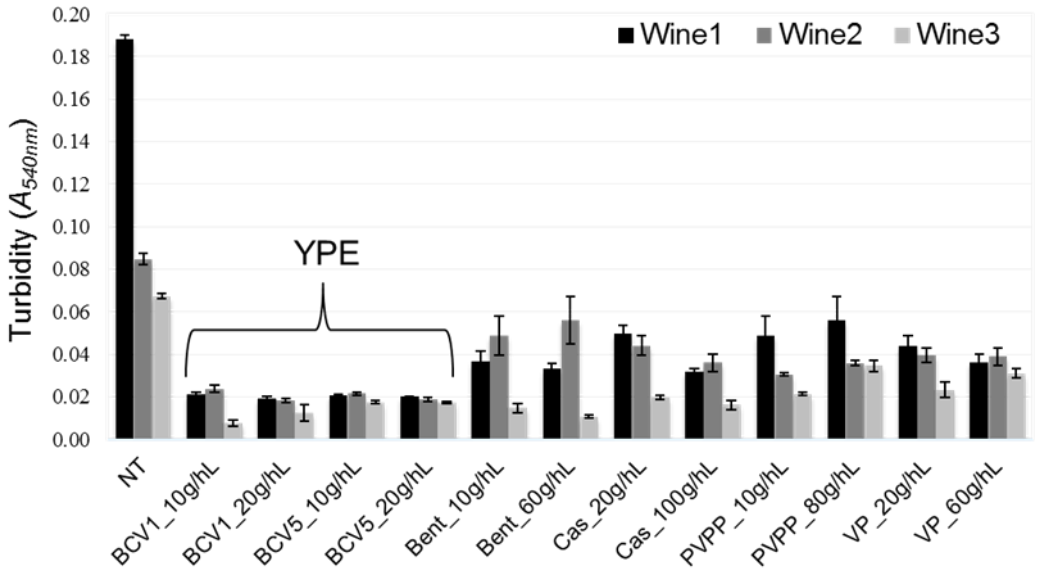

Fig. 3.2 - Final turbidity. Not treated wine: NT. Treated wine samples: Yeast protein extracts (BCV1 and BCV5); Bent (Bentonite); Cas (Casein); PVPP (Polyvinylpolypyrrolidon); VP (Vegetable protein). Bars indicate mean ± SD (n = 3).

Other study tested the effect of YPEs in red wines and also proved their ability to reduce wine turbidity to values that were comparable to gelatines (Iturmendi *et al*., 2010). Regarding the three different wines tested in this study, the final turbidity values obtained by YPE fining were located in a range of AbsA_{540nm} between 0.007 to 0.023 a.u. (absorbance units), a result that could not be obtained by the application of any reference fining agent herein tested. It is important to remark that the maximum dosage used for YPE (20 g/hL) was 3 to 5 times smaller than the reference products (60 g/hL – 100 g/hL).

The impact of the fining agents on the conventional oenological parameters is presented as supplementary information (***Annex III***, Tables S3.1 and S3.2 and Fig. S3.1). Concerning these results, no significant differences were identified between the effect of the common fining agents and YPE.

Colour characterization

As shown in **Figure 3.3**, the CIELab methodology used in this study revealed that when Wine 1 was treated with YPE presented a superior increase of brilliance (L*), along with a superior reduction of saturation (C*). Colour improvement was also verified for the wine treated with both yeast extracts, when compared to the reference fining agents. The final samples were found to be more greenish (-a*) and simultaneously less yellowish (b*), when compared with the other wine samples.

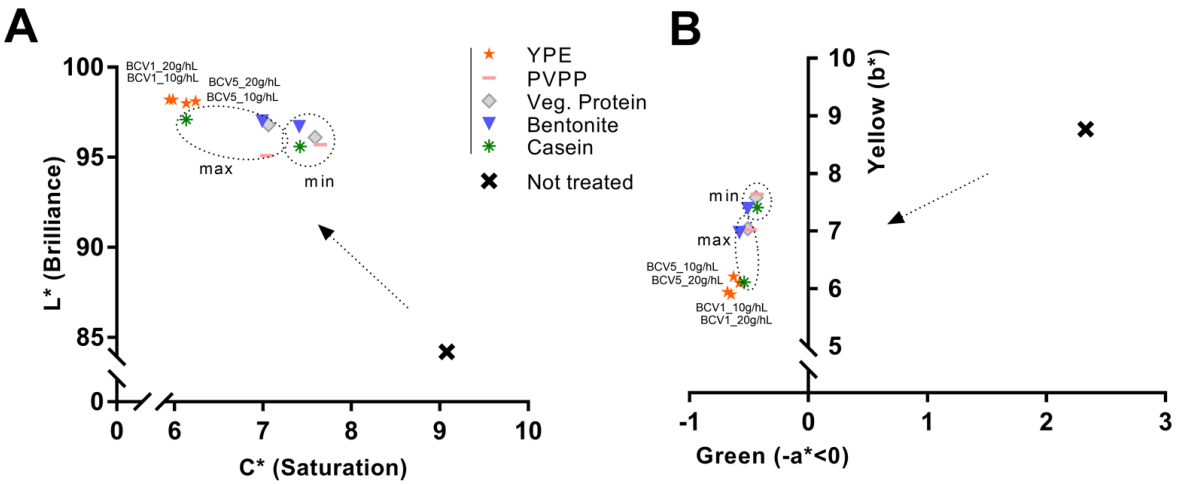

Fig. 3.3 - Chromatic characterization using CIELab system: A) Saturation (C*) vs. Brilliance (L*); B) Green (-a*) vs. Yellow (b*) values. Results were obtained before and after treatment of Wine1 with YPE and different fining agents. Two dosages were tested by treatment (Min.-minimum; Max.-maximum).

Lees volume

This also demonstrated that YPEs have the ability to remove undesired yellow pigments contained in the wines matter and that could cause oxidative problems after bottling. Only by applying the maximum dosage of casein comparable results were achieved and this could indicate that YPE might represent a good alternative to this animal origin product. CIELab data with the results obtained in terms of turbidity reduction and were also confirmed in the three different wine patterns tested in this study. See **Fig. S3.2** and **S3.3** of ***Annex III*** to consider the results

obtained in wine 2 and 3. As presented in **Figure 3.4**, the lees formed after the YPE application were visually more compact and homogeneous than the lees obtained by using of the reference fining agents. In terms of final volume and homogeneity, the YPE lees were found to be similar with those obtained with vegetable protein application. Accordingly, the volume percentage measured in relation to the wine initial volume was approximately 22%, 31% and 44% smaller than the lees respectively obtained with casein, PVPP and bentonite application (**Table 3.3**).

Table 3.3 - Lee volume percentage values obtained after fining trials with the different fining agents and YPE. Mean and standard deviation values were calculated concerning the three wines.

Lees volume *(v/v%) Mean ± SD*

Fining products	Min. dosage	Max. dosage
Bentonite	5,91% ± 0,12	6,87% ± 0,00
PVPP	5,00% ± 0,10	5,71% ± 0,01
Casein	4,29% ± 0,05	5,00% ± 0,05
Vegetable protein	2,86% ± 0,00	3,75% ± 0,02
BCV1	3,37% ± 0,02	3,53% ± 0,02
BCV5	3,42% ± 0,02	4,09% ± 0,05

This evidence agrees with the results reported by Iturmendi *et al*. (2010) in red wines fining, showing a correlation between lower lees volume and the proximity of protein pI to a value of 4.43 (isoelectric point of: BCV1 = 4.5 ± 0.2; BCV5 = 4.4 ± 0.2). This study also refers that YPEs produced smaller lees in red wines fining when compared with the impact of gelatine (Iturmendi *et al*., 2010). The final aspect and thickness of the lees produced by YPEs increase their potential use as alternative clarification agents, since are appreciated characteristics for the industrial clarification process implementation. Overall, YPEs seem to be promising technological tools that contribute to prepare wine for filtration and also reduce the wine loss on the vats bottom. In addition, since they are harmless and

biodegradable, have no constrains in terms of process handling or dregs disposal, contrary to bentonite or PVPP.

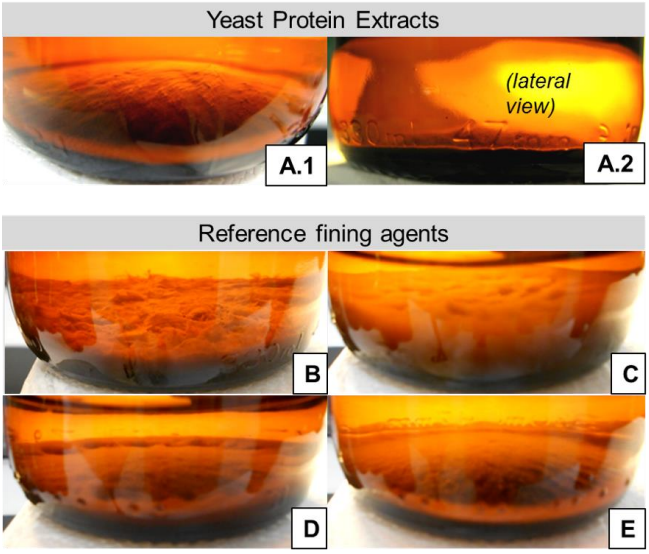

Fig. 3.4 - Aspect of the lees produced by the application YPE or fining agents. (A.1 and A.2) Yeast protein extract BCV1 - similar result was obtained with BCV5; (B) Casein - 100g/hL; (C) Bentonite - 60g/hL; (D) PVPP - 80g/hL; (E) Vegetable polymer - 60g/hL.

3.3.3. Stabilization potential

Protein haze protection

The results of the SDS-PAGE analysis performed in the wine samples show, as expected, that unstable proteins, specifically, thaumatin-like proteins (MW ~20-25 kDa) and chitinases (MW ~32 kDa), have only been removed from the wines treated with bentonite (**Fig. S3.4** of *Annex III*). By having an isoelectric point superior to wine pH, the yeast extracts became positively charge when added to wine and therefore, they were not able to interact with the positively charged unstable proteins, contrarily to the negatively charged bentonite clay. (Pocock *et al*., 2000; Marangon *et al*., 2011). Moreover, no bands of residual fining yeast proteins were detected in SDS-Page gels, which might indicate that most of the yeast proteins have been precipitated. This evidence correlates with the clarification potential results previously presented in this study, which indicate that flocculation seems to have

occurred very efficiently. Nevertheless, a more sensitive method as ELISA or mass spectrometry methodologies should be further used to analyze the probable presence of some residual yeast proteins on the final wines. Although the wine unstable proteins have not been removed by the YPE fining, the results of the heat test (**Fig. 3.5**) indicate that heat instability was significantly reduce (p<0.05) after the fining treatment in comparison to the untreated wines.

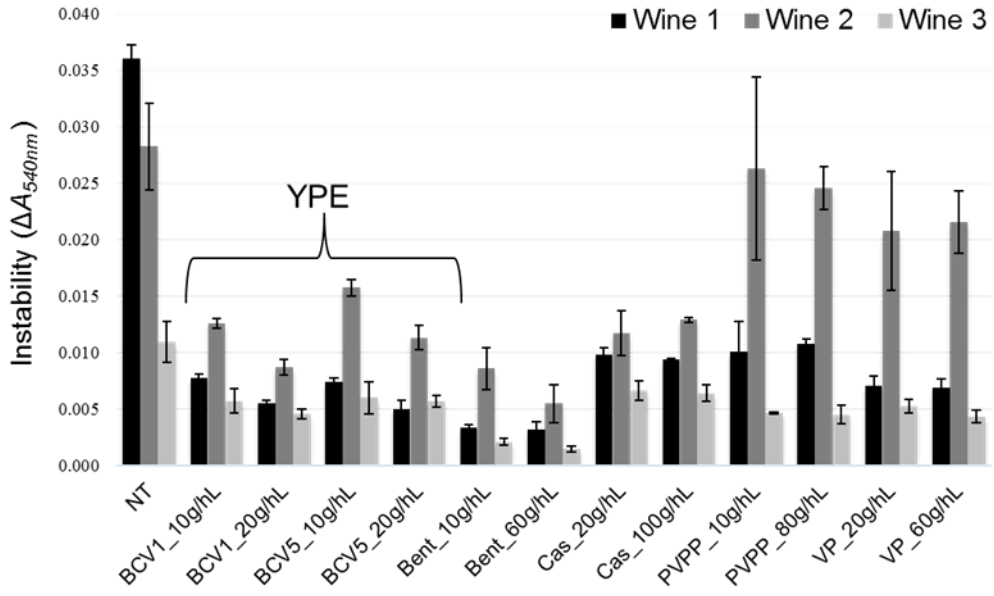

Fig. 3.5 - Heat test. Instability was measured according to the variation of absorbance at 540nm, by spectrometry methodology (ΔA_{540nm}). Not treated wine: NT. Treated wine samples: Yeast protein extracts (BCV1 and BCV5); Bent (Bentonite); Cas (Casein); PVPP (Polyvinylpolypyrrolidon); VP: (Vegetable protein). Bars indicate mean ± SD (n = 3).

Data obtained in wines treated with YPE and casein were not significantly different (p<0.05) and as expected, bentonite was the fining agent that promoted the higher instability reduction due to its capacity to flocculate the problematic wine unstable proteins. A recent study also tested the effect of reference fining agents on white wines protein haze and demonstrate that casein, egg albumin, isinglass, chitosan, chitin, and PVPP did not significantly affect the tendency of the wine to form protein haze (Chagas *et al*., 2012). Although the haze protection mechanism is still very

unclear in the literature (Moine-Ledoux and Dubourdieu, 1999), the stabilization effect promoted by YPEs application is possibly explained by the presence of some residual haze-protective material on our extracts. In particular it is probable that the YPEs still contain mannoproteins (glycoproteins containing 15 to 90% mannose by their weight) derived from the yeast cell walls which are already well described to prevent the haze phenomenon in white wines (Dupin *et al*., 2000a; Dupin *et al*., 2000b; Caridi, 2006). Indeed, some studies previously showed that wine aged on yeast lees presented reduced haze potential and lower bentonite requirements for protein stability than wine aged without lees but containing the same level of unstable protein (Moine-Ledoux and Dubourdieu, 1999). In addition, mannoproteins might also promote a positive impact in terms of wine body and volume enhancement (Caridi, 2006).

Browning prevention

Tests were performed to understand the tendency of the untreated and treated wines to oxidize. Casein was used as control to these tests, since it is a phenolic reactive agent often used to reduce oxidizable flavonoid and non-flavanoid fractions, and therefore to reduce the probability of the wine to oxidize (Schneider, 1995; Cosme *et al*., 2012). In the first test, wines were stored under natural oxidative conditions, without sulphur dioxide supplementation, during 5 and 9 months. Results show that YPEs could prevent the three wines to naturally oxidize (**Fig. 3.6**)

Their efficiency was significantly (p<0.05) similar or in some cases superior when compared with the results obtained by using casein. In particular, wines 2 and 3 became significantly less oxidized with YPE treatment than the ones treated with casein after the 9 months. To compare two distinct procedures, the untreated and treated wines were also submitted to the presence of a strong oxidizer, 3% (w/v) hydrogen peroxide during 3 days. In this case, results revealed no significant differences (p<0.05) between the impact obtained with YPE or casein. Both treatments herein presented seem to be efficient in preventing the browning phenomenon even after contact with extreme oxidation conditions. Wines 2 and 3 showed to be the more susceptive to the browning phenomenon.

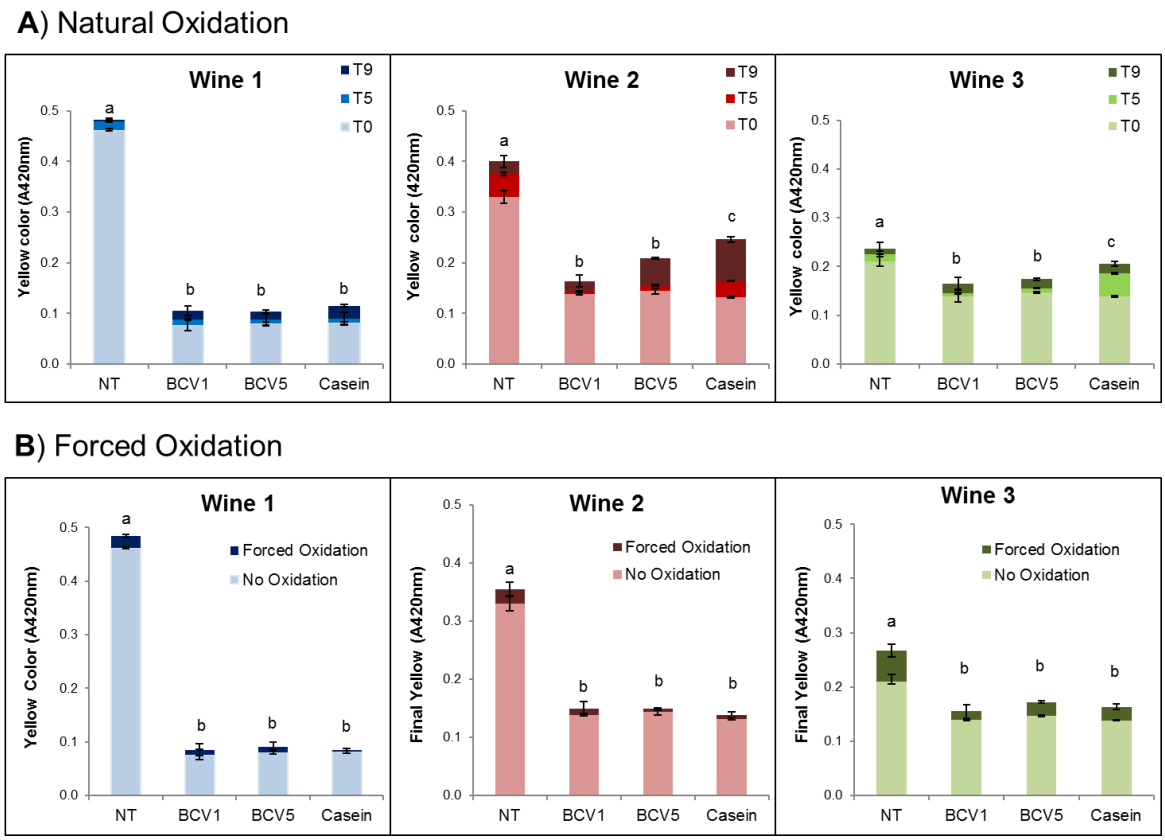

Fig. 3.6 - Browning evaluation after natural and forced wine oxidations. (A)Yellow colour (expressed as A_{420nm}) was measured after 0, 5, 9 months of oxidation, respectively: T0, T5, T9. (B) Oxidation was evaluated before and after oxidation using a solution of 3% (w/v) hydrogen peroxide. Different letters represent significant differences (p<0.05). Bars indicate mean ± SD (n = 3).

Curative capacity

In **Fig. 3.7** a comparison is presented between the effects of the fining treatments performed in the wines received after alcoholic fermentation (T0) and fining treatments regarding the intensively oxidized wines (after 5 months - browning).

In both fining trials, YPE promoted similar tendencies and achievements towards brilliance and colour improvement, proving that they efficiently correct wine browning, independently from the oxidation starting point or pattern of each wine. For both experiments, CIELab indexes show increase of Brilliance (L*) to values above 95 units along with saturation reduction to values below 10 units. In parallel, YPE treatment promoted a clear yellow colour reduction (b*) especially obvious in the extremely oxidized wines, 1 and 2. Moreover, a parallel increase in terms of

green (-a*) was verified in the case of the oxidized wine 3. Although browning is normally difficult to correct in excessively oxidized wines, casein is recognized by its curative capacity to uncolour the oxidized wines, contributing to colour refreshment while also refining gustatory characteristics (Braga *et al*., 2007). In comparison to casein, YPE revealed superior capacity to correct the browning regarding the three wines and with both dosages tested in our study (10 and 20 g/hL). Bonilla *et al*. (2001) previously tested a correction treatment using baker's yeasts in white wines and reported that the use of yeast at 1 g/L as a fining treatment was able to correct browning with similar results in terms of A_{420nm} decrease in comparison with a traditional treatment based on activated charcoal application (Bonilla *et al*., 2001). In agreement with this fact, other study revealed that whole yeasts or yeast cell walls present the capacity to efficiently adsorb phenolic compounds and browning products and thus decrease the yellow-brown colour levels after white wines fining (Razmkhab *et al*., 2002).

Overall, our data suggests that YPEs also have a preventive and curative capacity to correct white wines. Similarly to casein, yeast extracts might probably have the capacity to remove some oxidizable and oxidized species as phenolic compounds, in particular flavan-3-ol derivates, considered primarily responsible for oxidative aging of white wines, since small amounts of these molecules might generate browning (Schneider, 1995). The presence of other compounds as iron, copper, acetaldehyde or tartaric acid might also contribute to accelerate different types of condensation and oxidative reactions, contributing to the production brown compounds (Razmkhab *et al*., 2002). Based on this, further research is necessary to better understand the protective and corrective mechanism behind the YPE fining potential.

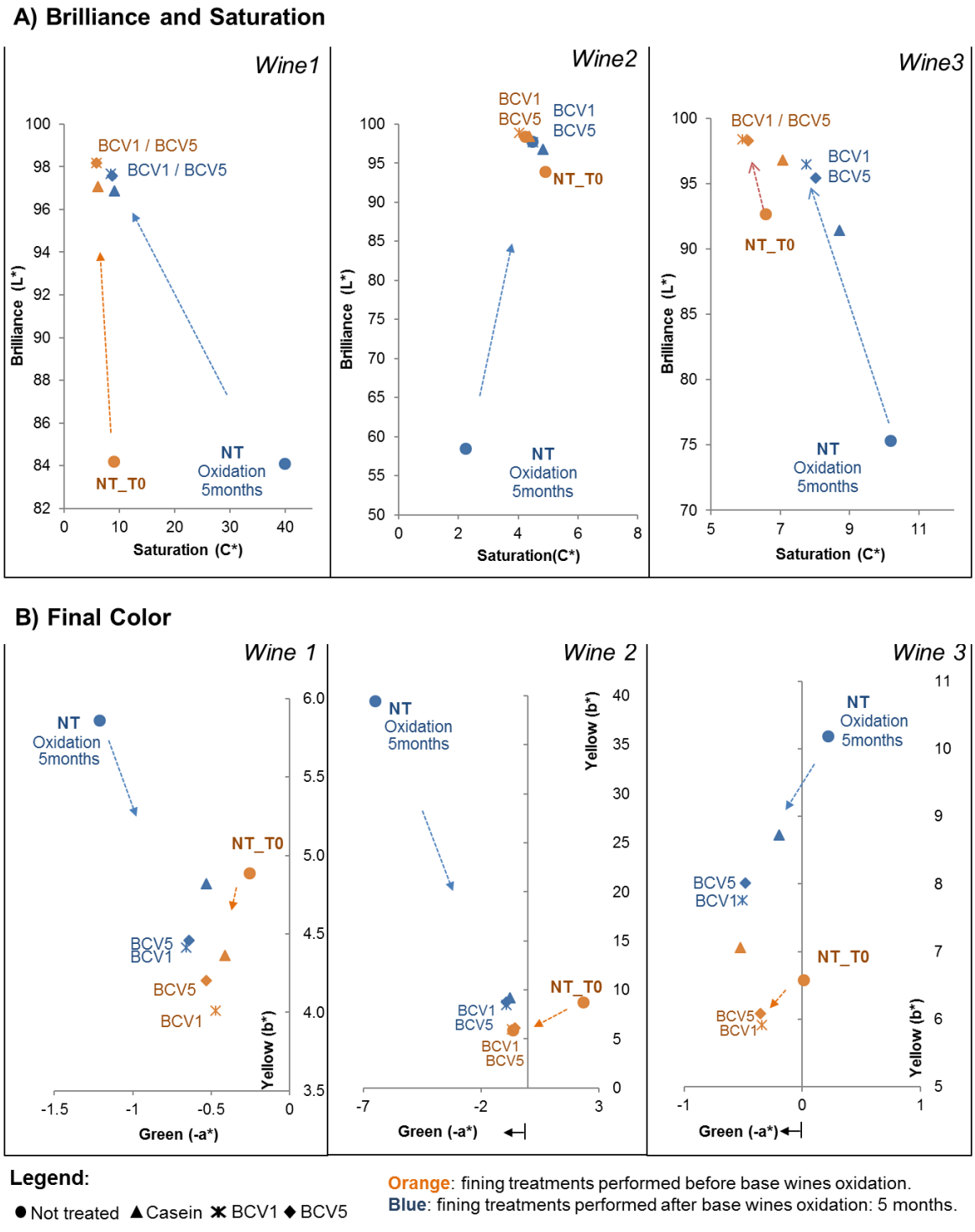

Fig. 3.7 - Test of curative potential using chromatic characterization. Not treated samples were fined after 5 months oxidation and effects were compared with the previously obtained results (T0). Arrows indicate the direction of the impact obtained after the respective treatments.

3.4. Conclusions

In this work, we revealed the potential of novel yeast protein extracts towards white wine clarification, stabilization and curative processes. Particularly, two yeast protein extracts previously selected and optimized for this study, could promote a significant brilliance increase, turbidity reduction and final colour improvement when compared with the reference fining agents available on the market. The lees produced after the YPE treatment were indeed found to be more compact and thick than the obtained with reference formulations, conferring to these yeast by-products an extra technological advantage in terms of the industrial fining validation. A protective effect to protein haze was also verified in samples fined with YPEs, although they were not able to interact and remove unstable proteins as bentonite did. YPE treatment also revealed a superior effect on browning prevention and curative ability when compared to casein, an animal origin product, already well recognized in the literature to efficiency reduce oxidation and browning in white wines (Cosme *et al*., 2008).

Overall, in this study we present an innovative fining alternative that can efficiently contribute to improve white wines final quality. Indeed, YPEs represent a novel tool for the wine sector, since they are obtained from a completely native source. This treatment represents a more sustainable alternative when compared to the exogenous substances from mineral, animal proteins or wheat origins. In future work, is our aim to further investigate the impact of these YPEs in terms of sensorial analysis and to test their potential implementation at industrial scale.

Author Contributions

R.N. developed the yeast protein extracts, J.F. and F.C. performed the experiments; J.F, M.F.T. and A.C.G. designed the experiments; J.F. and A.C.G. wrote the paper.

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Chapter 4

New immunological-based methods for detection of unstable proteins in white wines

New immunological-based methods for detection of unstable protein in white wines

This Chapter aims at fulfilling the specific objective of this thesis: “to develop a specific immunological tool to target unstable proteins in white wines as chitinases and thaumatin-like proteins, which cause haze problems after bottling in this wine type”.

Abstract

White wine unstable proteins, specifically chitinases and thaumatin-like proteins, cause stability and clarity problems mainly during the transportation and storage of wines. Indeed, traditional predictive methods such as the heat test have been applied in order to forecast the haze in bottled wine caused by such unstable proteins, however, this is a rather imprecise approximation, which often leads to the use of over-estimated dosages of bentonite clays causing technical, environmental and health concerns. To tackle this, we have developed immunology-based techniques to specifically detect the unstable proteins causing haze in white wines, allowing for its precise quantification.

The results herein presented confirm that these methods are valuable tools to quantify the amount of unstable proteins in white wines, helping winemakers to determine the lowest dosage of bentonite required to stabilize the wine. Moreover, the developed method is fast, simple and intuitive, and thus could be readily transferable to wineries.

Keywords: Chitinase (CHT), ELISA, heat test, haze, stability, thaumatin-like proteins (TLP), *VVTL1*

4.1. Introduction

Pathogenesis-related (PR) proteins are present in grapes to counter fungal infection and possibly other plant stresses (Falconer *et al*. 2010; Ferreira *et al*. 2004). During grape berry maturation (*vérasion*), sugar accumulation increases along with the rapid production of this type of proteins. Typically, PR proteins consist of chitinases (CHT) and thaumatin-like proteins (TLP), which have low molecular weight (12–30 kDa), low pl (4.1-5.8) and are highly resistant to proteolytic degradation (Ferreira *et al*. 2004).

In red wines, due to their high content of tannins, all proteins are precipitated. Conversely, white wines preserve a protein content of 10-500mg/L (Santoro 1995), which are the major cause of protein haze (Hsu *et al*. 1987; Dawes *et al*. 1994). Denaturation of these proteins and formation of insoluble aggregates might occur under the storage and transportation of wines (e.g. storage at higher temperatures) (Marangon *et al.* 2011). As a result, haze occurs, resulting in an unpleasant appearance for consumers of white wine.

To solve this, haze-forming proteins are generally removed by applying bentonite, a mineral fining agent that interacts electrostatically with unstable proteins, promoting flocculation (Ribéreau-Gayon *et al*. 2006). However, bentonite treatments are not selective and might cause a decrease of the quality in final wines. Further, previous studies highlight several problems concerning the usage of bentonite such as losses of wine volume (1-3%), environmental costs of bentonite disposal, wine colour alteration and aroma losses (Falconer *et al*. 2010; Sauvage *et al*. 2010). It fact, a mass spectrometry study has recently confirmed that bentonite also has affinity for β-glucosidases, which are crucial enzymes for the hydrolysis and consequent release of aroma precursors (Jaeckels *et al*. 2015).

At present, researchers are investigating possible alternatives for bentonite treatments based in ultrafiltration, addition of pectins or incubation with specific proteases associated with a heat treatment (Pocock & Waters 2006; Fernando N. Salazar *et al*. 2006; Marangon *et al*. 2012). Despite these efforts, there are still no authorized methods available on the market that might efficiently substitute bentonite.

Predictive assays have been explored in order to give better haze estimation and to avoid the application of extreme bentonite dosages (e.g. heat test, *Bentotest*, ethanol precipitation test and tannin precipitation test). These procedures normally force the protein aggregation and precipitation phenomena to occur faster (Pocock & Waters 2006, Harbertson 2008). However, some authors consider these tests biased towards the over-estimation of required bentonite dosages and expose concerns about the technical inaccuracy of the protocols (Pocock & Waters 2006; Sauvage *et al*. 2010).

Herein, immunological methods were developed to detect unstable proteins in white wines, which were based on western and dot-blotting. These procedures enable producers to rapidly and specifically identify and quantify the presence of unstable proteins in white wines, facilitating a more accurate determination of the necessary dosage of bentonite to apply during the stabilization step.

4.2. Material and Methods

4.2.1 Wines

White wines were provided from Portuguese producers and received in closed glass bottles. Wine samples were collected before and after clarification treatment with bentonite, performed by the producer (using unknown concentrations) and named, 'Untreated wines' and 'Treated wines', respectively. Wines were stored at room temperature for a short period of time (one-week maximum) and opened just prior the analysis.

4.2.2 Bentonite fining trials

Laboratorial fining assays were performed in 15 ml Falcon tubes. For each wine sample, a group of 9 falcon tubes were prepared with 10ml of total wine volume and accordingly, 9 different concentrations of bentonite were tested (0, 2, 4, 8, 12, 20, 40, 60 and 100 g/hL). A bentonite solution at a concentration of 50 g/L was prepared immediately before its addition by dissolving the clay in distilled water. After

bentonite addition, fining trials were simultaneously performed during 48 hours, protected from light and kept at room temperature.

4.2.3. Heat Test

Heat instability was assessed by the heat test methodology described in other studies (Fusi *et al*., 2010; Benucci *et al*., 2014) and also recommended by The International Organisation of Vine and Wine (OIV) [Oeno 494-2012] with small modifications. In brief, wine samples of 10 ml volume were set in test tubes and closed using screw caps. The tubes were heated at 80 ºC in a water bath for 30 min and then allowed to cool to room temperature during 2 hours. The increase in turbidity was detected by spectrophotometry (Cary 100 BIO UV-Visible spectrophotometer and Cary WinUV software) at 540 nm in 1 ml quartz cuvettes and expressed as ΔA_{540nm}. All measurements were performed in triplicate.

4.2.4. Protein precipitation

As described by Vincenzi *et al.* (2005), a 10% stock solution of sodium-dodecyl sulphate (SDS) was prepared and then added to wine to achieve final concentrations of 0.1%. Samples were gently mixed for 2 min and then heated in a boiling water bath for 5 min. Potassium chloride (KCl) (2 M) was added to each sample to attain a final concentration of 200 mM. Samples were incubated for 45 min and KDS-protein pellets were recovered by centrifugation at 14,000 g for 15 min at 4°C (Vincenzi *et al.* 2005). After removing the supernatant, pellets were dissolved in 1x phosphate buffered saline, pH 7.4 (PBS buffer).

4.2.5. Protein analysis

Protein Quantification

The protein content of wine samples was measured using the BCA kit methodology (Bio-Rad), using bovine serum albumin as standard. The protein molecular weight pattern of each wine sample was assessed by SDS-PAGE electrophoresis, performed after protein precipitation with the KDS-method, according to Vicenzi *et al*, 2005 (Vincenzi *et al*., 2005).

SDS-PAGE

The molecular weight profile of wine proteins was determined by SDS-PAGE electrophoresis in accordance with the OIV resolution [OENO 452-2012]. Gel electrophoresis was performed on a Bio-Rad Protean II apparatus with power supply set at 100 V/gel for the stacking gel and 150 V/gel for the resolving gel. Protein samples were prepared in *Laemmli* Sample Buffer (5X) and boiled at 95ºC for 5 min. 12.5% polyacrylamide resolving gels were used to resolve proteins. Coomassie staining was performed using Blue R250 reagent.

4.2.6. Immunological methods and detection

Antibodies

Polyclonal primary antibodies against chitinase and thaumatin-like proteins were acquired from Abcam (*AT2*; Chicken polyclonal anti-thaumatin2) and Agrisera (*AC1*; Rabbit Polyclonal Anti-Chitinase Class I), respectively. The anti-VVTL1 antibody was in-house produced by the Structural Biotechnology Laboratory (Center for Neuroscience and Cell Biology, Coimbra). Briefly, the antibody was generated in *Coturnix japonica* using as immunogen a recombinant version of *Vitis vinifera* thaumatin-like protein-1 (VVTL-1). Specific anti-VVTL1 antibodies were collected and purified from egg-yolk extracts of immunized hens using standard chromatographic methods. The antibody efficiency was characterized against both recombinant VVTL1 and native (wine) samples.

Goat anti-Chicken IgY H&L (Agrisera) (#as09606) and Goat Anti-Rabbit IgG H&L (abcam) (#ab6722), both ALP conjugated, were used as secondary antibodies.

Western Blotting

Proteins in polyacrylamide gels were transferred to a Polyvinylidene difluoride (PVDF) membrane (Trans-Blot® Turbo™ Midi PVDF Transfer Packs). A Trans-Blot® Turbo™ Transfer System was used and a current of 2,5 A x cm^2 was applied for 7 min. To prevent non-specific interactions, saturation was performed using a solution of 3% non-fat dry milk in Tris-buffered saline (TBS), for 2 hours with gentle agitation. Afterwards, membranes were washed twice for 5 minutes in 1x TBS with gentle agitation. Following this, the *VVTL-1* primary antibody was added to the

membranes in a solution of 3% non-fat dry milk in 1x TBS, for 2 hours at 4 ºC with agitation. Membranes were washed twice for 5 minutes with 1x TBS to remove unbound primary antibody. A secondary antibody conjugated with alkaline phosphatase was then added and incubation occurred for 1 h at room temperature (RT) with agitation. Protein-immunoreactive bands were developed using the Enhanced Chemifluorescence (ECF) detection system (GE Healthcare) and visualized in a Molecular Imager FX System (Bio-Rad).

Dot-Blotting

A volume of 5 µl of wine protein samples was placed on a Polyvinylidene difluoride (PVDF) membrane. After 15 min drying, the membrane was saturated with 3% non-fat dry milk in TBS at RT with agitation. Primary detection was performed against the target proteins using commercial antibodies (*AT2, AC1*) or alternatively, the new *VVTL-1* antibody in a 3% non-fat dry milk in 1x TBS solution for 2 h at RT. After washes with TBS-Tween (TBS-T), a secondary antibody conjugated with alkaline phosphatase was applied in 1x TBS solution for 1hour at RT. Afterwards, membrane was washed again 3 times with 1x TBS-T. Revelation was made with BCIP/NBT substrate, which contains 5- bromo-4-Chloro-3'-Indolyphosphate (BCIP) p-Toluidine Salt and Nitro-Blue Tetrazolium Chloride (NBT). BCIP/NBT 1-Component AP Membrane Substrate reacts with alkaline phosphatase, and a bluish-purple reaction product is developed. Images of the membrane with coloured spots were registered using a Gel Doc™ EZ System. The signal of the background area around the dots was collected, and dot signal intensities were then normalised against that mean. Each analysis was performed in triplicate.

4.3. Results and Discussion

Comparison: traditional (heat test) and immunological-based methods

Twenty-seven white wines were subjected to immunological detection of TLP and CHT unstable proteins, using commercial antibodies. In parallel, the traditional heat

test was also performed to measure heat instability. Results of the dot-blotting experiments revealed lower intensities of the dots for most the 'treated wines'. Contrarily, some of the 'untreated wines' exposed the highest intensity values, indicating greater contents of the unstable proteins in the wines (**Fig. 4.1A**).

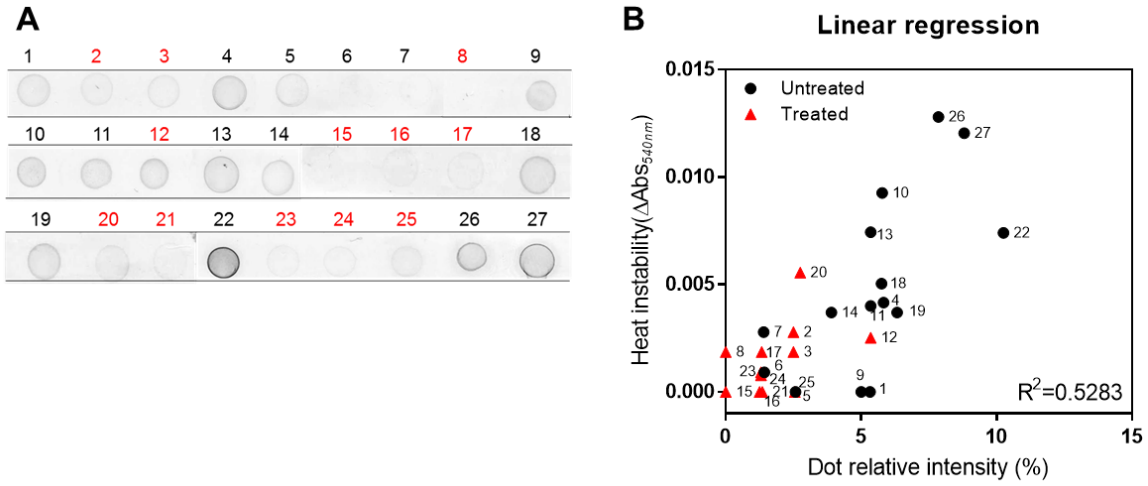

Fig. 4.1 - Comparison of methods to access protein instability in white wines. A) Dot blotting detection using both commercial antibodies against TLP and Chit; **B**) Linear regression between results obtained in both tests: heat test (instability A_{540nm}) and dot blot analysis (dot relative intensity (%).

These tests have exposed the existing low correlation between intensity of dots (unstable proteins) and haze formation after heat test (heat instability) (**Fig. 4.1B**). In fact, this information exposes the limitations of the heat tests to predict the existence of unstable proteins and reinforces empiric findings obtained at the wineries that suggest biased results caused by an accelerated temperature-induced oxidation and condensation between phenolic compounds and proteins (Sarmento *et al*. 2000). Indeed, these results are also consistent with other studies that demonstrated the interaction between haze-forming proteins and other wine components, affecting their denaturation, precipitation and consequently, the haze development (Hsu *et al*. 1987; E.J. Waters *et al*. 1995; Falconer *et al*. 2010). In particular, phenolic compounds, polysaccharides and metal ions (e.g. sulfate cations) might play an important role in protein stability and aggregation

mechanisms. Moreover, the haze phenomenon is known to be influenced by additional chemical and physical factors such as ethanol concentration, wine pH, protein molecular weight and protein pI (Waters *et al*. 2005). Altogether, these results revealed that the existence of TLP and CHT proteins could not be directly assessed by a haze estimation.

Optimization of the immunological detection of TLP – New VVTL1 antibody

Under the framework of the previous observations, and given the inexistence of a reliable method for detection of unstable proteins in white wines, we have developed a new immunological-based method for analysis of protein instability, which involved the development of a new VVTL1 antibody.

To optimize the immunological methodologies required for TLP-detection with the *in-house* developed antibody, four untreated white wines - not submitted to any clarification or stabilization treatment - were collected immediately after fermentation. Protein wine samples were subjected to SDS-PAGE analysis followed by Coomassie blue staining.

Results of the SDS-PAGE (**Fig. 4.2A**) indicated varied amounts of total protein between the wines and all samples presented two bands in common: a main band at 22-24 kDa (TLP) and a minor band with higher MW (~65 kDa). No CHT proteins were detected using SDS-PAGE (band ~32 kDa) (Le Bourse *et al.* 2011).

Western blots were then performed using the anti-*VVTL-1* antibody against TLP as a primary detection antibody at an optimal dilution of 1:2000. A band corresponding to TLP was detected, especially for wines 1, 2 and 4. Although barely visible using SDS-PAGE analysis, several smaller proteins were also present in wines 1 and 4, which might correspond to degradation products of the main TLP (Falconer *et al.* 2010). Variations on the TLP content might be likely caused by several factors such as grape varieties used, distinct winemaking processes, bottling and posterior storage conditions (Sarmento *et al.* 2000). Further, the same samples were applied on a PVDF membrane for dot blot detection, using different concentrations of primary antibody (1:4000; 1:2000; 1:500). A dilution 1:500 of *VVTL1* was required to detect the range of protein concentrations, previously observed by Western blot.

Using optimal concentrations, a positive correlation was found between the signal intensity obtained through both dot and western blotting techniques, revealing a consistency between the methods.

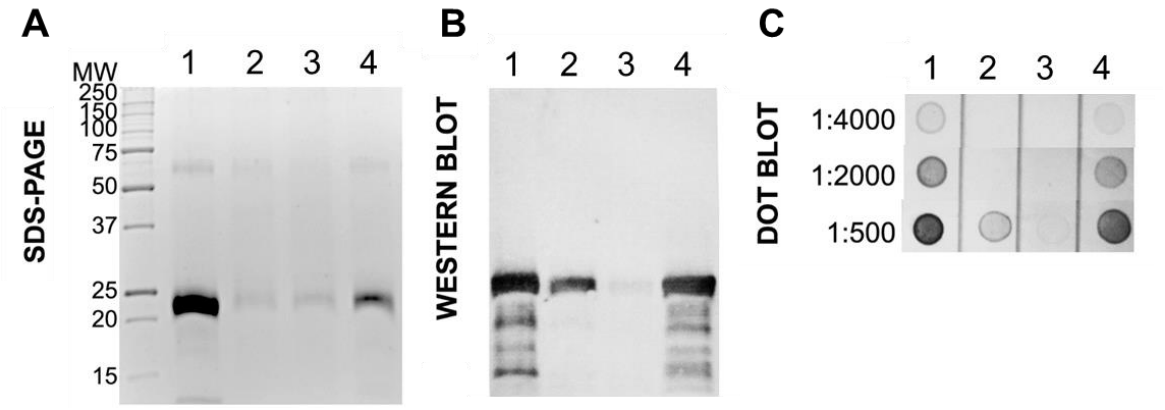

Fig. 4.2 - Optimization of Thaumatin-like proteins detection and comparison between methodologies. Four different untreated wines (1, 2, 3 and 4) were subjected to protein precipitation followed by protein detection analysis using the three different presented methods: **A**) SDS-PAGE; **B**) Western blotting and **C**) Dot blotting. Dot blot primary detection using antibody for VVTL-1 was optimized for different concentrations: 1:4000, 1:2000, 1:500 (with secondary antibody 1:5000 dilution to all tests).

Wine protein samples were applied on a PVDF membrane for dot blot detection, using different concentrations of primary antibody (1:4000; 1:2000; 1:500). A dilution 1:500 of *VVTL1* appears to be required to detect the range of protein concentrations, previously observed by Western blot. Using optimal concentrations, a positive correlation was found between the signal intensity obtained through both dot and western blotting techniques, revealing consistency between the methods.

These results demonstrate that its methodology could be easily transferable to a production environment through the usage of dot-blotting, which requires only simple equipment and faster steps, thus overcoming the technical requirements required to carry out Western-blots at the wineries.

Effect of increasing application dosages of bentonite in different white wine matrices

The previously optimized immunological-based methods were herein applied to assess the impact of increasing dosages of bentonite in different wine matrices and therefore, to understand their capacity to expose different protein removal profiles and efficiencies.

Two untreated wines were tested against one final bottled wine, which was previously treated with bentonite by the winemaker (unknown dosage). After receiving these three wines, increasing dosages of a commercial bentonite (0-100 g/L) were applied, simultaneously, to all the wines through laboratorial fining trials.

Protein analysis was then performed on all samples using the immunological methods. Again, total protein analysis revealed a major band at 22-24 kDa in both untreated wines, corresponding to TLP (**Fig. 4.3**). In contrast to the untreated wines, and as expected, no signal of these specific proteins was detected in the wine previously treated at the winery. Faint bands at ~65-70 kDa were also observed in all SDS-PAGE gels, probably corresponding to invertase proteins (Sauvage *et al.* 2010). Through this experiment, it was possible to demonstrate that the increased application of bentonite resulted in a progressive reduction of total protein in the untreated wines 1 and 2. These tendencies are also clear trough the measurement of the main band intensity in the SDS-PAGE gels, along the gradual increase of bentonite dosages (Graphs in **Fig. 4.3**). Indeed, the pattern of this reduction slightly varied between the two previously untreated wines. In untreated wine 1, protein loss seems to be more efficient as the main band was almost fully removed after addition of 40g /hL of bentonite (~85% removal).

Although, the untreated wine 2 required 60 g/L bentonite to achieve that removal percentage, 100 g/L of bentonite (maximum dosage normally applied in the sector) seems to efficiently remove a greater proportion of protein (~85-95%).

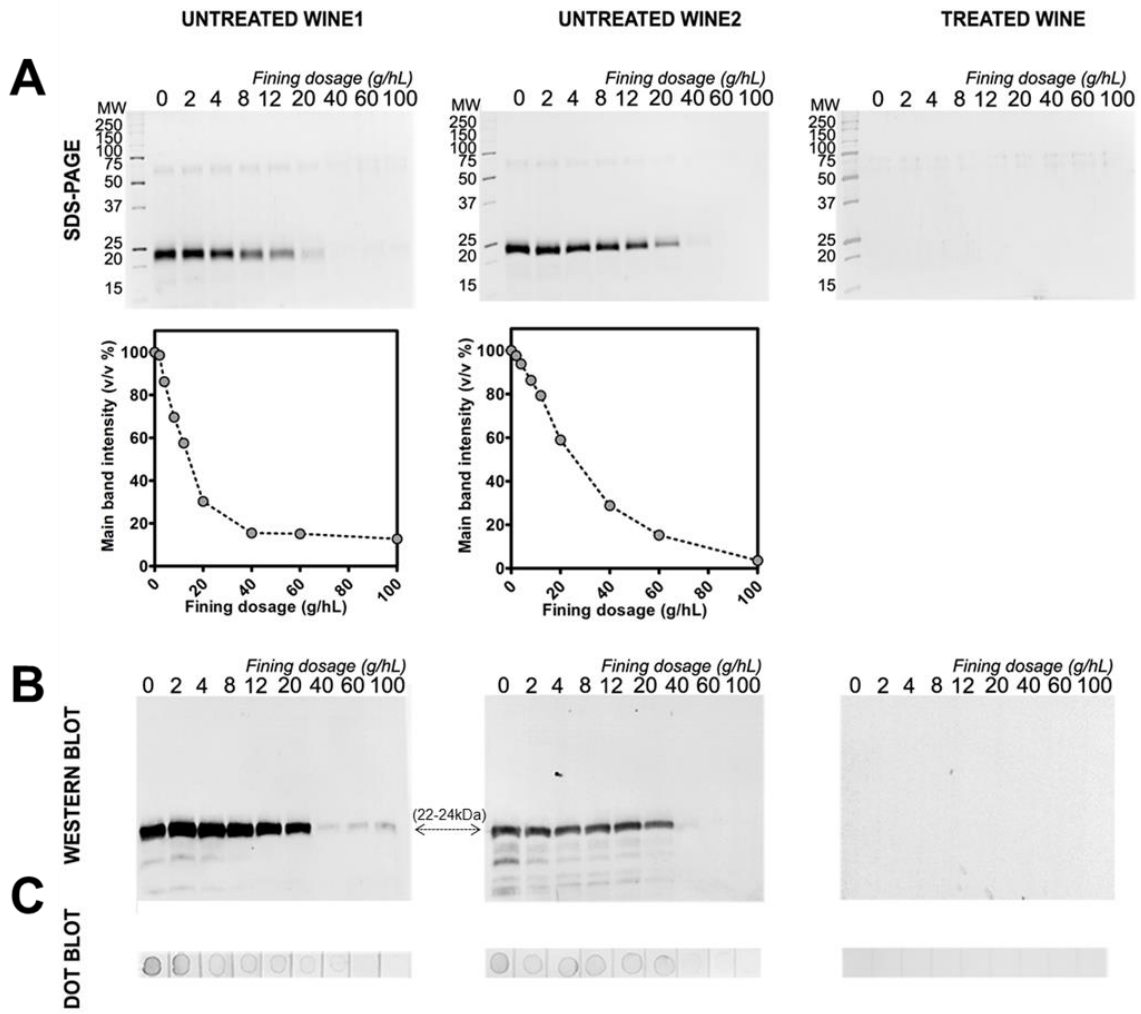

Fig. 4.3 - Detection of total protein and Thaumatin-like proteins after addition of different dosages of bentonite. A) Two untreated wines (1 and 2) and a Treated wine were fined with 0-100 g/hL of a commercial bentonite prior to analyses. **B)** SDS-PAGE gels were stained using Coomassie and bands intensities are respectively shown (intensity v/v%). Each band intensity was calculated in relation to the first band (0 g/hL). Due to the lack of bands in the Treated wine, no graph is presented for that experiment. **C)** Both Western and Dot blotting experiments were performed using the primary antibody against *VVTL-1*.

Western and dot blotting of the targeted TLP exposed that one of the wine matrices (untreated wine1) originally contained higher signal intensity for the main TLP and both revealed similar removal efficiencies after bentonite treatment of the untreated wines. Consistent with the previous results, it was also possible to detect TLP of lower molecular weights and the pattern and amount of these proteins/protein fractions also differed between the two untreated wines. No signal of unstable protein was detected in the treated wine.

Taken together, these results indicate that different wine matrices have different protein content and respectively, different precipitation behaviours after addition of bentonite. Indeed, these findings highlight the importance to implement immunological-based methods at the wineries that could more accurately follow-up the removal of unstable proteins when different application dosages of bentonite are applied in distinct wine matrices and/or under different production conditions (eg. temperature, light exposure, sedimentation period). Accordingly, our results demonstrated that these tools enable a more accurate assessment of the essential amount of bentonite required to eliminate the unstable proteins in the untreated wines.

4.4. Conclusions

Heat instability tests traditionally performed at the wineries are influenced by complex chemical and physical factors, leading to biased predictions on the amount of bentonite required to remove unstable proteins. In this study, we implemented specific immunological-based assays to specifically target this type of proteins in white wines. Indeed, a novel antibody was raised to target Vitis vinifera TLP, which causes major problems after denaturation in bottled wines.

Our results revealed that immunological-based assays allowed for an accurate and simple detection of these haze-forming proteins and consequently, providing useful tools to determine the minimum amount of bentonite to eliminate them, with evident efficiency and environmental gains. In particular, we believe that the dot-blotting approach could be easily transferred to the winery to facilitate the detection of TLP and CHT in a wide-range of samples and conditions in parallel, either from white wines or grape juices. Moreover, the protein removal efficacy of different treatments (bentonite clays) and amounts can be rapidly tested as a complement of each fining trials performed in the wineries, avoiding the application of over-estimated dosages at large scale.

Author Contributions

J.F. and R.C. performed the experiments; J.F, R.P. and A.C.G. designed the experiments; J.F. and A.C.G. wrote the paper.

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PART II

BENEFITS OF WINE MODERATE CONSUMPTION ON CONSUMERS' HEALTH

Chapter 5

PART II - General Introduction and Objectives

5.1. Benefits of wine polyphenols intake for consumers' health

The "French Paradox" was firstly perceived in the 90's, through the study of the Mediterranean culture, stating that wine moderate consumption plays a direct role on human's health (Renaud S *et al*. 1998). Indeed, Serge Renaud and his co-authors observed that despite the pronounced consumption of high saturated fats, the myocardial infarction rate among the French population was found to be 40% lower when compared to other European countries (Renaud S *et al*. 1998; Fernández-Mar *et al*. 2012; Katalinić *et al*. 2004; Schrieks *et al*. 2013). This inconsistency was then attributed to the regular and moderated red wine consumption (1 or 2 glasses of wine per day) registered in France, which was later found to induce cardiovascular protective effects (Katalinić *et al*. 2004; He *et al*. 2008). Since then, successive evidences confirmed the potential of wine moderate consumption in the prevention of cardiovascular and neurodegenerative diseases and certain types of cancer (Giacosa *et al*. 2014; Kuppusamy *et al*. 2014).

Although the health benefits of wine moderate consumption might be attributed to a wide-ranging variety of bioactive molecules, ranging from sugars, vitamins, minerals to volatile compounds (Mas *et al*. 2014), recent evidences support a fundamental role for wine polyphenols in cardio-protection, cognitive, anti-inflammatory, anti-carcinogenic, antiviral and antibacterial functions (Arranz *et al*. 2012a; Ružić *et al*. 2011; Cíz *et al*. 2008). Moreover, some polyphenols were shown not only to stimulate the growth of beneficial bacteria in gut, but also to inhibit the pathogenic ones (Dueñas *et al*. 2015).

5.1.1. Wine polyphenolic content

Polyphenols represent the most abundant, diverse and widespread group of secondary metabolites produced among the Plant kingdom and are recognized to be the 'archetype of bioactive phytochemicals' due to their recognized benefits for human health. (Lorrain *et al*. 2013). Contrarily to other alcoholic beverages, wine encloses a large variety of polyphenols, naturally originated from the skins, seeds

and stems of the white and red grapes - 4 g and 5.5 g per kg of bunches, respectively (Basha *et al*. 2004).

In the grapevine, these molecules not only play an active role in the primary metabolism and function, protecting the plant against biotic and abiotic stresses, but also contributing to its fertility and growth. Other biological functions include bactericide and antioxidant properties, which are of high value to the nutraceutical and pharmaceutical industries. (Ribéreau-Gayon *et al*. 2006; Lorrain *et al*. 2013). Depending on the structural arrangement of the molecules, phenolic compounds can be categorized as flavonoids or non-flavonoids (**Fig. 5.1**) (Cartea *et al*. 2011).

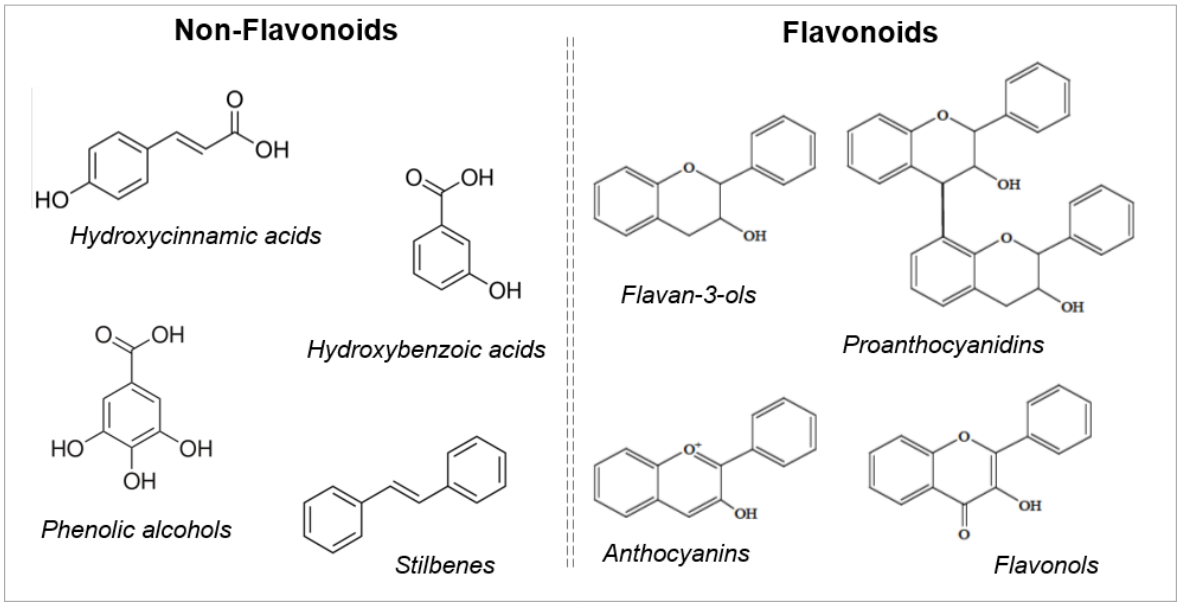

Fig. 5.1 - Principal polyphenols present in wine. Image adapted from Dueñas *et al*, 2015

- *Flavonoids* are the most concentrated natural antioxidants in the grape berry, including the sub-families of anthocyanins and proanthocyanidins (or 'condensed tannins'), which influence the wine colour and mouthfeel, respectively (Kennedy *et al*. 2002).

- *Non-flavonoids* include important stilbenoids, as resveratrol or piceatannol and phenolic acids as benzoic, caffeic and cinnamic acids (Moss *et al*. 2013; Zhu *et al*. 2007; Arranz *et al*. 2012). Indeed, hydroxycinnamic acids are the major phenolics in white wine and might be present in their free or esterified forms.

So far, the health benefits of wine moderate consumption have been mostly connected to flavonols (myricetin and quercetin), flavan-3-ols (catechin and epicatechin), proanthocyanidins, anthocyanins and phenolic acids (Giacosa *et al*. 2014). However, resveratrol, a stilbene largely present in the skins of red grapes is still being considered the "Golden Bullet" among the wine bioactive components. Resveratrol is known to stimulate cardio- and neuroprotection, antioxidant and anti-inflammatory responses (Athar *et al*. 2009; Moss *et al*. 2013; Beher *et al*. 2009; López-Vélez *et al*. 2003; Kovářová *et al*. 2010; Artero *et al*. 2015; Schrieks *et al*. 2013). In fact, its therapeutic potential was already exposed towards numerous illnesses, such as ischemia, cardiovascular diseases (CVD) and cancer (López-Vélez *et al*. 2003; Moss *et al*. 2013; Artero *et al*. 2015). These effects have been linked with biological functions as cell signalling, enzyme modulation, metal chelation and antioxidant potential (Rodrigo *et al*. 2011).

5.1.2. Variability factors: from grapevine to winemaking

The polyphenolic profile is markedly distinct among different wines. Overall, differences are dependent on several factors as varietal and clonal variability within *Vitis Vinifera* (Mitić *et al*. 2010; Katalinić *et al*. 2004). Moreover, the *terroir* and phenolic maturity of the grapes during harvest play a crucial role on the final physicochemical characteristics and polyphenolic profiles of wines (Liu *et al*. 2016). Importantly, during the crushing of the grapes at the winery, oenological processes or reactions occurring during aging and storage, polyphenols are prone to structure change due to winemaking conditions related to temperature, pH, SO_2, ethanol and possible application of protein fining agents.

Despite their metabolic variety, red wines contain approximately 10-fold more total polyphenols (~2g/L) than white wines (0.2-0.3g/L) due to a superior polyphenolic content in red grapes, which is leveraged by the maceration with the grape skins, a process that can last for several weeks (Arranz *et al*. 2012). Anthocyanins, flavonols, monomeric and phenolic acids are the most concentrated polyphenols in red wines (**Fig. 5.2**). Conversely, though having a much lower total polyphenolic content, white wines contain higher amounts of specific polyphenols, such as hydroxycinnamic acids, tyrosol and hydroxytyrosol (Fernández-Mar *et al*. 2012; Giacosa *et al*. 2014). However, white wines can bear increased concentrations of polyphenols by optimizing oenological practices as the pre-fermentative maceration to extract more phenols from the grape seeds, skin and stalks (Hernanz *et al*. 2007; Ružić *et al*. 2011).

Wines with higher polyphenolic concentrations have been demonstrated to have increased antioxidant activities in humans and each polyphenol has been shown to have different antioxidant capacities (Venturini *et al*. 2010). Moreover, studies have unveiled synergistic reactions within complex matrices of polyphenols (Pignatelli *et al*. 2000). These findings highlight the importance to further explore the antioxidant benefits of different wine polyphenols and respective mixtures.

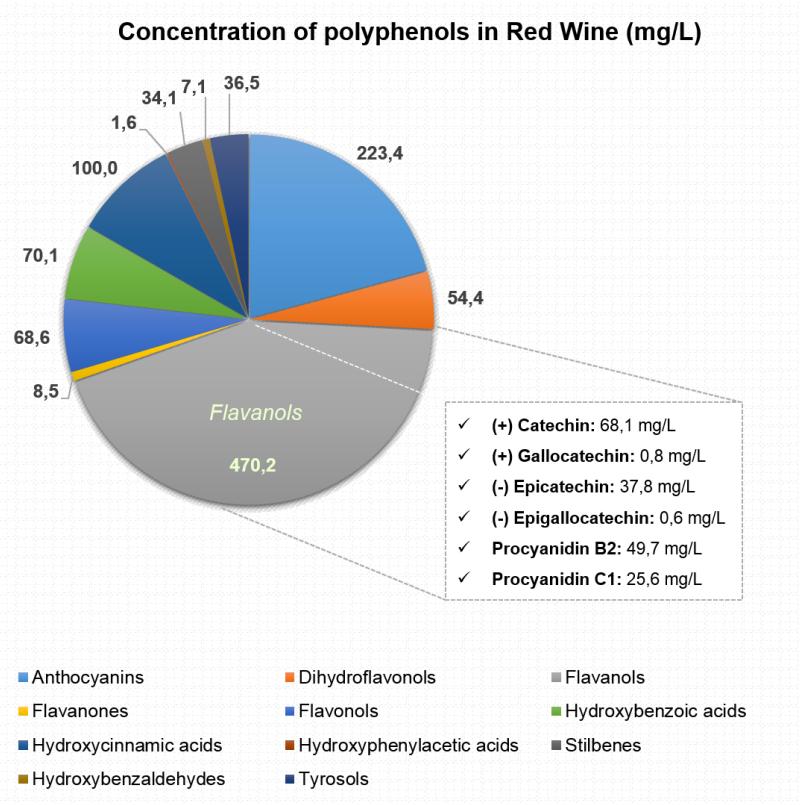

Fig. 5.2 - Diagram showing the concentration (average of polyphenols in red wine). Concentration values obtained from Arranz, *et al*, Nutrients (2012).

5.1.3. Polyphenolic profiling

Modern analytical techniques provide rapid, accurate and reliable methods for the analysis and identification of these biomolecules (Zhu *et al*. 2007). Due to its high sensitivity, mass spectrometry (MS) has been the most widely used tool for both targeted and non-targeted approaches and it can be performed either alone or coupled with liquid chromatography (LC-MS).

In particular, these advanced methodologies have been used for wine authentication and to unveil the terroir impact on wine molecular composition (Rubert *et al*. 2014; Roullier-Gall, Boutegrabet, *et al*. 2014; Forcisi *et al*. 2013; Tarr *et al*. 2013). Indeed, those are useful tools to quantify important monomeric stilbenoids as resveratrol, piceatannol, and their glycosides, piceid and astringin (Moss *et al*. 2013). It is also

possible to unravel the structure of these molecules using tandem mass spectrometry (MS/MS) or multi-stage mass spectrometry (MSn) (Zhu *et al*. 2007). Furthermore, electrospray ionization mass spectrometry (ESI-MS) coupled with HPLC has also been increasingly used to identify and characterize anthocyanins, flavonols, proanthocyanidins (PAs), pyranoanthocyanins and phenolic acids (Lopes-Lutz *et al*. 2010).

Modern holistic approaches require highly sensitive and powerful tools (Roullier-Gall, Witting, *et al*. 2014) and it is still recognized that no method can describe the whole wine metabolome by itself (Delcambre & Saucier 2013). Novel approaches are currently being applied in order to give a rapid access to each wine polyphenol fingerprint and a novel extension of systems biology was recently proposed: 'Polyphenomics'. As reported by Saucier *et al*. (2013), the data obtained by this non-targeted high-throughput methodology is not only highly reproducible, but also provides more information than previous methods, such as direct injection-ESI-MS.

In future, novel tools are expected to be developed to provide more significant data about the different metabolites existing in grapes and wine, followed by reduced requirements in terms of sample preparation or analysis time. Altogether, these new approaches should not only help improving the winemaking practices, but also to better characterize human diets and link wine signatures to health benefits and potential biomarkers (Kay 2010; Engskog *et al*. 2016).

5.2. Antioxidant capacity

The antioxidant activity of a molecule is related with its capacity to react with reactive oxygen species (ROS) or to interfere with ROS production (Artero *et al*. 2015). Previous studies have indicated that resveratrol and other polyphenols act like ROS-scavengers, by preventing the oxidation of low density lipoprotein Cholesterol (LDL) *in vitro* and, consequently, retarding the development of atherosclerosis and coronary heart disease (CHD) (De Whalley *et al*. 1990; Osada *et al*. 2001).

In particular, proanthocyanidins were referred to have 20 and 50 times greater antioxidant potential than Vitamin E and C, respectively (Rodrigo *et al*. 2011). Moreover, model solutions of three flavonoids and resveratrol have been proved to promote the decrease of ROS production in a culture of murine macrophages (Cíz *et al*. 2008). The same study has suggested that the number of hydroxyl groups existing on each molecule is probably correlated with the different levels of scavenging activity. Indeed, in a human study, polyphenols were proved to act synergistically with each other and consequently, benefits are surely dependent on the specific composition of each wine (Pignatelli *et al*. 2006).

Several studies have been focused in differentiating wines as regards to total and specific polyphenolic composition and antioxidant capacity (Andrade *et al*. 2001; Peña-Neira *et al*. 2000; Mitić *et al*. 2010; De Beer *et al*. 2003; Ivanova-Petropulos *et al*. 2015). Actually, various *in vitro* methods might be used to evaluate the antioxidant ability of different wines (Zulueta *et al*. 2009; Re *et al*. 1999; Shimamura *et al*. 2014). These approaches might consist in simple scavenging assays, allowing a rapid high-throughput screening of isolated compounds or mixtures of polyphenols or even wines (Muselík *et al*. 2007). Amongst others, the 2,2´-azinobis-(3-ethyl-benzothiazoline-6-sulphonate (ABTS•+) cation radical method and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (**Fig. 5.3**) evaluate the capture of free radicals via spectrophotometry (De Beer *et al*. 2003; Liang & Kitts 2014).

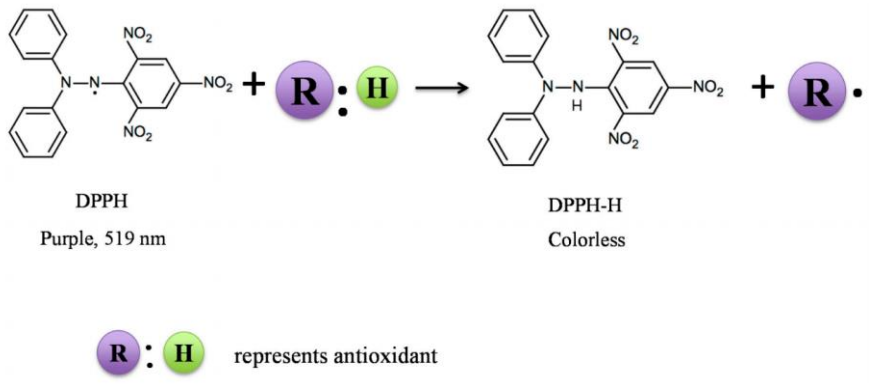

Fig. 5.3 - Scavenging reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. R:H - Antioxidant radical scavenger; R - Antioxidant radical (Liang & Kitts 2014).

Cellular models for oxidative stress and antioxidation protection

Animal models and human clinical studies are highly expensive and not suitable to perform initial antioxidant potential screening of bioactive compounds or mixtures. Therefore, cell culture models are promising tools to access the bioactivity of several antioxidants at wide concentration ranges.

The selection of a specific cellular model is critical to the success of the bioassay. The properties and sensitivity of the cells and their growth status are critical factors that affect antioxidant assessment, but also the lack of cytotoxic effect and a realistic concentration range in human diet and serum. Over the last decades, various types of cancer cell lines from lung, neurons, liver, colon and breast have been used to set up cell-based bioassays for assessing antioxidants activity of foods and dietary supplements. Nevertheless, none of those can mimic an aging ROS-generating environment *per se*. In this study, we have assessed antioxidant effects of wine polyphenols and complex mixtures by using Hutchinson-Gilford Progeria Syndrome (HGPS) vascular cells, which have been recently acknowledged as a reliable aging model (Brassard *et al*. 2016; Pitrez *et al*. 2015).

Hutchinson Gilford Progeria Syndrome (HGPS)

HGPS is a rare, genetically-encoded condition that causes premature aging in children, as at the age of 18-24 months they exhibit characteristics associated with accelerated aging. Phenotypic effects include body damage feature, such as hair lost, low body fat, bone density reduction, alopecia and weak muscle development (Pitrez *et al*. 2015).

Recent studies have demonstrated that progeroid cells suffer from a persistent attack of macromolecule-damaging reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\bullet}$) (Singh & Jialal 2006; Viteri *et al*. 2010; Seco-Cervera *et al*. 2014; Richards *et al*. 2011). The reactive oxygen species (ROS) damage is highly connected with chronic degenerative diseases (Pelicano *et al*. 2004; Chen & Keaney 2012) and could be

formed either by enzymatic or non-enzymatic pathways in mammalian cells (Kamenisch & Berneburg 2009). In general, those highly reactive species cause oxidation of amino acid chains, formation of protein-protein cross-linkages and protein fragmentation. Importantly, the accumulated DNA damages induced by ROS are normally unrepairable causing mutations, chromosomal translocations, or genetic information loss (Richards *et al*. 2011).

Progeria is described as one of the most severe degenerative disorders from the laminopathies group, which includes rare genetic disorders caused by mutations in genes encoding proteins of the nuclear lamina (Richards *et al*. 2011). In particular, this disease is caused by a single mutation in the *LMNA* gene (1824→T) (Viteri *et al*. 2010) that encodes the intermediate filament protein, lamin A. This *de novo* mutation causes alternative splicing and as result, a truncated form of this protein is produced, termed prelamin A or progerin (Ghosh *et al*. 2013). Normal lamin A is a major component of the nuclear matrix (NM) in differentiated cells (Richards *et al*. 2011; Ghosh *et al*. 2013) and it is important to maintain the nuclear structure through nuclear support and chromatin organization (Viteri *et al*. 2010). Previous *in vivo* and *in vitro* studies proved that lamin A directly binds and activates the human sirtuin1 (SIRT1) (Ghosh *et al*. 2013; Liu *et al*. 2012). SIRT1 is an important deacetylase, since it regulates several vital metabolic pathways (Liu *et al*. 2012) and functions such as genomic stability, inflammatory response, stress regulation and tumour suppression. Contrarily, the interaction between STIR1 and the dysfunctional prelamin A, (progeroid cells), is highly compromised, leading to a rapid cellular degeneration (Liu *et al*. 2012). As result, cells become more prone to senescence and neoplastic transformation (Seco-Cervera *et al*. 2014).

Treatments of Progeria cells using ROS-scavengers have been recently proposed (Richards *et al*. 2011; Liu *et al*. 2012). Results indicated that a standard antioxidant, N-acetyl cysteine (NAC), was able to reduce the levels of DSB and improve cell grow (Richards *et al*. 2011). Further, a study from Liu *et al* (2012) demonstrated that Resveratrol (3,5,4'-trihydroxystilbene) improved the binding between prelamin A and SIRT1, enabling its proper localization in the NM and consequently, enhancing its activation (Liu *et al*. 2012). Indeed, it has also been suggested that the resveratrol

treatment might contribute to stress resistance, consequently ameliorating progeroid symptoms and life span in mice (Ghosh *et al*. 2013; Liu *et al*. 2012; Demidenko & Blagosklonny 2009). Still, the efficiency of both molecules is limited by their toxicity at high concentrations (Demidenko & Blagosklonny 2009) and thus, it is important to study the impact of a large range of drugs, dosages or even different treatments combinations and therapeutic strategies.

5.3. Anti-carcinogenic effects

Cancer is one of the leading cause of death worldwide (Sancho & Mach 2015) and it may occur when damaged cells fail to undergo apoptosis and proliferation or when the replaced cell is also mutated and proliferates. Both cases result in a cellular unbalance, as mutated cells proliferate at a higher rate than apoptosis.

Recent studies demonstrated that wine moderate consumption may have beneficial effects on cancer prevention and treatment. These effects have been mainly associated with the wine content in polyphenols (Sancho & Mach 2015; Arranz *et al*. 2012b; Angel-Morales *et al*. 2012; Mazué *et al*. 2014). Indeed, several authors demonstrated that flavonoids such as resveratrol present anti-carcinogenic effects, due to their ability to interfere with the multi-step process of carcinogenesis (Slamenova & Horvathova 2013; Athar *et al*. 2009; Giovannelli *et al*. 2000). Several anti-carcinogenic activities have been previously linked to cell signal modulation, control of cell cycle proteins as cyclin B1 and D1, and progress along the cell cycle (Gómez-Alonso *et al*. 2012). The consumption of flavonoids has been widely recognized to protect against chemically induced colon cancer, in cell and animal models (Alshehri & Elsayed 2012; Lambert & Yang 2003; Zaveri 2006). Further, phenolic acids as benzoic, caffeic and gallic acids have been related to anti-carcinogenic activities in prostate, breast, colon, renal and thyroid cancer cells (Sahpazidou *et al*. 2014a).

Grape stems extracts (Sahpazidou *et al*. 2014a), grape extracts (Shrotriya *et al*. 2012), wine extracts (Briviba *et al*. 2002; Gómez-Alonso *et al*. 2012; Luceri *et al*. 2002) and wine compounds (Gómez-Alonso *et al*. 2012) have been reported to

inhibit human cancer cell growth. In fact, a recent study identified an inhibitory activity of grape stem extracts against growth of colon, breast, renal and thyroid cancer cells (Sahpazidou *et al*. 2014a). In particular, quercetin and kaempferol are referred to inhibited A549 cell proliferation derived from a lung carcinoma by activating an extracellular signal-regulated kinase (Hertzog & Tica 2012). On the other side, a study suggests that catechin and some of its metabolized products have cancer chemo-preventive effects by inhibiting cyclooxygenases (COX-1 and COX-2) activities (Seeram *et al*. 2003).

Although these results suggest that polyphenols or grape/wine extracts might act as promising chemo-preventive and/or chemotherapeutic agents, some authors state that there is no statistically significant association between flavonoids intake and bladder (Neuhouser 2004) or breast cancers risk (Kilkkinen *et al*. 2004). Besides, other denote inconsistent results derived from a genetic basis (McCann *et al*. 2002). Concerning this contradiction and the current lack of clinical randomized trials, more studies are needed to confirm these benefits for each specific type of cancer.

Human studies to assess anti-carcinogenic effects

Human interventional studies are critical research tools to assess the definite anti-carcinogenic effects of different molecules and mixtures in the human body. Nevertheless, they are currently scarce and highly restricted due to short intervention periods, low number of individuals and sampling points. Furthermore, human interventions performed thus far consist in single dose experiments that only investigate the compound absorption rate after 4 to 24 h of consumption (Boocock *et al*. 2007; Yiu *et al*. 2015). Indeed, clinical trials of longer intervention periods mostly focus the clinical benefits after intake, not confirming the existence of their metabolites in plasma (Fujitaka *et al*. 2011; Tomé-Carneiro *et al*. 2012; Bhatt *et al*. 2012).

For these reasons, a larger number of human studies are required to support the current cancer research, particularly concerning the most aggressive forms of the

disease, such as colon cancer - one of the most dangerous types of cancer in the world due to a poor response to the currently available chemotherapeutic agents.

Colorectal cancer

Although its early development in the colon/rectum, this cancer type has the capacity to spread to other gastrointestinal organs, liver, lung and ovaries (Kim *et al*. 2006). Most colorectal cancers result from older age and lifestyle factors, but also from some genetic disorders.

Whereas excessive alcohol consumption, obesity, a high-calorie diet, and a lack of physical activity also promote colon cancer, several nutraceuticals have potentially shown benefits for its prevention and treatment and their classes and uses are presented in **Fig 5.4**. In fact, previous evidences have indicated that a human diet rich in folates, selenium, Vitamin D, dietary fibre, garlic, milk, calcium, spices, fruits and vegetables is protective against this type of cancer (Aggarwal *et al*. 2013).

In particular, the plant-derived nutraceuticals, such as polyphenols from fruits and vegetables, have been previously connected to a significant decline of colon rectal cancer growth and presented additional benefits of improving overall human health with fewer adverse effects, when compared to the other types of treatments (Kuppusamy *et al*. 2014). Despite this, some authors still consider that the colonic effects originated from polyphenolic intake are much less studied than the ones promoted by other compounds, such as carbohydrates or proteins (Serra *et al*. 2012; Kuppusamy *et al*. 2014). Nevertheless, important chemopreventive effects of dietary polyphenols in colorectal cancer was previously reported by using cancer cell lines. In particular, quercetin has been shown to inhibit proliferation, induce apoptosis and cell-cycle arrest in colon cancer cells (Shan *et al*. 2009). Later, other authors confirmed that flavonols are able to inhibit colon adenocarcinoma cell proliferation through the blocking of G2/M cell cycle (Gómez-Alonso *et al*. 2012).

Epidemiological studies revealed that the specific administration of *trans*-resveratrol might contribute to the prevention of colon carcinogenesis (Juan *et al*. 2012;

González-Sarrías *et al*. 2011). Previous studies support this evidence, showing that the wine phenolic compounds could reduce the incidence of colorectal cancer (Arranz *et al*. 2012; Sancho & Mach 2014; Sahpazidou *et al*. 2014).

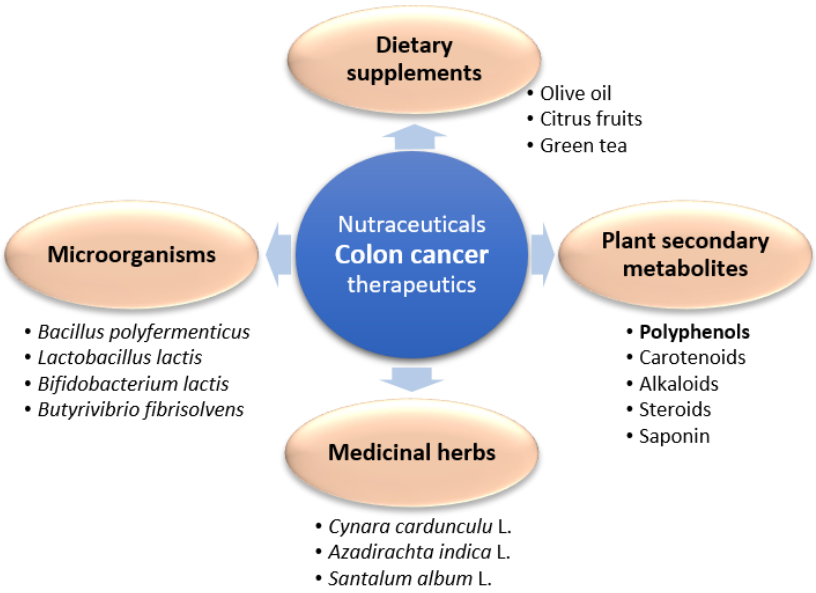

Fig. 5.4 - Different nutraceuticals used in colon cancer therapeutics (image adapted from Kuppusamy *et al* 2014).

5.4. Bioavailability and metabolism

Biological effects of polyphenols are permanently dependent on their bioavailability, a factor that highly diverges among these bioactive molecules. Still, limited information exists about the pharmacokinetics of polyphenols in humans.

In previous epidemiological experiments, some polyphenols, such as procyanidins, quercetin and flavanols have proved to be successfully absorbed into plasma through a passive transport process across membranes (Rodrigo *et al*. 2011). Still, this absorption was found to be dependent on a previous hydrolysis of the molecules by intestinal enzymes or colonic microflora, followed by an extensive modification pathway (**Fig. 5.5**). Other studies in humans and rats revealed that even though

resveratrol is highly absorb into the human blood, its molecules have poor bioavailability, water solubility and are chemical unstable (Walle *et al*. 2004; Wenzel *et al*. 2005; Neves *et al*. 2013), which suggests that health benefits might be more associated with the whole pool of antioxidants existing in wine. Hence, other authors verified a high inter-individual variability of *trans*-resveratrol glucuronides in human serum, rather than its free form (Vitaglione *et al*. 2005a). Indeed, there is a clear need to test these metabolites in *ex vivo* and *in vitro* model systems rather than just the native compounds that occur in food or plant extracts (Ottaviani *et al.* 2016).

Altogether, while these polyphenolic molecules successfully enter in the blood, the underlying mechanisms are still not fully understood among the scientific community and more information on bio-distribution and metabolism of wine polyphenols is still needed (Cíz *et al*. 2008).

Indeed, current research is focused in discovering analog molecules of polyphenols, as methylated and glucoside derivatives of resveratrol that could have an improved bioavailability and thus, be useful for medicine and pharmaceutical purposes (Walle 2011; Choi *et al*. 2014). Further, large-scale biotechnological approaches have been developed to produce such molecules, as opposed to chemical synthesis or plant extraction (Khanduja & Bhardwaj 2003; Kang *et al*. 2014; Choi *et al*. 2011). Also, nano-delivery systems are being investigated as conceivable carriers to enhance oral administration of resveratrol molecules (Neves *et al*. 2013).

The ethanol content in wine is also described as an important feature for this research field. In general, patterns of regular moderate consumption are associated with lower risk of diseases, while episodic heavy consumption contradicts any beneficial health effect (Conigrave *et al*. 2001; Murray *et al*. 2002; Wharton & Harris 2007; Ruidavets *et al*. 2010). These patterns confirm the J-shaped association between alcoholic beverages consumption and risk of diseases, such as dementia, obesity, cardiovascular diseases, and/or risk of mortality. In particular, the moderate ethanol intake might benefit the absorption of quercetin and its 3-O-glucoside (Dragoni *et al*. 2006) and it seems to indirectly contribute to the antioxidant capacity of wine by increasing the polyphenols bioavailability (Serafini *et al*. 1997).

Additionally, while dealcoholized or low-alcohol wines might have significant antioxidant effects in humans, the results seem to be inferior to the ones achieved using wines with higher alcohol levels (Guilford & Pezzuto 2011). Contrary to these outcomes, a human intervention study described that a 4 weeks consumption of red wine, increases the plasma levels of a oxidative lipid damage marker, isoprostane 8-iso-PGF2α, when compared with the dealcoholized red wine (Schrieks *et al*. 2013).

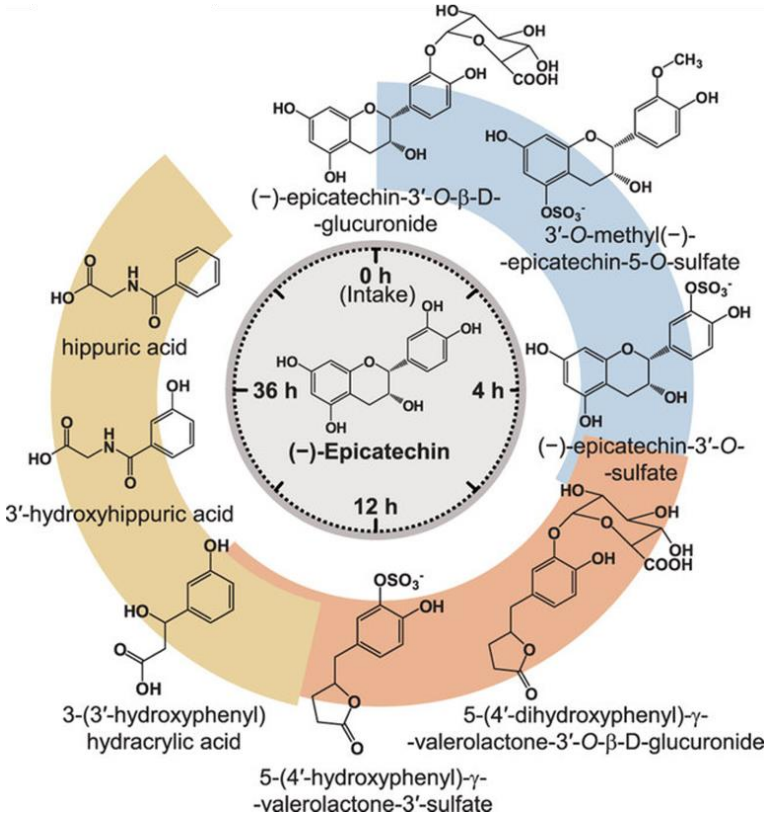

Fig. 5.5 – Representation of the main epicatechin metabolites existing in plasma after oral human intake of red wine. Adapted from Ottaviani *et al.* 2016.

On the other side, the matrix also plays a key role in bioavailability. *trans*-resveratrol seem to be better absorbed from red wine or juice than from grape extract tablets (Stockly *et al*. 2012). Indeed, parameters such as pH, intestinal fermentations, biliary excretion and transit time have consequences for the polyphenols absorption. Also, enzymes and carriers involved in the absorption and metabolism may affect the bioavailability of such molecules.

Human studies focused on the bioavailability of the resveratrol are still scarce and in most of them it was administrated in the form of tablets, capsules or caplets (Brown *et al*. 2010; David J. Boocock *et al*. 2007; David J Boocock *et al*. 2007), which raises the need to further study bioavailability of resveratrol and its metabolites after intake.

5.5. Objectives

Main objective

In the Part II of this thesis, the main objective was to understand the potential health benefits of individual polyphenols, polyphenolic mixtures and wines through the application of innovative methodologies and strategies.

To achieve the main objective of Part II of this thesis, the following specific objectives were defined:

a) to evaluate the antioxidant potential of different polyphenols and polyphenolic complex mixtures;

b) to explore the bioavailability of potentially important anti-carcinogenic polyphenols;

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Chapter 6

Wines and individual polyphenols promote antioxidant benefits on a human iPSC model of Hutchinson Gilford Progeria

Wines and individual polyphenols promote antioxidant benefits on a human iPSC model of Hutchinson Gilford Progeria

This Chapter aims at fulfiling the specific objective of this thesis “to evaluate the antioxidant potential of different polyphenols and polyphenolic complex mixtures, by using a novel model based in Progeria cell lines, which originally have high levels of ROS production”, whose results were submitted in “Scientific Reports - Nature”, an international reviewed journal, from the Nature group.

Abstract

Red wine moderate consumption has been reported to prevent age-related diseases, including Alzheimer and dementia. A variety of different wine polyphenols has previously demonstrated antioxidant benefits against the DNA damage caused by reactive oxygen species that cause these aging conditions. Still, their individual effects and possible synergies in complex mixtures are poorly understood.

In this work, the cellular impact of twenty-two red wines and individual polyphenols was assessed in a cell model for oxidative stress, based on human Hutchinson-Gilford Progeria Syndrome (HGPS) cells. Indeed, HGPS cells mimic an aging environment, rich in reactive oxygen species (ROS). In parallel, the physicochemical properties and polyphenolic composition of the different wines were also evaluated.

Results indicate significant impacts as regards to cell proliferation and oxidative stress reduction, after cellular exposure to red wines and polyphenolic solutions (0.05% (v/v)). Among all the polyphenols measured in the wines, procyanidin B and (-)-epicatechin revealed the greater impact on the cell growth of the progeroid cells. Further, most of the red wines promoted greater levels of stress reduction, when compared to the effect of individual polyphenolic compounds, thus unveiling potentially important synergic effects within the components of the wine complex chemical mixture.

Overall, this study clearly demonstrates that red wines and individual polyphenols present strong antioxidant benefits on human cell lines with aging potential, stressing out the importance to further explore them to develop new strategies to fight against aging-associated conditions.

Keywords

Antioxidant potential, Hutchinson-Gilford Progeria Syndrome (HGPS), oxidative stress, polyphenols, radical oxidative species, red wines, smooth muscle cells (SMCs).

6.1. Introduction

Since the acceptance of the French paradox, it has been known that wine moderate consumption – 1/2 standard glasses of wine per day - provides health benefits to humans. Both epidemiological and laboratory research studies confirmed benefits associate with cardio-protection, anti-inflammatory, anti-carcinogenic and antiviral effects (Arranz *et al*. 2012; Ružić *et al*. 2011; Cíz *et al*. 2008). Moreover, others reported that moderate red wine drinking promotes benefits for the brain and nervous system, causing a delay in aging symptoms associated to Alzheimer's disease and dementia (Rege *et al*. 2014; da Luz *et al*. 2012).

Red wines normally contain 2.0 to 6.0 g/L of total polyphenols (Quideau *et al*. 2011), 10-fold more than white wines (Pignatelli *et al*. 2006), which is explained by the presence of anthocyanins (pigments) and higher quantities of catechin compounds extracted during the maceration steps. Polyphenols have been reported to exhibit antioxidant activity through various endogenous and exogenous mechanisms (Obrenovich *et al*. 2010). In particular, polyphenolic compounds such as (+)-catechins, (-)-epicatechins or stilbenes are widely recognized for preventing the incidence of pathological disorders associated with DNA damage caused by reactive oxygen species (ROS) (Singh & Jialal 2006; Viteri *et al*. 2010; Seco-Cervera *et al*. 2014; Richards *et al*. 2011; Liu *et al*. 2012; Villaño *et al*. 2005). The main ROS species include superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$).

Importantly, different wines have different metabolomic fingerprints and, consequently, polyphenolic compositions, leading to different health effects in humans (Gruz *et al*. 2008; Delcambre & Saucier 2013; Katalinić *et al*. 2004). This variability is explained by several features: (i) the nature of the grapes (soil, grape varieties, appellation and climate); (ii) the vinification techniques and (iii) the wine evolution during storage (Mitić *et al*. 2010; Andrade *et al*. 2001; Zafrilla *et al*. 2003). Indeed, previous authors have demonstrated a high metabolic variability between different *Vitis vinifera* varieties, particularly as regards to polyphenols and volatile compounds (Perestrelo *et al*. 2014; Câmara *et al*. 2004; de la Cerda-Carrasco *et al*.

2014). This is of high importance and value, as world-wide more than 10000 grape varieties are known, which exposes a vast array of possible wine metabolic compositions (This *et al*. 2006).

Wine is composed by ~80-85% of water, ~9-15% ethanol, and about only 3% of it corresponds to its metabolite fraction, which may be of more than 1000 different molecules (Revi *et al*. 2012). Given its chemical nature, wine can be regarded as a matrix of high complexity, where it is important to understand possible synergistic or antagonistic interactions between the different polyphenols or between polyphenols and other molecules, such as volatiles, organic acids, sugars or proteins (Kurin *et al*. 2012; Pignatelli *et al*. 2000). Such complexity, already present in monovarietal wines, is specially leverage in blended wines, from regions where it is a common practice to mix different grape varieties, thus increasing the metabolic complexity within wines and the probability of synergistic and antagonistic events to occur at unknown levels.

However, a bottleneck for the research of this topic is the existing experimental methods as animal and human assays present several concerns and limitations. Indeed, it is important to stablish simple and reliable methods to measure antioxidant activities *in vitro*. Chemical methodologies have been commonly applied to measure the total antioxidant potential of different compounds or mixtures *in vitro* (e.g. 1,1 –diphenyl-2-picrylhydrazyl (DPPH)) by estimating their capacity to act as a free-radical scavenger in a ROS-rich medium (Van Den Berg *et al*. 1999; Ružić *et al*. 2011; Villaño *et al*. 2005). Previous studies have applied these methods to estimate and compare the antioxidant capacity of different chemical compounds (Villaño *et al*. 2005); different wines matrices obtained from different grape varieties (Mitić *et al*. 2010); and obtained by different oenological techniques Cellular *in vitro* approaches using rat and human cell cultures have also been widely used to expose potential cellular impacts of wine. Indeed, these systems are closer to *in vivo* natural systems, contributing to a more accessible acquisition of knowledge So far, research on the cellular impact of wines has been focused on neuronal and cancer cell lines, such as prostate, breast, lung, colon rectal (Venturini *et al*. 2010; Gómez-Alonso *et al*. 2012; Wallenborg *et al*. 2009; Leifert & Abeywardena 2008). All these

cell lines are suitable models for degenerative and age-related diseases, but cannot be regarded as aging models *per se*.

Hutchinson-Gilford Progeria Syndrome (HGPS) vascular cells have been acknowledged as a reliable aging model (Brassard *et al*. 2016; Pitrez *et al*. 2015). HGPS is a rare, genetically-encoded condition that causes early-onset premature aging in children, whose origin remains to be fully understood, but evidences point towards the involvement of factors as reduced DNA-damage-repair response; progerin-mediated stem cell pool exhaustion; mesenchymal lineage differentiation defects and nuclear fragility (Zhang *et al.* 2011; McClintock *et al.* 2006). As result, higher levels of free reactive oxygen species (ROS) are produced, causing an excessive accumulation of DNA damages in the cells (Pitrez *et al*. 2015; Chi *et al*. 2009). To alleviate progeroid symptoms derived from Hutchinson-Gilford Progeria Syndrome, antioxidant treatments such as resveratrol and N-acetyl cysteine (NAC) have been suggested in previous reports (Richards *et al*. 2011; Liu *et al*. 2012).

Herein, we have used HGPS cell cultures as cellular models to mimic a unique ROS-generating environment and to test the potential ROS-scavengers among the different red wines, complex polyphenolic matrices and individual polyphenols as regards to their (i) direct effect on cells (*Direct test*); (ii) their ability to rescue cells from an additional oxidative stress (*Rescue test*); and (iii) potential synergistic effects between polyphenols present on different wine matrices.

6.2. Material and Methods

6.2.1. Reagents and solutions

Acetonitrile (ACN), methanol (MeOH) and water (LC-MS Grade) were obtained from Fisher Chemical. Formic acid (FA) was obtained from Amresco®. The internal standards (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, procyanidin B2, procyanidin C1, *trans*-caffeic acid and *trans*-resveratrol-(4-hydroxyphenyl-13C6) (purity ≥ 98%) were purchased from Sigma-Aldrich®. Standard stock solutions used for LC-MS/MS analysis were prepared in MeOH except for Ellagic acid that was prepared in ethanol.

All solutions were stored at -20°C until used. In each working day, freshly diluted calibration standard solutions were prepared by diluting the stock solutions in 2% ACN: 0.1% FA. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), tert-Butyl hydroperoxide (TBHP) and 37% Formaldehyde solution were purchased from Sigma (St. Louis MO). DAPI (4′,6-diamidino-2-phenylindole) and CellROX® Deep Red Probe were acquired from Thermo Fisher Scientific (Waltham, Massachusetts, EUA) and the cells Medium (Bullet kit SMGM-2) from Lonza (Basel, Switzerland).

6.2.2. Individual polyphenol solutions

Preparation of solutions

Single stock solutions of (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, procyanidin B2, procyanidin C1, *trans*-caffeic acid and *trans*-resveratrol were prepared in absolute ethanol and stored at -80 ºC. The respective dilutions of the individual polyphenols and the polyphenolic mix (*Poli mix*) were freshly prepared using a hydroalcoholic solution (13% ethanol). Final concentrations were stablished as the average concentration of these compounds in red wines, according to the mean value of bibliographic data from Phenol-Explorer Database, Version 1.5.7 (Arranz *et al*. 2012). A polyphenolic mixture (*Poli mix*) containing the referred polyphenols at their respective average concentrations was prepared.

6.2.3. Wines preparation

Selection

Wine selection was performed to include a high heterogeneity of wines. The final wine samples included: (i) different grape varieties, including of national and international origin (ii) mono-varieties and blends; (iii) distinct appellations; (iv) different producers; and (v) different price ranges (from 2 to 50€). All wines were certified by their *Denomination of Controlled Origin (DOC)* and their complete list is presented in ***Annex VI*** as Table S6.2.

Storage

All tested wines were received in closed glass bottles of 750 mL. Each wine was homogenized and divided into both 500 µL and 15 mL aliquots and stored at -80 ºC until used. To avoid repetitive thawing and freezing, a new aliquot was always used for each experiment. The necessary dilutions for the cellular assays were performed just prior to experiments using a hydro-alcoholic solution (13% ethanol).

6.2.4. Wines analysis

Physicochemical characterization

Wines were characterized according to their oenological parameters. Enzymatic methods were used in a Miura200 chemical analyzer from TDI (Barcelona, Spain) to access the wine total polyphenolic content (TPC), tartaric acid, anthocyanins and glucose + fructose. Other parameters as Free and total SO_2, density, alcohol content, titratable acidity (TA), volatile acidity (VA), malic acid, tartaric acid, pH, and glycerol content were measured using a FOSS Wine-Scan (FT-120) infrared Fourier-transform spectrometer and a WineScan software Version 2.2.1 (FOSS, Hillerod, Denmark).

Solid-Phase Extraction (SPE) of the wine samples

Internal standards - (±)-catechin-2,3,4-13C3 (0.235 ng/µL) and resveratrol-(4-hydroxyphenyl-13C6) (0.281 ng/µL) - in a 2% FA solution (MilliQ water) were added to 500 µL of each wine sample. Solid-phase extraction was performed with Oasis HLB SPE cartridges (60 mg). Briefly, the cartridges were conditioned with 1.5 mL of methanol and 1.5 mL of water (both LC-MS grade). Samples were passed through the cartridge 3 times. The cartridges were then rinsed with 2 mL of H2O: 5% MeOH. The retained polyphenols were eluted with 2 mL of MeOH, partially evaporated in a Speedvac (Concentrator Plus, Eppendorf), and readjusted to 500 µL of 2% ACN: 0.1%FA. Wine samples were diluted 2 and 100 times with 2% ACN: 0.1%FA, after centrifugation (MiniSpin Plus, Eppendorf) at 14.000×g for 5 min.

Quantification of Polyphenols by LC-MS/MS

Samples were analyzed on a CTC PAL/ekspert ™ ultraLC 10 (Eksigent®) coupled to a hybrid triple quadrupole/linear ion-trap 4000 QTRAP® mass spectrometer and acquisition was performed by Analyst® (version 1.6.2, AB Sciex). The separation in the chromatography system was performed with a 2.6 µm Kinetex® F5 (50 x 2.1 mm, 100 Å, phenomenex) column with a 2.1 mm SecurityGuard™ ULTRA cartridge for F5 UHPLC (phenomenex) as guard-column. The column was set at 40 °C. The mobile phase, with a flow rate of 250 µL/min, was composed by solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) with a gradient of 2-30% B (0–9 min), 30–95% B (9–11 min) and 95% B (11–15 min) followed by cleaning and re-equilibration steps with a run of 4 min from 90 to 2% of B.

The mass spectrometer equipped with a turbo V™ source was operated in positive mode for (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, procyanidin B2, procyanidin C1, *trans*-Caffeic Acid and p-coumaric acid detection; and in the negative mode for the analysis of ellagic acid and *trans*-resveratrol. The ion spray voltage was 5500 V for positive and 4500 V for the negative mode. The other source parameters were the same for both ionization modes: curtain gas (CUR), 30 psi; ion source gas 1 (GS1), 30 psi; ion source gas 2 (GS2), 20 psi; source temperature, 450 °C. Parameters were optimized for each compound in multiple reactions monitoring (MRM) mode by direct infusion of a standard solution of each polyphenol at 9 µL/min. The values of declustering potential (DP), collision energy (CE), collision exit potential (CXP), and transitions are presented in ***Annex VI*** as Table S6.1. The sample volume of injection was of 10 µL, for all but the red wine in the negative mode, which was of 2 µL. Data was processed using MultiQuant™ software (version 2.1.1, AB Sciex). Quantification of each polyphenol was performed using calibration curves prepared in solvent with criteria for acceptance of r^2 value of at least 0.99, and the calibrators accuracy within 80-120%.

In vitro free radical scavenging by DPPH

The free radical scavenging capacity of the polyphenol standards and wines was accessed using the 2,2-Diphenyl-1-picrylhydrazyl methodology (DPPH) (Katalinić *et al*. 2004). All determinations were performed in triplicate and the inhibition percentage of the DPPH radical by the phenolic compounds was calculated according to the following equation (Yen & Duht 1994):

Equation 6.1.

$$\%\ inhibition\ =\ \left[\frac{\left(A_{C(0)} - A_{A(t)}\right)}{A_{C(0)}}\right] \times 100$$

6.2.5. Cell assays

Cell culture

Progeria smooth muscle cells (Progeria-SMCs) were firstly differentiated from Progeria-iPSCs. Briefly, Progeria vascular progenitor cells ($CD34^+$ cells) were isolated from human embryoid bodies at day 10 using magnetic activated cell sorting, as reported by Ferreira, L., 2007. The $CD34^+$ cells (30,000 cells/cm^2) were plated in a 24-well plate and grown in EGM-2 media supplemented with $PDGF_{BB}$ (50 ng/mL) for Progeria-SMCs induction, followed by a maturation step in SmGM-2. Cell cultures were maintained at 37 ºC, 5% CO_2 in a humidified atmosphere, with media changed every 2 days (Ferreira *et al*. 2007). Normal human vascular SMCs (Lonza) were used as controls and named Control-SMCs.

Preceding the oxidative assays, cells were cultured in 96-well plates using SmGM-2 rich medium (Lonza). Progeria-SMCs were cultured at a concentration of 10^4 cells/well, while Control-SMCs were cultured at 6.5 x10^3 cells/well. Concentrations were previously optimized for each cell line to guarantee the same level of confluency in culture well (Progeria-SMCs are smaller when compared to the Normal cell line). Culture media was changed for both cell lines every 2/3 days until testing.

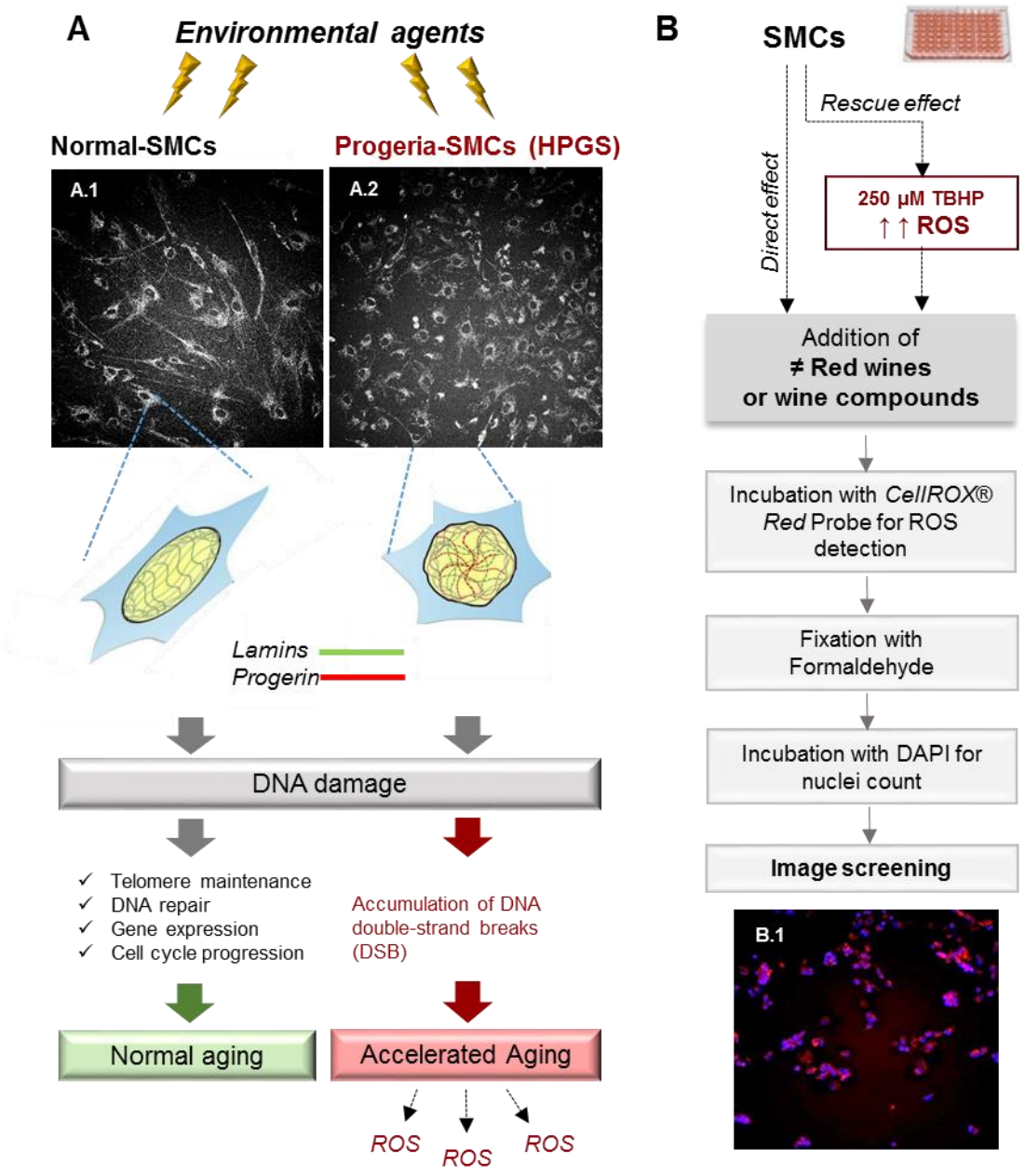

Fig 6.1 - Progeria-SMCs as a ROS-generating model. A) The differences between of nuclear integrity on Normal and Progeria-SMCs. In normal cells, damage is repaired by the DNA repair machinery. In progeroid cells, accelerated aging ensues, resulting in a ROS-generating environment (adapted from Ya-Hui Chi, *et al*, 2009. **B)** Cell-based assays implemented in this study. Experiments were performed to access the direct effect of wines and their Rescue capacity (after stress induction with 250µL TBHP, 2h).

Oxidative cellular assays

Direct test – The cellular impact of either wines or the individual polyphenols was firstly investigated without any induced oxidation to cells. On each well, their final concentration was of 0.05% (v/v). Assays were performed in both Normal and Progeria-SMCs. A hydro-alcoholic solution of 13% EtOH served as a blank control and was added to the cells at the equivalent final concentration of 0.05% (v/v)), to consider alcohol contribution.

Rescue test - The Rescue capacity of both wines and individual polyphenols was also investigated. After 12h seeding, oxidative stress was induced by adding 250 µM of tert-Butyl hydroperoxide (TBHP) (a standard oxidant), followed by 2 hours of incubation at 37 ºC. Cells were then exposed to 0.05% (v/v) of the different wines, the different individual polyphenols, the blank control solution of 13% EtOH and a 50 µM NAC standard antioxidant control. For oxidative stress determination, cells were stained with 5 µM of CellROX® Deep Red (Thermo Fisher Scientific) by adding the probe to the complete medium, followed by incubation at 37 °C during 2 hours. Cells were then fixed using a 2.5% Formaldehyde solution during 30 min at room temperature and washed twice with PBS. To access cell proliferation, nuclei were stained with 4', 6'-diamidino-2-phenylindole (DAPI). Final plates were examined with a high-content fluorescence microscope (IN Cell 2200, GE Healthcare). Assays were performed in quadruplicates.

Image scan

Images were recorded in each well (12/well) using a 20X objective in the IN cell Analyzer 2200 Imaging System (IN Cell 2200, GE Healthcare) and analyzed using the Analyzer Workstation 3.7.2 software (GE Healthcare). For cell viability analysis the number of cell nuclei were counted for each condition. For oxidative stress, the mean intensity of CellROX® Deep Red (Thermo Fisher Scientific) was registered in both cell nucleus and cytoplasm. The total value of intensity was used and normalized according to cell area. The variations of nuclei count and oxidative stress (%) were then determined, as follows:

Equation 6.2. Oxidative stress variation - ΔOS (%)

$$\Delta \mathrm{OS}(\%) = 100 \times (\mathrm{OS}_t - \mathrm{OS}_c)/\mathrm{OS}_c$$

- $\mathbf{OS_c}$**:** Oxidative Stress registered in cells with addition of a hydro-alcoholic solution (13% ethanol) as control.
- $\mathbf{OS_t}$**:** Oxidative Stress registered in cells with addition of each antioxidant sample (wine or polyphenol).

Equation 6.3. Nuclei count variation - ΔNC (%)

$$\Delta \mathrm{NC}(\%) = 100 \times (\mathrm{NC}_t - \mathrm{NC}_c)/\mathrm{NC}_c$$

- $\mathbf{NC_c}$**:** Nuclei count registered in cells with addition of a hydro-alcoholic solution (13% ethanol) as control.
- $\mathbf{NC_t}$: Nuclei count registered in cells with addition of each antioxidant sample (wine or polyphenol).

6.2.6. Statistical analysis

Statistical analysis was mostly performed in the *GraphPad Prism* software, version 5.0. Standard deviation calculations, linear regression and t-test analysis were performed. Differences of $p<0.05$ were considered significant. Principal Component Analysis was performed in the *Primer* software, version 6.1.16 (Clarke and Gorley, 2006).

6.3. Results and Discussion

Metabolic characterization of red wines

The twenty-two different red wines used in this study were characterized using enzymatic and Fourier-transform spectrometry (FTIR) methodologies, followed by mass spectrometry quantification of different polyphenols. As reported by previous

authors, the chemical composition of the grapes and wines is highly linked to specific factors, such as grapevine variety, location, soil, climate and technical practices (Roullier-Gall, Lucio, *et al*. 2014; De Andrés-De Prado *et al*. 2007; Tarr *et al*. 2013). Nonetheless, the wine polyphenolic composition and concentration has been reported as being mostly dependent on the vineyard (Lampíř & Pavloušek 2013). Herein, a broad variety of red wines - including monovarietal and blends, in a total of 22 wines - was tested in order to unravel the existing physicochemical heterogeneity of wines and their polyphenolic profile. Further details about the wines and respective characterization is presented in ***Annex VI*** as Figs. S6.1 and Table S6.3.

Red wines: Impact on oxidative stress reduction

At first, cellular assays were performed in Progeria-SMCs, using both white and red wines, in triplicate. The objective was to ascertain their potential antioxidant effect in the cells, testing a larger range of wine concentration (0.005 to 4% (v/v)). Results indicated that red wines had a higher potential to decrease the cellular oxidative stress, when compared to white wines (**Fig. 6.2**). This evidence was confirmed through the *Direct* and *Rescue* cellular tests and it became more evident at higher concentrations. In fact, this evidence supports previous reports showing that red wines originate greater antioxidant effects when compared to white wines (Kurin *et al*. 2012; Arranz *et al*. 2012).

According to this, only red wines were then selected for downstream experiments and an optimal dosage of 0.5%(v/v) was stablished as being the required concentration to achieve the same or better results when comparing to the control (NAC) at its standard concentration.

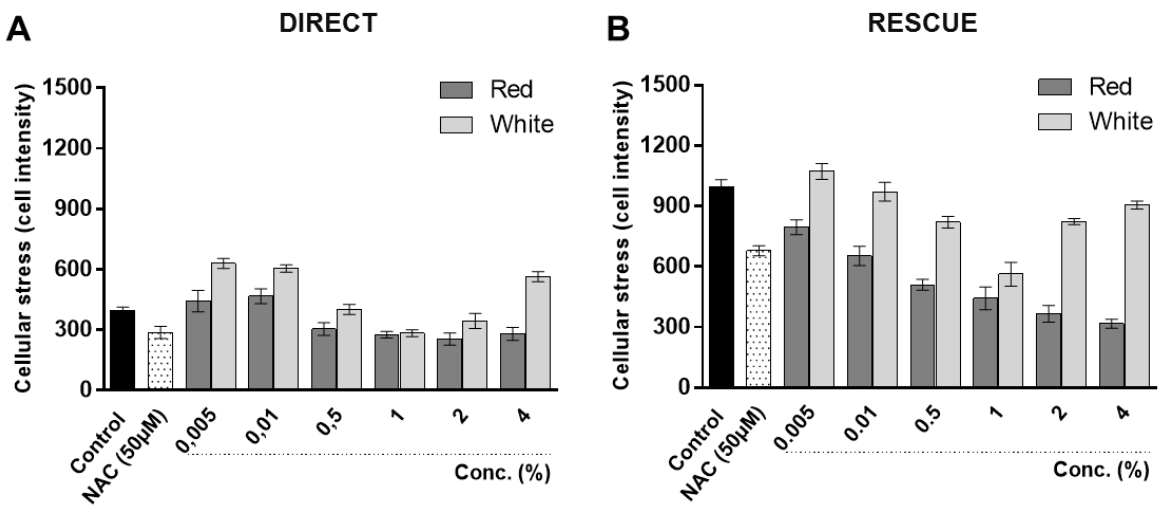

Fig 6.2 - Primary cell assays performed in Progeria-SMCs. Two wines (white and red) were firstly tested in the **A)** Direct and **B)** Rescue cellular assays, using a wide range of concentrations (0.005 to 4% (v/v)). NAC 50µM was used as a standard antioxidant control. Bars indicate mean ± SD and experiments were performed in triplicates.

Still, this concentration was 10 times lower than the one applied in a previous study that directly tested red wine matrices on progenitor and cancer cells (Wallenborg *et al*. 2009). The cellular impact of red wines was tested in normal- and Progeria-SMCs to unravel the potential of the latest as a ROS-generating model. Prior the assessment of the antioxidant effects, bright-field captions were registered showing the initial cellular morphologies (**Fig. 6.3 A**). Dissimilar characteristics were initially observed, namely Control-SMCs presented a fusiform shape and a normal nuclear morphology, whereas, Progeria-SMCs were characterized by an irregular, rounded and smaller cell shape, whose nuclear envelope integrity was found to be highly heterogeneous.

Initial cell cultures were then incubated with the ROS-detecting probe (*CellROX Red*) and the cell fluorescence signals were registered before and after the addition of a standard oxidant (250µM TBHP). In both cases, Progeria-SMCs presented superior levels of fluorescence intensity. (**Fig. 6.3 B-C**). Moreover, more signs of cellular damage were observed after the induced oxidation in Progeria-SMCs (**Fig. 6.3 C2**). Altogether, these evidences confirm recent reports that suggest higher

levels of ROS production and DNA damage in progeroid cells (Pitrez *et al*. 2015; Seco-Cervera *et al*. 2014; Ghosh, Liu, and Zhou 2013).

The full set of red wines (n=22), with average ethanol content of 13.67% ± 0.69 (v/v); and a total acidity of 5.79 ± 0.37 g/L (H_2SO_4), was equally tested in both SMCs lines. The direct application of the wines to the cell cultures at the concentration of 0.5% (v/v) (*Direct test*) caused significantly different responses between the cell lines, concerning the reduction of oxidative stress (p<0.0001) (**Fig. 6.3 D**). Contrarily to what was verified for Normal-SMCs, all red wines promoted a decrease of oxidative stress levels in Progeria-SMCs. Importantly, these cells were shown to be significantly more benefited by the direct addition of the antioxidant wine samples when compared to the Normal-SMCs. In a parallel experiment under rescue conditions (*Rescue test*), wines were applied to the initial cells after induction of high ROS production - with 250μM TBHP (standard oxidant). This assay also unveiled different responses concerning the stress reduction verified on the two tested cell lines (**Fig. 6.3 E**). In fact, the oxidative stress reduction was significantly different (p<0.0024) in Progeria-SMCs - ranging from 45% to 63%. Still, all wines tested also caused important stress reduction in the Normal-SMCs.

Indeed, a previous study has showed that a red wine extract protected against the oxidative stress in endothelial cells, after being induced by TBHP senescence (Botden *et al*. 2012). Herein, we present the first evidence that red wines directly promote a reduction of oxidative stress in a different cell line (Progeria) and confirmed this evidence with a considerably higher number of bioactive samples. These effects support the association of Progeria-SMCs with increased levels of intrinsic oxidative stress, which are currently being suggested as potentially relevant to the pathogenic mechanisms of HGPS (Trigueros-Motos *et al*. 2011; Pitrez *et al*. 2015).

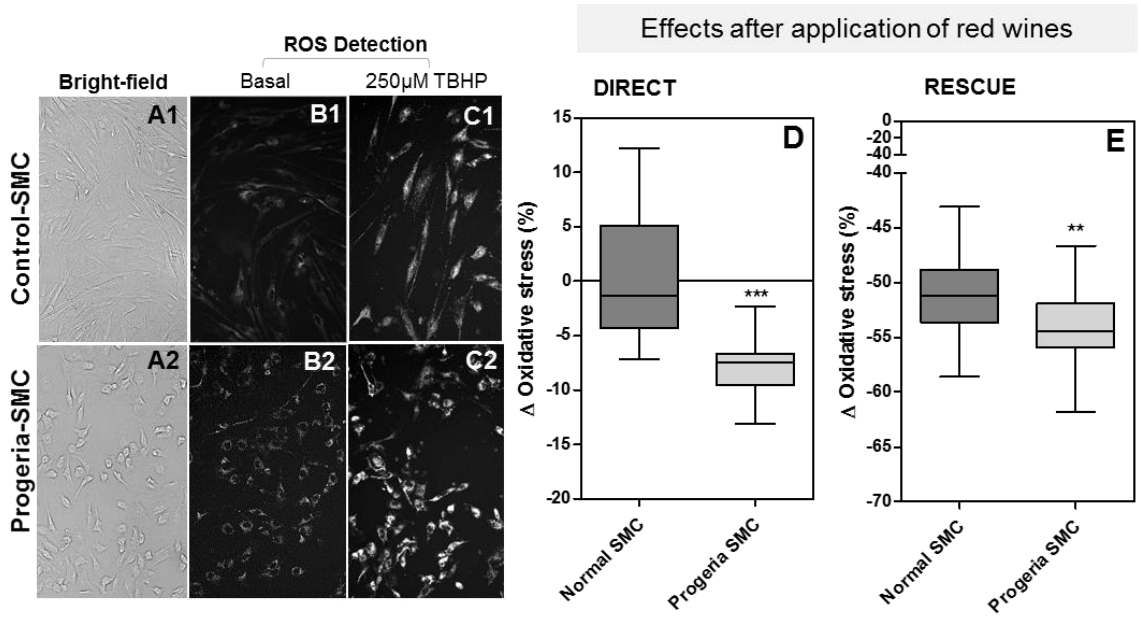

Fig 6.3 - Comparison of the wine effects on the oxidative stress of both Normal and Progeria-SMCs. A-C) Cellular captions obtained for both cell lines during the experiments. A: Bright-field images; B: Cell intensity of cells without ROS induction; C: Cell intensity of cells after induction of ROS with an oxidant (TBHP). **D-E)** Boxplots with whiskers representing the variability of stress impacts caused by application of the red wines (n=22) on each cell line. t-test shows significant differences and asterisks represent: ** p<0.005, *** p<0.0001. Experiments were performed in quadruplicates.

Correlation between total polyphenols and antioxidant capacity

The antioxidant capacity of the red wines was assessed using the DPPH assay (**Fig 6.4 A**) and through the oxidative stress reduction obtained through Progeria cell assays (**Fig 6.4 B**), to examine the existence of possible correlations between these parameters and the polyphenolic content of the red wines tested.

Interestingly, the radical scavenging capacity (RSC) was apparently correlated with the total polyphenolic content of the red wines (r^2=0.5448) (**Fig. 6.4 A**), which supports previous studies (Katalinić *et al*. 2004; Mitić *et al*. 2010). As regards to the cell assays, much lower correlation values were acquired between polyphenolic content and oxidative stress reduction. In fact, when red wines were directly added to the Progeria cells, the measurement of total polyphenols had no correlation with stress reduction (r^2=0.0761) (**Fig. 6.4 B**) suggesting that other factors might contribute to these antioxidant effects through possible synergistic reactions.

Surprisingly, when cells were rescued, results showed a slightly higher correlation between total polyphenols and stress reduction (r^2=0.3602), supporting the fact that the polyphenolic total content might be more relevant when rescuing cells that have been previously primed with high levels of oxidative stress.

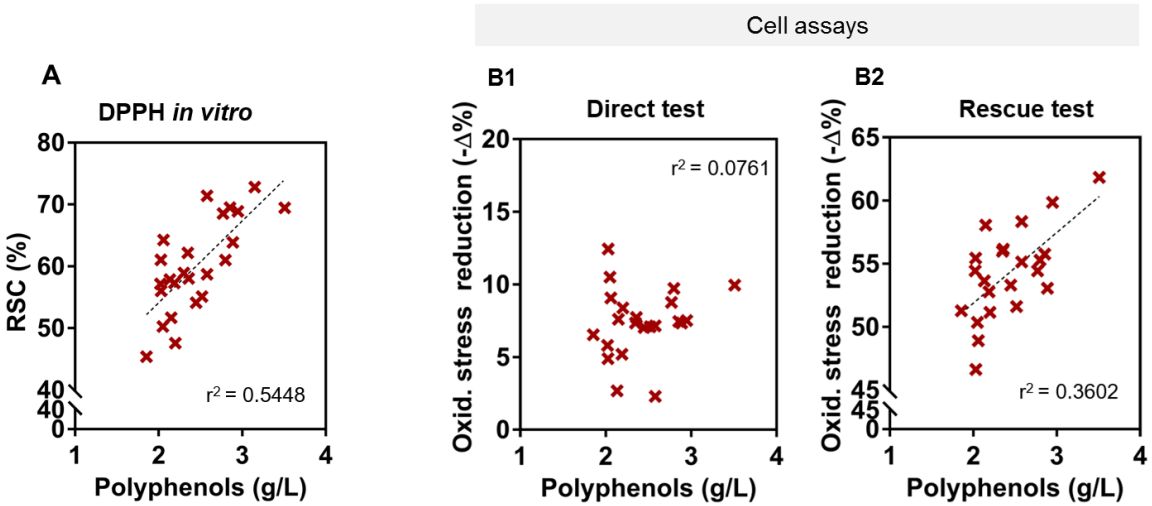

Fig 6.4 - Correlation between wine total polyphenols and the wine antioxidant capacity evaluated in different assays: A) DPPH scavenging test (RSC%), measured through a chemical experiment. **B)** Cell assays performed using Progeria-SMCs: B1. *Direct test*; B1. *Rescue test.* Linear regression R-squared values are presented for each scatter plot.

Polyphenolic content: Impact on cellular proliferation

To assess the impact of the specific wine polyphenolic content on cellular proliferation, polyphenols were quantified in all the red wines tested, by using targeted LC-MS. Results unveiled that the polyphenolic content had a relevant impact on proliferation assessed through nuclei count variation registered for Progeria- and Normal-SMCs (**Fig. 6.5**). Indeed, when cells were directly exposed to antioxidants, the total polyphenolic content had a positive effect on the variation of nuclei count, which was especially evident on Progeria-SMCs. Nevertheless, no statistically significant correlation was identified (**Fig. 6.5 A1**). The highest correlations were found to be related with the content of (+)-Procyanidin B2 and (-)-epicatechin, which are some of the most abundant polyphenols in red wines. Particularly, their concentrations were significantly associated with an improvement in cell proliferation in this cell line (p=0.0001 and p=0.0029, respectively) (**Fig. 6.5 B1** and **C1**)

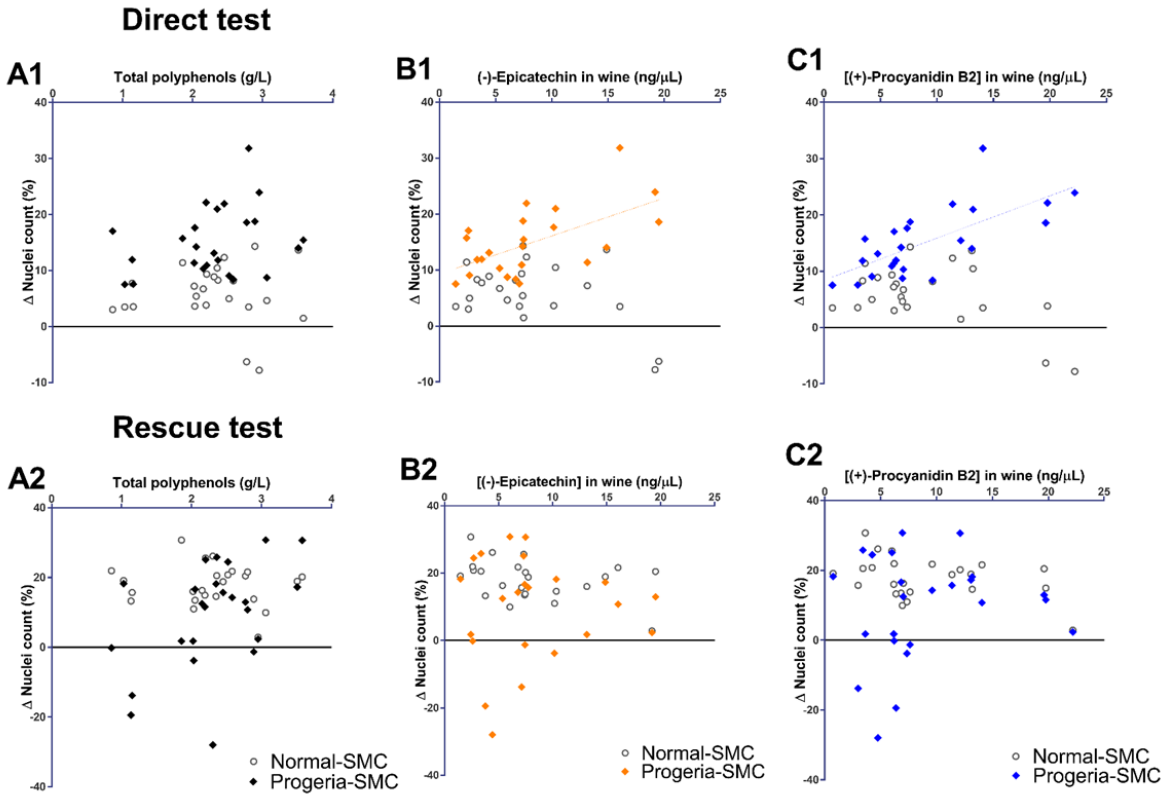

Fig 6.5 - Relation between the wine polyphenolic content on the cellular proliferation of Progeria-SMCs and Normal-SMCs. **1**) Direct and **2**) Rescue assays were performed. Results were plotted according to **A**) Total polyphenols; **B**) (-)-Epicatechin; **C**) Procyanidin B2 measured in all red wines. In the case of direct assays B1 and C1, orange and blue lines show significant correlation tendencies.

Contrarily, Normal-SMCs did not expose the same level of proliferation improvement and two of the wines have even reduced proliferation (<10%). Again, these results indicate that Progeria-SMCs benefit the most with the direct addition of the wines when compared to Normal-SMCs. As far as we know, this is the first study that clearly demonstrates that the wine matrices have a potential beneficial effect, with enhanced results on already stressed cells.

The polyphenolic mixtures exposed a general positive effect on the cellular proliferation under rescuing conditions, but this tendency was not linear (**Fig. 6.5 A2**, **B2** and **C2**). Still, Normal and Progeria-SMCs revealed a general increase on

proliferation levels (up to 30%) after being exposed to the different red wines, which confirms that their application also caused a positive cellular response under rescue conditions.

On the other side, some wines (n=6, representing 27% of our samples) caused a reduction of cellular proliferation on Progeria-SMCs, which may perhaps be caused by other wine features, such as pH, acidity, sugar concentration, or due to the presence of possible undesirable compounds in these wine matrices. Such variability on the observed cellular impact of the different wine matrices highlights the importance for testing a large number of wines and to further study the whole wine matrices as possible synergistic or antagonistic reactions might also occur.

Red wines vs. individual polyphenols

Solutions of the 8 individual polyphenols and their respective Polyphenol mix (*Poli mix)* were prepared at a specific initial concentration, based on their average concentration in red wines (Arranz *et al*. 2012). Accordingly, as performed for the red wines, these polyphenolic solutions were equally added to cells at 0.5% (v/v), enabling a simultaneous evaluation of the cellular effects. As shown in **Figures 6.6 A-B**, at the tested concentration, individual polyphenols are not as efficient as red wines matrices regarding the reduction of oxidative stress. Indeed, most of them, when added individually may increase the oxidative stress, thus having a negative impact on cells.

Only 3 individual polyphenols, (+)-catechin, procyanidin B2 and (-)-epigallocatechin, and the *Poli mix* could slightly reduce the basal oxidative stress of the cells. Differently, all red wines demonstrated benefits, by reducing the oxidative stress (-2.29 to -13.088%) and promoting proliferation (maximum of 31.804%). In particular, cellular benefits of adding (-)-epigallocatechin (0.6 mg/L) to cells were revealed to be superior when compared to resveratrol, which exists in higher amounts in red wine (2.7 mg/L), thus bringing to the limelight the cellular importance of other minority polyphenols.

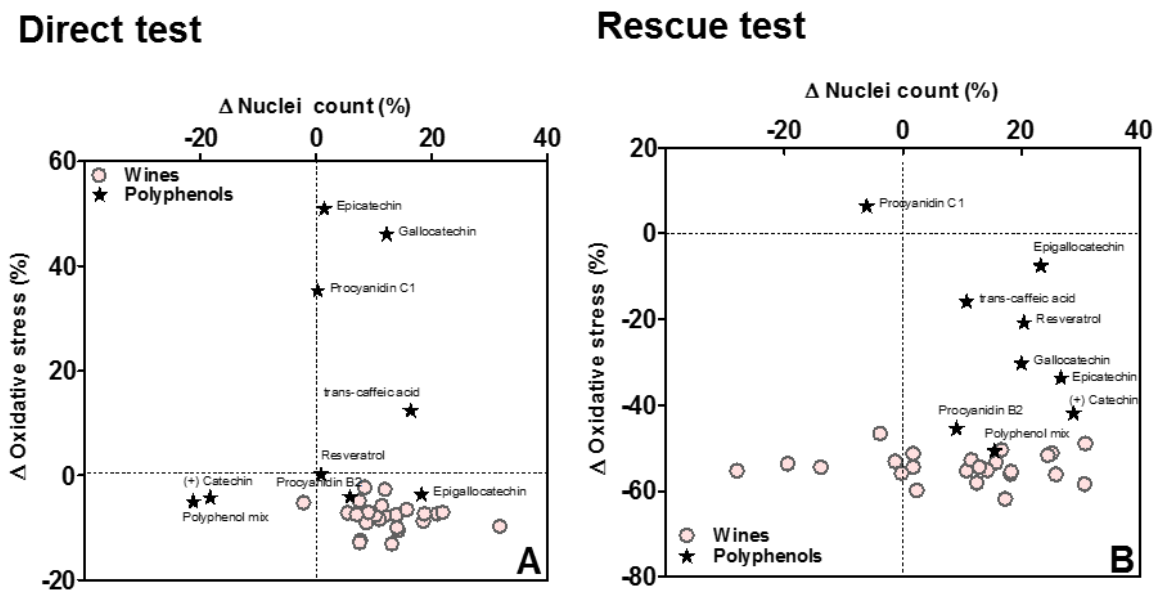

Fig 6.6 - Cellular impact of potential antioxidants. A-B) Comparison of the cellular effects obtained using individual polyphenols and all set of red wines. C-D) Closer evaluation of the cellular effects promoted by the red wines. Direct and Rescue tests were performed in Progeria-SMCs. Variations of oxidative stress (%) and nuclei count (%) were calculated according to a control experiment where antioxidants were substituted by a hydro-alcoholic solution (13% ethanol) applied in the same proportion. Experiments were performed in quadruplicates.

Indeed, red wines were largely more efficient than individual polyphenols in decreasing induced levels of oxidative stress (**Fig. 6.6 B**), which unveils the importance and the impact of synergistic action between polyphenols. Nevertheless, almost all the tested solutions promoted stress reduction resulting in enhanced proliferation. Procyanidin C1 was the only polyphenol with a slightly tendency to cause cell death at the tested conditions.

These results indicate that specific wine polyphenols might be more relevant than others in reducing cellular oxidative stress, which is in line with previous findings (Mitić *et al*. 2010). Nevertheless, since the individual polyphenols were tested based on their average concentrations in red wines, it is important to take that factor into consideration. Under rescuing conditions, it was observed that procyanidin B2 (49.7 mg/L) or (+)-catechin (68.1 mg/L) reduced higher levels of oxidative stress, when compared to resveratrol (2.7 mg/L), but these benefits might be influenced by the superior concentration of these polyphenols in red wines.

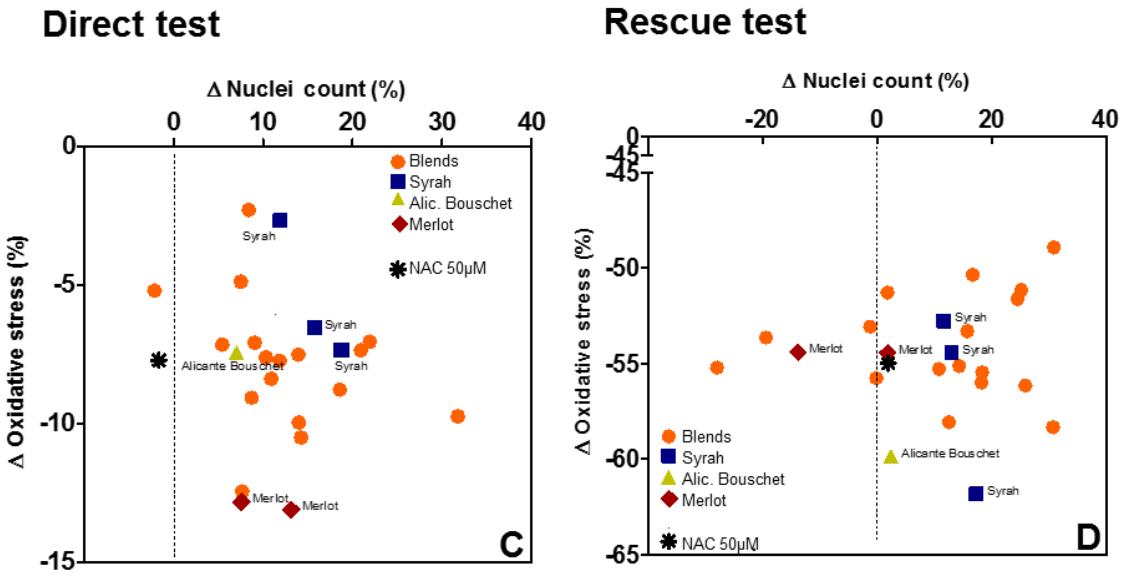

Fig 6.7 - Cellular impact of potential antioxidants. C-D) Closer evaluation of the cellular effects promoted by the red wines. Wines produced using a main foreign variety were identified. Direct and Rescue tests were performed in Progeria-SMCs. Variations of oxidative stress (%) and nuclei count (%) were calculated according to a control experiment where antioxidants were substituted by a hydro-alcoholic solution (13% ethanol) applied in the same proportion. Experiments were performed in quadruplicates.

These data also indicate that it would be important to carry out further metabolomics analysis of the wine matrices to better explore possible synergies between different polyphenols and other bioactive molecules. Indeed, previous studies demonstrated important synergistic effects between specific polyphenols as quercetin and catechin (Pignatelli *et al*. 2006; Pignatelli *et al*. 2000). Nonetheless, other types of wine bioactive molecules, such as anthocyanins and aroma compounds, were recently associated to health benefits for the wine consumers (Antonella *et al*. 2013; Zengin & Baysal 2014; Pojer *et al*. 2013). For example, some terpenes were recently described to increase the permeability of the cell membrane, which might facilitate the entrance of other important molecules into the cells. (Mendanha & Alonso 2015; Camargos *et al*. 2014; Videira *et al.* 2014).

The cellular impact of the red wines was also presented according to their main variety (≥ 50 v/v%) – Blend and non-Blend (Syrah, Alicante Bouschet, Merlot) (**Fig. 6.7 C-D**).

6.4. Conclusions

Wine moderate consumption has been associated to positive anti-aging effects, such as cell damage, dementia and cognitive function. Recent studies have been tested on the cellular impact of bioactive matrices, such as food-derived polyphenols and extracts, from tea, oils fruit and wines. Nevertheless, most of these were focused on cancer cell models, such as hepatomas, prostatic and lymphoma cell lines.

This study describes the cellular effects of polyphenolic mixtures and red wines on a cellular aging model, based on Progeria-SMCs, a natural ROS-generating environment. Indeed, we have clearly demonstrated that, at the testing conditions, the application of polyphenols and red wines on these aged cells has caused a significant reduction of oxidative stress and improvement of cellular proliferation.

Further, our results exposed that the average wine content in (-)-epicatechin and procyanidin B2 seem to have the highest correlation with the improvement of Progeria-SMCs proliferation. Possible synergies between different polyphenols and other bioactive compounds in wines should be further investigated, since our results present, for the first time, compelling evidences that the whole wine matrix causes a higher stress reduction in the ROS-generating environment than the individual polyphenols.

Altogether, our results stress out the importance of the wine bioactive matrices and indicates that its polyphenols should be further explored to possible develop new strategies to fight against aging-associated conditions.

Author Contributions

J.F., P.P. performed the cell assays; J.F performed the wine physicochemical analysis; J.P and B.M. performed the LC-MS experiments; J.F., P.P, L.F. and A.C.G. designed the study; J.F., P.P. and A.C.G. wrote the paper; J.S. performed a final correction of the manuscript.

Acknowledgments

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Chapter 7

Metabolites in human plasma following consumption of de-alcoholised red wine enriched with resveratrol

Metabolites in human plasma following consumption of de-alcoholised red wine enriched with resveratrol

This Chapter aims at fulfiling the specific objective of this thesis: “to explore the bioavailability of potentially important anti-carcinogenic polyphenols in human plasma after a repetitive moderate consumption of dealcoholized wine”.

This study was integrated in a 4-years project entitled ‘Resveratrol in the chemoprevention of colorectal neoplasia’, undertaken at Australia by the Royal Melbourne Hospital in conjunction with the Department of Colorectal Medicine and Genetics and the Australian Wine Research Institute.

Further manuscripts resultant from this study are in preparation for peer-review publication.

Abstract

The consumption of resveratrol, either as a dietary supplement or as a component of certain foods and beverages, has been associated with potential health benefits for consumers. This evidence has been mostly obtained from *in vitro* or animal studies and limited human *in vivo* studies, which have observed only traces of resveratrol in plasma post consumption below proposed efficacious concentrations. Hence, the bioavailability of resveratrol and presence of its metabolites in human plasma is still under debate.

In this study, subjects were recruited to a dietary intervention, based on their family history of bowel cancer and hence increased risk for developing colorectal cancer. Over a six-week period, one group of the subjects received a daily dosage of 100 mL de-alcoholised red wine containing less than 0.5 mg of *trans*-resveratrol and the other group received 100 mL of de-alcoholised wine containing 50 mg of *trans*-resveratrol. A non-targeted metabolomics experiment was performed using HPLC-QTOF-MS to explore the plasma composition of resveratrol conjugates and other compounds after the intervention. Results indicate that under the specific study conditions, significantly increased amounts of resveratrol conjugates and other metabolites were found in plasma after six weeks of daily resveratrol intake. Free resveratrol could also be identified, but only in trace amounts. No gender differences were observed.

These findings demonstrate the bioavailability of resveratrol and changes to the plasma concentrations of a range of compounds related to the ingestion of resveratrol. The findings highlight the need for further investigations into i) indirect impacts of resveratrol ingestion on metabolite profiles in human plasma and ii) the underlying mechanism(s) of the potential health benefits associated with resveratrol and its metabolites and wine consumption.

Keywords Bioavailability, de-alcoholised red wine, dihydro-resveratrol, metabolomics, plasma, glucuronides, resveratrol, sulfates.

7.1. Introduction

Over the last three decades, research has been highly focused on the effects of wine consumption on human health (Arranz *et al*. 2012; Rodrigo *et al*. 2011; Mazú *et al*. 2014). Moderate consumption of wine - approximately one to two glasses per day - is suggested to have health benefits for consumers associated with its relatively high content of phenolic compounds compared to other foods and beverages (Lippi *et al*. 2010; Artero *et al*. 2015). Previous studies have shown phenolic compounds to be associated with a reduced incidence of cardiovascular and cognitive diseases, diabetes, and a reduced risk of certain cancers such as lung, colorectal, endometrial and ovarian as well as lymphomas (Sancho & Mach 2014; Arranz *et al*. 2012; Angel-Morales *et al*. 2012). In particular, some epidemiological studies have demonstrated that moderate wine consumption may be protective against colorectal cancer, in both males and females (Patel *et al*. 2010; Park *et al*. 2009) and this protective effect has been confirmed in different countries, and associated with different diets (Fira-Mladinescu *et al*. 2008; Kontou *et al*. 2012; Mazú *et al*. 2014).

Resveratrol is one of the most commonly studied phenolic compounds in red wine in the context of its diverse biological effects. For example, some recent studies have suggested that resveratrol may inhibit cancers such as lymphoma, breast cancer and colorectal cancer by interference with different cell signalling pathways associated with cell proliferation, motility and survival (Patel *et al*. 2011; Frazzi *et al*. 2013; Du *et al*. 2016). Also anti-proliferation and apoptosis-inducing effects have been shown for certain cancers (Varoni *et al*. 2016). *In vitro* data also suggest that 5 µmol/L resveratrol is the minimum concentration required for the chemopreventive effects of the compound to be elicited (Boocock *et al*. 2007).

Bioavailability studies have previously indicated that some wine-derived phenolic compounds such as resveratrol are efficiently orally absorbed (Walle 2011; Vitaglione *et al*. 2005), but it is still unclear to what extent these molecules reach the target organs and where they perform a biological activity (Manach *et al*. 2005). In fact, considerable amounts of potentially bioactive dietary compounds are lost along

the human digestive system, due to low absorption of the molecules, degradation in specific organ environments, or extensive metabolic modifications which mostly occur in the liver, and small and large intestine (Serra *et al*. 2011; Wenzel *et al*. 2005; D'Archivio *et al*. 2010). These extensive modification pathways might cause degradation, excretion and generation of multiple and different conjugated metabolites, compromising the final biological activity of phenolic compounds. While high rates of absorption have been observed for *trans*-resveratrol, only trace amounts of the free form have previously been found in the plasma and urine (Walle *et al*. 2004). The main metabolites identified include different conjugates, such as resveratrol mono glucuronides and sulfates, but also dihydro-resveratrol (Goldberg *et al*. 2003; Patel *et al*. 2010). There are few studies on the bioavailability of resveratrol in humans (Tomé-Carneiro *et al*. 2013), and most performed thus far administrated the compound in tablets, capsules or caplets (Brown *et al*. 2010; David J Boocock *et al*. 2007). This practice avoids constraints related to alcohol consumption, but may also alter the normal rate of absorption and excretion when compared to the wine matrix intake (Rotches-Ribalta *et al*. 2012). Moreover, most studies in humans are also highly restricted by a low number of individuals (normally n<25) (Gresele *et al*. 2008; Goldberg *et al*. 2003), which may limit the reliability of the information obtained from the study. In fact, previous reports were found to ignore the individual variation, leading to insufficient statistical power and consequently, biased conclusions (Sanson-Fisher *et al*. 2007).

As part of a broader study to determine if resveratrol administered in a de-alcoholised red wine can reduce the risk of colorectal cancer in susceptible subjects, a double-blind, placebo-controlled dietary intervention experiment was undertaken. One of the additional aims of the intervention was to explore the existence of resveratrol and its metabolites in plasma. Here we report the results from the targeted profiling of resveratrol metabolites in human plasma by HPLC-MS following daily ingestion over a period of six weeks of de-alcoholised red wine supplemented with resveratrol. In addition, we discuss the results from the non-targeted metabolomics analysis of resveratrol-related and other metabolites in human plasma.

7.2. Material and Methods

7.2.1. Chemicals

Reference standards of *trans*-resveratrol, *trans*-resveratrol-3-glucuronide, *trans*-resveratrol-4'-glucuronide, *trans*-resveratrol-3-sulfate, *trans*-resveratrol-3-sulfate, *trans*-resveratrol-3-glucuronide and *trans*-resveratrol-4'-glucuronide were acquired from the Institute of Food Research, Norwich Research Park, UK. All reference analytes and internal standards were stored in the dark at –20°C to avoid degradation. HPLC-grade (purity>99.99%) acetonitrile (ACN) and methanol (MeOH), 2-propanol, ammonium acetate and formic acid (FA) and were purchased from Merck Millipore.

7.2.2. De-alcoholised wine

Volunteers ingested a red wine (Tempranillo, 2008), previously de-alcoholised to ca. 0.00% alcohol v/v by a commercial winery using 'spinning cone' technology. The resveratrol content of the de-alcoholised wine was <0.5 mg as determined by targeted reverse phase HPLC-QTOF analysis. To half of the de-alcoholised red wine, resveratrol was added in a concentration of 50 mg per 100 mL of wine, immediately prior to bottling into 375 mL claret bottles. The added resveratrol consisted of 99% resveratrol extracted from grape skins by Changsha Organic Herb Inc., China. The dose of resveratrol was chosen after consideration of the studies of Goldberg *et al*. (2003) and Walle *et al*. (2004), which detected measurable amounts of resveratrol in plasma and urine after consumption of this dose (Walle *et al*. 2004; Goldberg *et al*. 2003).

The Australian Wine Research Institute coordinated and supervised the production of the treatment and control products, ensuring that they complied with the specifications outlined in the Australian New Zealand Food Standards Code.

7.2.3. Study participants

Subjects, 27 males and 43 females born between 1930 and 1985, were recruited to this dietary intervention, based on their family history of bowel cancer and hence increased risk for developing colorectal cancer. All subjects completed the ethics-approved study after providing their informed consent.

7.2.4. Interventional study design

This intervention study was conducted by the Department of Colorectal Medicine and Genetics at The Royal Melbourne Hospital, Victoria according to the National Statement on Ethical Conduct in Human Research (2007) produced by the National Health and Medical Research Council of Australia.

De-alcoholised wine (100 mL per serve) was administrated to the subjects in a double-blind, placebo-controlled dietary intervention. Thirty-nine subjects were randomly selected to receive the control wine (non-spiked resveratrol - NS) and the other 31 consumed the test wine (containing 50 mg of spiked resveratrol - RS). Wine intake occurred once per day with the evening meal over the six-week intervention period. Subjects were also placed on a low plant polyphenol diet for three weeks prior to the intervention and for the following six-week intervention period (**Fig. 7.1**). Blood samples were collected in heparinised tubes immediately after the first intake (T0) and after 30, 60, 90, 120 min post intake.

After six weeks (T6w) of daily wine intake, blood was again collected from all the patients, 24h after the last wine intake. Samples were then centrifuged at 2000 rpm, straight after collection, and the plasma was aspirated and transferred to polypropylene cryotubes. Plasma samples were stored at -80ºC until further analysis.

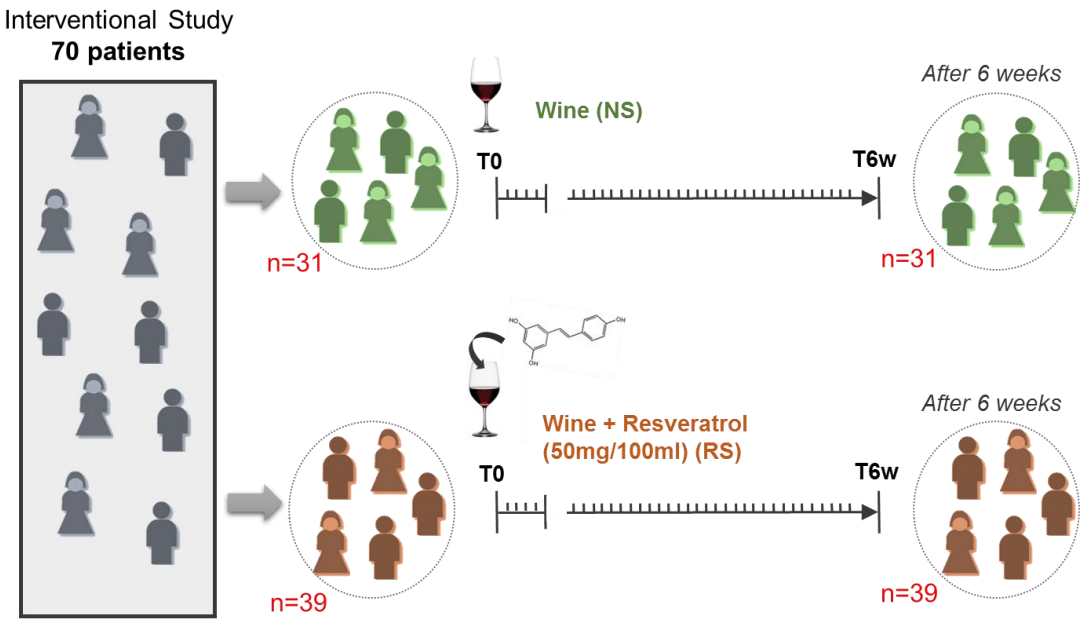

Fig. 7.1 - Scheme of the interventional human study design.

7.2.5. Samples preparation

De-alcoholised wines

Polyphenols were extracted from de-alcoholised wine samples (with and without resveratrol added) using solid-phase extraction (SPE) with Phenomenex Strata-X 33 µm 85Å polymeric reverse-phase cartridges (60 mg adsorbent in 3mL). A pooled mix containing an equal aliquot of each sample was also prepared to monitor instrument performance. Cartridges were conditioned using 1 mL methanol followed by 1 mL Milli-Q water. Samples were diluted 1:4 in Milli-Q water and a volume of 10 mL was loaded on the column. Wash was performed using 1 mL of 2% methanol, followed by full vacuum during 2min. Both samples and calibrants were eluted with 1mL methanol, which was then evaporated to dryness. (TurboVap® LV Zymark). The pellets were redissolved in 100µL of LC solvents (75%A, 25%B). Solvent A (2% formic acid, 0.5% methanol in Milli-Q water) and Solvent B (2% isopropanol, 2% Milli-Q water, 40%acetronitrile in methanol).

Plasma

Polyphenols were extracted from plasma samples using solid-phase extraction (SPE) with Phenomenex Strata-X 33µm 85Å polymeric reverse-phase cartridges (60 mg adsorbent in 3mL). A calibrant mix was prepared using 100 µL of a 1 mg/mL solution of each standard metabolite (*trans*-resveratrol, resveratrol-3-sulfate, resveratrol-4'-sulfate, resveratrol-3-glucuronide, resveratrol-4'-glucuronide) in a final volume of 10 mL of 10% methanol to give 10 mg/L of each analyte. Cartridges were conditioned using 1mL methanol followed by 1 mL Milli-Q water. Samples were diluted 1:1 in Milli-Q water and a volume of 750 µL was loaded on the column. Wash was performed using 1 mL of 10% methanol, followed by full vacuum during 10 min. Samples were eluted with 1mL methanol, which was then evaporated from the respective fractions using the evaporation system, during 30 min. After evaporation, the pellets were redissolved in a volume of 150 µL of starting LC solvents 10%B, 90%A. Solvent A (5mM ammonium acetate, 2% isopropanol in MilliQ water, pH adjusted to 5 with acetic acid) and Solvent B (2% isopropanol in methanol). A Master mix was prepared by mixing equal amounts of all the plasma samples.

7.2.6. Reverse phase metabolite profiling by HPLC-QTOF

De-alcoholised wines

Metabolite profiling of the de-alcoholised wines was acquired on an Agilent 1200 SL HPLC coupled to a Bruker microTOF-Q II. Instrument control was performed with Bruker HyStar software and LC fitted with a Phenomenex Kinetex PFP column (150mm x 2.1mm ID) coupled with a SecurityGuard PFP guard column. The column was set at 30°C and the DAD acquisition range 190-400λ. For the mobile phase, Solvent A was 2% formic acid, 0.5% methanol in Milli-Q water and solvent B was 2% formic acid, 2% *Milli*-Q water, 40% acetonitrile in methanol. A flow rate of 220 µL/min was used and the gradient was as follows: 1% B (0–25min), 1-7.5% B (25–80min), 7.5–60% B (80–125min), followed by cleaning and re-equilibration. The mass spectra was acquired using ESI in negative mode and the capillary voltage

was 3500 V. Other parameters were optimized: nebulizer pressure, 2.0 Bar; Dry gas flow, 8.0 $L.min^{-1}$; Mass range, 50 ─ 1650 m/z; Acquisition rate, 0.5 Hz; Source temperature, 200 °C. Using Bruker's DataAnalysis (v4.3, SP4) software, mass spectra (line) were calibrated on ions in the range 90-1000 *m/z* using an in-house sodium formate calibration solution (5mM sodium hydroxide, 0.2% (v/v) formic acid in 50% (v/v) 2-propanol) introduced immediately before the sample injection via an inline post-column switching valve and sample loop. Bruker's enhanced quadratic algorithm was used for mass calibration.

Non-targeted metabolomics experiment

Separation was performed on an Agilent 1200 SL HPLC coupled to a Bruker microTOF-Q II, but in this case, separation was obtained using a Waters Atlantis T3 3 µm 150 mm x 2.1 mm column. The column was set at 45°C and the DAD acquisition range 190-400λ. The mobile phase, with a flow rate of 200 µL/min, was composed by solvents A (5mM ammonium acetate, 2% isopropanol in Milli-Q water, pH adjusted to 5 with acetic acid) and B (2% isopropanol in methanol) with a gradient of 10% B (0–2 min), 10–55% B (2–16 min), 55–98% B (16–17.5 min), 98% B (17.5–28 min) and 10% B (29–40 min) followed by cleaning and re-equilibration steps with a run of 4 min from 90 to 2% of B.

The mass spectrometer was operated in negative mode and the ion spray voltage was 3500 V. Other parameters were optimized: nebulizer pressure, 2.0 Bar; Dry gas flow, 6.0 $L.min^{-1}$; Mass range, 50 - 1000 m/z; Acquisition rate, 0.5 Hz; Source temperature, 200°C. Using Bruker's DataAnalysis (v4.3 SP4) software, mass spectra (line) were calibrated on ions in the range 90-1000 *m/z* using an in-house sodium formate calibration mix (5mM sodium hydroxide, 0.2% (v/v) formic acid in 50% (v/v) 2-propanol) introduced immediately before the sample injection via an inline post-column switching valve and sample loop. Bruker's enhanced quadratic algorithm was used for mass calibration.

MS/MS parameters

MS/MS analysis was acquired by using an Agilent 1200 SL HPLC coupled to a Bruker microTOF-Q II. Experiments were performed using the following conditions: Negative ESI mode; Nebulizer pressure of 2 Bar; Capillary voltage of 3500V; Dry temperature set to 200ºC. According to the targeted compounds, a collision energy of 7-40V was defined. Fragmentation data was registered for m/z 417.3202, 403.3052, 331.0451, 449.0820, 192.9882, 419.1344, 315.0306, 375.0138, 308.0338, 377.0330, 493.2410.

7.2.7. Data analysis

The files were exported in mzXML format and processed under a 64-bit Linux environment using the R scripting language (v3.1) and the BioConductor packages XCMS and CAMERA.

The existence of possible resveratrol metabolites in the plasma samples was explored using an non-targeted metabolomics approach. After analysis of the samples, the initial data was processed using RStudio software (Version 0.98.1103). Parameter settings were optimised for the data acquired. Tolerance windows of mass (*m/z*) and retention time (RT) values were set to 0.01 and 30s. For each molecular feature (MF) a representation was obtained (png file) showing the appearance of the respective chromatogram. All the files were manually examined and signals with low intensity (<500) were generally excluded. After data curation, the results were exported as a tab separated base peak matrix that included the retention time, *m/z* and intensity for each molecular feature, for all samples. Principal component analysis (PCA) was performed using The Unscrambler.

Fold change calculations, paired t-tests ($p<0.05$) and one-way ANOVA ($p<0.05$). Putative MS/MS identification was performed through databases as METLIN, FoodDB and Human Metabolome Database (HMDB). Statistical analyses were performed using The Unscrambler® software, version 10.4 (CAMO Software AS,

2016, Oslo Science Park, Oslo, Norway) and Minitab® software version 17.3.1 (Minitab Inc., United States).

7.3. Results

To explore the bioavailability of resveratrol, other polyphenols and their metabolites in the plasma samples, the non-spiked resveratrol (NS) and the resveratrol-spiked wines (RS) were respectively administrated to two separated groups of individuals through a double-blind experiment. Consumption (100 mL) of the de-alcoholised red wine, with or without added resveratrol, occurred once per day with the evening meal, over the six-week of intervention.

7.3.1. De-alcoholised wines

Both de-alcoholised wines used in this study were first characterised using targeted reverse phase analysis of polyphenols by HPLC-QTOF analysis. As shown in **Fig. 7.2**, the overall phenolic profile of both wines was typical for a red wine and the resveratrol-spiked de-alcoholised wine (RS) has substantially higher concentrations of *cis*- and *trans*-resveratrol (53.1 and 16.4-fold higher, respectively), compared to the non-spiked (NS) de-alcoholised red wine.

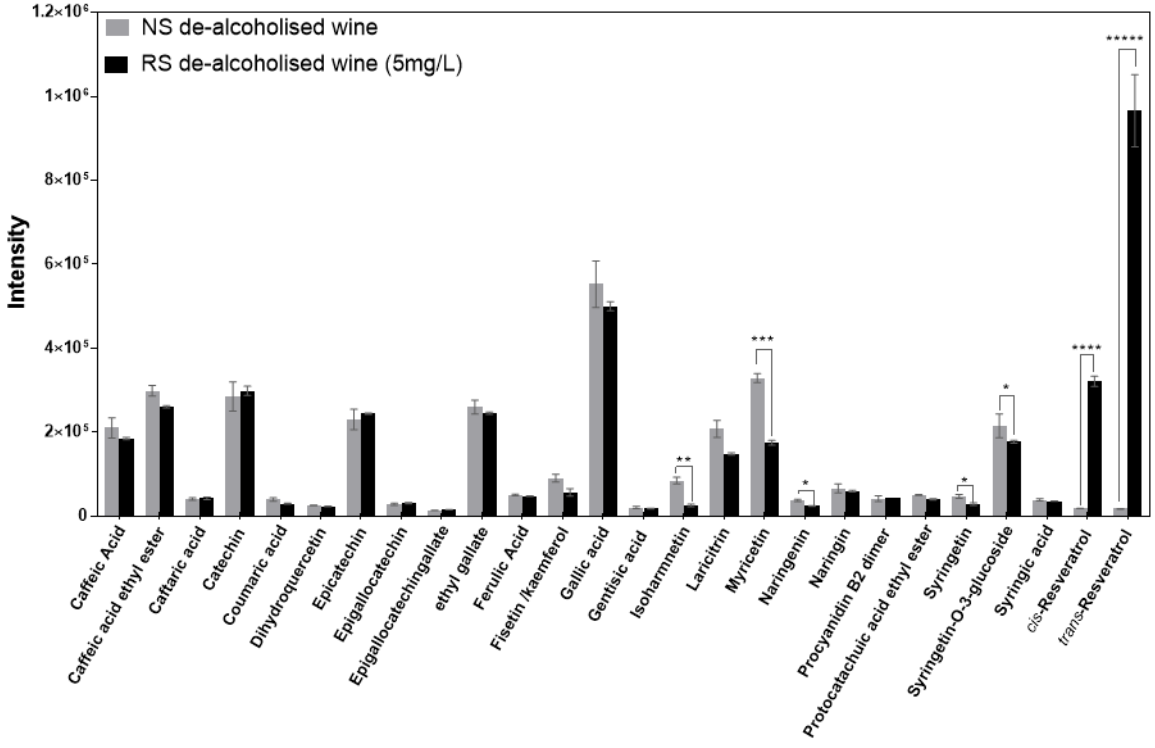

Fig. 7.2 - Relative abundance of wine polyphenols in both wines: NS and RS de-alcoholised red wines. Mean values of intensity and the respective standard deviations are presented. Asterisks indicate significant differences between the two tested wines (p<0.05). Samples were analysed in triplicate.

7.3.2. Non-targeted analysis of data from quantitative HPLC-MS analysis of resveratrol metabolites

Data had been previously acquired for the targeted analysis of resveratrol metabolites from 50 participants (25 from group RS and 25 from group NR) at T0 and T6w timepoints. This dataset was explored further using a non-targeted approach to reveal discriminating variables between the RS and the NS groups, after six weeks of regular de-alcoholised wine consumption. After alignment of the chromatograms, a total of 1582 molecular features were extracted using the R studio software. After this step the data were manually curated, resulting in 544 molecular features (66% removed) that were subsequently submitted to statistical analysis. The filtered features where used to compare molecular features between

the RS and NR groups, but also to assess differences in molecular features between the two time-points of blood collection for each group. Based on one-way ANOVA and multiple t-tests, a total of 9 significantly different molecular features of interest (MFOI) ($p<0.05$, fold change>2) could be identified in this dataset (**Table 7.1**).

After six weeks of regular consumption of de-alcoholised wine (T6w), as expected higher concentrations of several well-known resveratrol conjugates were detected in the plasma of individuals that consumed the RS-de-alcoholised wine. In contrast, none of these resveratrol metabolites were detected in any T0 sample and the NS-de-alcoholised control wine at T6w. As shown in **Fig. 7.3**, the resveratrol metabolites detected in the plasma samples of these patients after six weeks included the three isomeric mono sulfates, two di-sulfates and a putative glucuronide of a resveratrol sulfate ester. Two mono-glucuronides of resveratrol were also detected, and tentatively assigned as –3 and –4' isomers. Free *trans*-resveratrol was also detected in plasma, but only in trace amounts.

The identification of resveratrol-3-sulfate, resveratrol-4'–sulfate, resveratrol-3-glucuronide and resveratrol-4'-glucuronide was confirmed through the comparison of retention times and mass spectra with reference compounds. Identity of the remaining resveratrol metabolites was tentatively assigned based on MS/MS experiments, and a comparison to related metabolites detected in this study and published data (Menet *et al*. 2014). Particularly diagnostic in the MS/MS spectra of the resveratrol conjugates were 307.027, 227.073, 185.061, 143.050 for resveratrol mono-sulfates and 403.103, 227.071, 185.061, 143.050 for resveratrol mono-glucuronides – neutral loss of *m/z* 80.00 and *m/z* 176.01 from sulfate or glucuronide moieties respectively (**Table 7.1**). In addition, the characteristic fragment ion *m/z* 227.07 from the resveratrol moiety was detected for all the resveratrol conjugates.

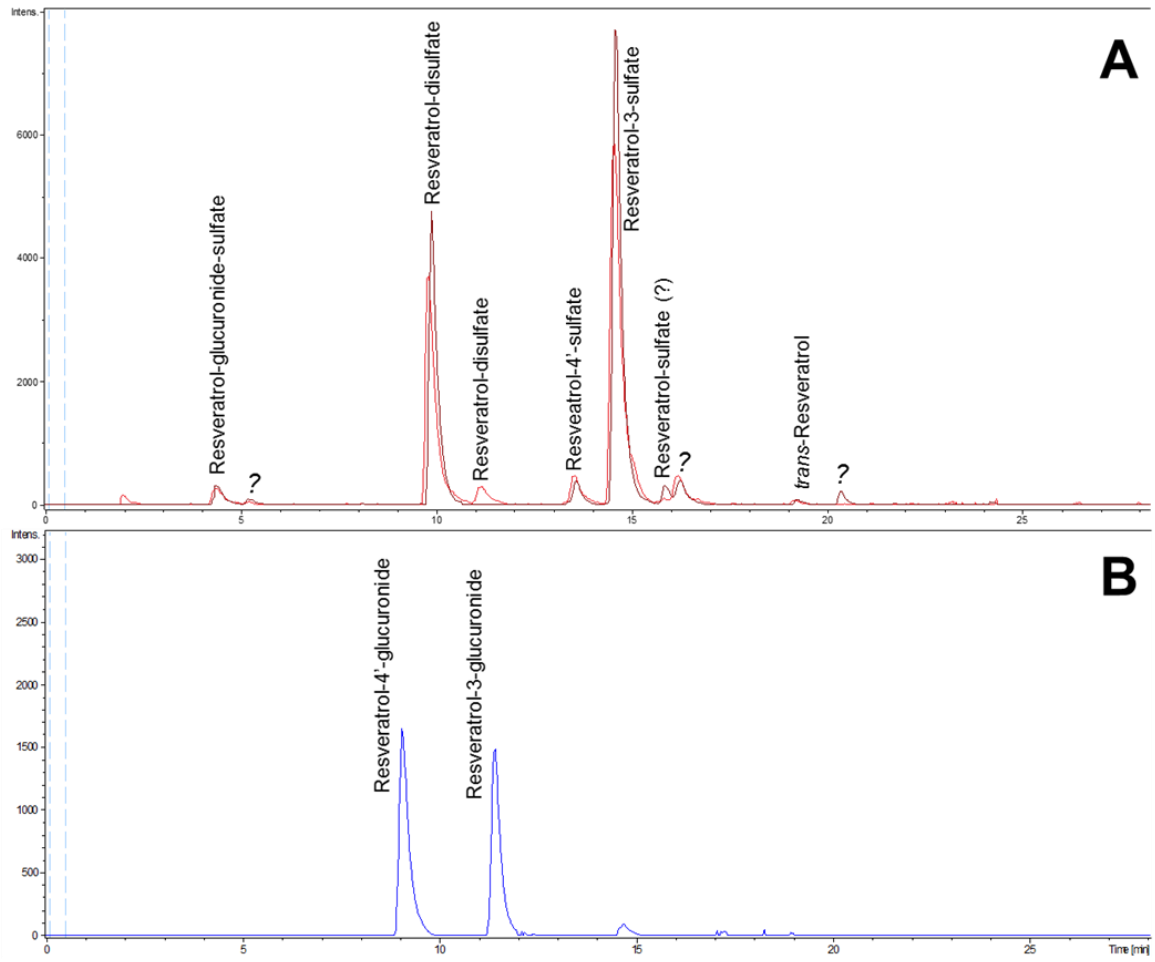

Fig. 7.3 - Examples of Extracted Ion Chromatograms of A) Resveratrol-sulfates (chromatograms from 2 samples are presented) and B) Resveratrol-glucuronides. Results obtained from plasma samples of RS patients after 6 weeks of de-alcoholised wine consumption.

Table 7.1 - MS/MS information for each resveratrol metabolite identified in plasma through preliminary LC-MS scan analysis.

Compound	Rt (min)	$[M-H]^-$	MS/MS fragments	Formula
Resveratrol glucuronide-sulfate	4.4	483.058	483.058, 386.985, 307.027, 227.071	C20H19O12S
Resveratrol di-sulfate1	9.8	386.985	386.985, 307.027, 227.071, 185.060, 143.050	C14H11O6S
Resveratrol-4'-glucuronide	10.1	403.103	403.103, 227.071, 185.061, 143.050	C20H19O9
Resveratrol di-sulfate2	11.2	403.1028	386.985, 307.027, 227.071, 185.060, 143.050	C14H11O6S
Resveratrol-3-glucuronide	12.5	403.103	403.103, 227.071, 185.061, 143.050	C20H19O9
Resveratrol-4'-sulfate	13.6	307.027	307.027, 227.073, 185.061, 143.050	C14H11O6S
Resveratrol-3-sulfate	14.6	307.027	307.027, 227.073, 185.061, 143.050	C14H11O6S
Resveratrol sulfate (?)	15.9	307.027	307.027, 227.073, 185.061, 143.050	C14H11O6S
trans-Resveratrol	20.3	227.070	227.070, 143.052	C14H11O3

7.3.3. Non-targeted profiling of metabolites in human plasma samples

Having established the presence of resveratrol and its key metabolites in the plasma samples after six weeks of resveratrol consumption in de-alcoholised red wine, further differences between the groups were characterised through a non-targeted metabolomics experiment. In this case, all 140 plasma samples from 70 patients at T0 and T6W were analysed in random order random by HPLC-MS/MS. This metabolomics experiment resulted in the extraction of 1741 features from aligned chromatograms, which were reduced to 1010 after manual curation of the data. Again, Molecular Features of Interest (MFOI) were selected by filtering using a cut-off of $p<0.05$ and larger than two-fold change.

Increased MFOI in plasma after intake for six weeks of resveratrol with de-alcoholised red wine

Through paired-comparisons, most differences were found between RS and NS groups after six weeks of intervention, with the data analysis identifying 96 significant different MFOI. Similarly, 54 significantly different MFOIs were detected when comparing the two time-points, T0 and T6w, for the RS group of patients. In total, 31 features were common between both comparisons. In addition, principal component analysis (PCA) was performed with all the identified MFOIs, resulting in a clear separation between the RS group after six weeks and all the other defined categories (**Fig. 7.4**).

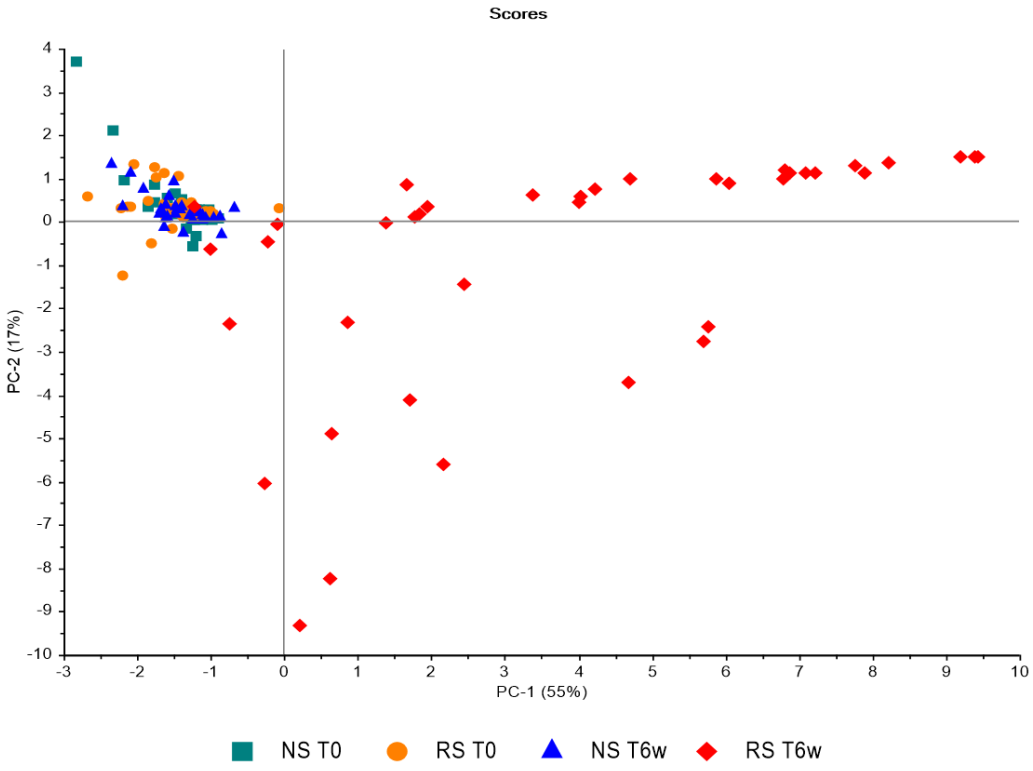

Fig. 7.4 - Principal components analysis (PCA) scores plot of the plasma samples of 70 individuals, considering the two different groups (RS and NS) at different time-points of de-alcoholised wine intake (T0 and T6w). PC1 55%; PC2 17%; PC3 15%.

These results confirmed the data obtained earlier on resveratrol conjugates by HPLC-MSMS analysis of plasma of a subgroup of participants in this study. Once more, identification of these compounds was performed using standards, but also by MS/MS experiments. In **Table 7.2** information is presented for detected resveratrol metabolites, including the MS/MS fragments and the t-test results after comparing the groups (RS vs. NS after six weeks) or time-points (T0 vs. T6w for RS group). Notably, the resveratrol mono sulfates and disulfates showed the most significant increase in the RS plasma samples (T6w), followed by the mono dihydro-sulfates. Free *trans*-resveratrol and a putative dihydro-resveratrol were also identified in these samples, but only in trace amounts.

Table 7.2 - Resveratrol metabolites increased in plasma after 6 weeks of RS de-alcoholised wine intake.

Compound	Rt (min)	$[M-H]^-$	Expected mass	Error (Da)	Average MW	Molecular formula	MS/MS	CV (%)	RS T0 Vs. T6w t test (p-value)	T6w NR Vs. RS t test (p-value)
Resveratrol-3-sulfate	15.08	307.0276	307.0282	0.0006	308.31	C14H12O6S	307.027, 227.071, 185.060, 143.050	23.779	7.41187 E-10	6.99288 E-10
Resveratrol-4'-sulfate	14.34	307.0304	307.0282	0.0023	308.31	C14H12O6S	307.027, 227.071, 185.060, 143.050	20.073	0.00111	0.00182
Resveratrol-4'-glucuronide	10.40	403.1028	403.1035	0.0006	404.37	C20H20O9	403.103, 227.071, 185.060, 143.050, 113.024	17.806	0.00786	0.00744
Resveratrol-3-glucuronide	12.98	403.1014	403.1035	0.0021	404.37	C20H20O9	403.103, 227.071, 185.060, 143.050, 113.024	18.593	0.03067	0.03976
Resveratrol-dihydro-sulfate	16.53	309.0410	309.0427	0.0117	310.05	C14H14O6S	309.044, 229.087	14.693	8.72917E-08	9.97918E-08
Resveratrol-disulfate	17.60	386.9847	386.9839	-0.0008	387.99	C14H12O9S2	386.985, 307.027, 227.071, 185.060, 143.050	10.169	6.0164 E-09	7.36045 E-09
trans-Resveratrol	19.40	227.0713	227.0714	0.0008	228.23	C14H12O3	227.0713, 147.0816	-	-	-
Dihydro-Resveratrol	19.40	229.0864	229.0865	0.0001	230.26	C14H14O3	229.0864, 149.0961	-	-	-

Apart from resveratrol conjugates, a number of other MFOIs were also present in higher concentrations in the RS plasma samples after six weeks and were unchanged or non-existent in all the other samples. A list of putative metabolites is presented in **Table 7.3**. The obtained m/z values of these putative resveratrol-related metabolites were searched in databases, finding possible matches for 6 MFOI. However, when compared to commercially-available standards, no match was found between those standards and the targeted molecular features.

Table 7.3 - Other molecular features of interest (MFOI) increased in plasma after 6 weeks of RS de-alcoholised wine intake. *p*-values are registered for each significant difference found between groups.

Tentatively identified compound	Rt (min)	[M-H]-	Expected mass	Error (Da)	Average MW	Molecular formula	CV (%)	RS T0 Vs. T6w t test (p-value)	T6w NR Vs. RS t test (p-value)
Pentahydroxy-methoxyflavone	15.76	331.0451	331.0459	0.0008	332.26	C16H12O8	15.26	2.04166E-07	2.88268E-07
Myricetin-arabinoside	14.11	449.0820	449.0725	0.0095	450.35	C20H18O12	22.04	2.31845E-07	1.72589E-07
Trihydroxydihydrochalcone-glucoside	18.00	419.1344	419.1348	0.0004	420.40	C21H24O9	21.36	7.70057E-05	3.75301E-05
Myricetin-galloylglucoside (M-2H)	19.13	315.0306	315.0433	0.0127	632.48	C28H24O17	13.03	0.00063	0.00073
Dihydronaringenin-sulphate (M+Na-2H)	16.29	375.0138	375.0156	0.0018	354.33	C15H14O8S	14.05	0.00047	0.00030
Indoxyl glucuronide	17.30	308.0338	308.0387	0.0049	309.08	C14H15NO7	14.63	0.02629	0.00050

Additionally, the results were analysed by gender PCA to assess if gender contributed to differences detected in the RS group (T6w). As shown in **Fig. 7.5**, no correlation was found between gender and the presence of any particular MFOI in plasma.

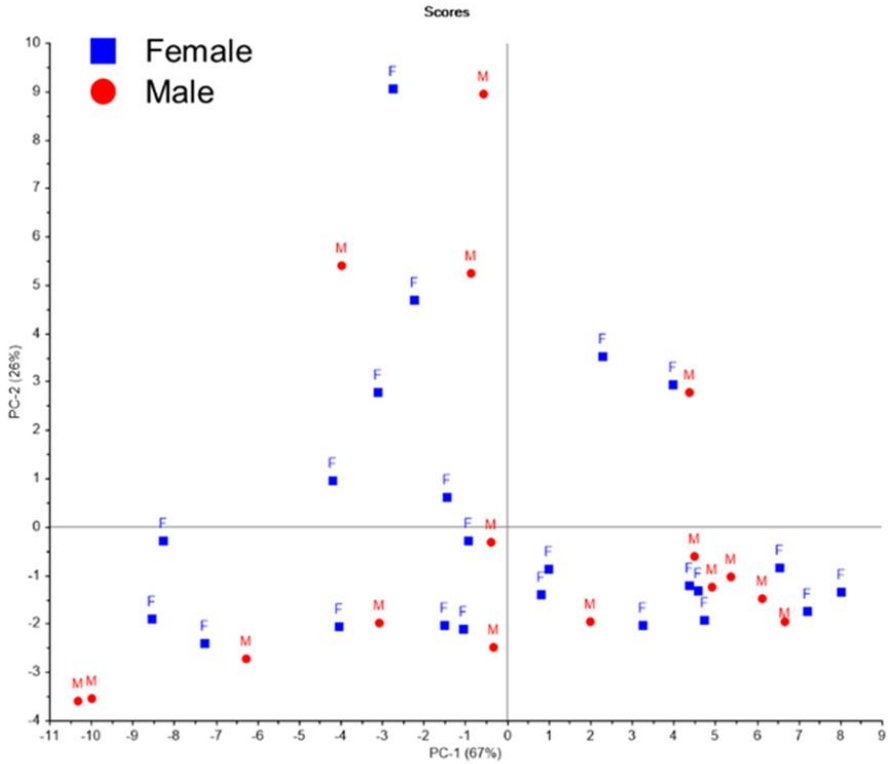

Fig. 7.5 - Principal component analysis (PCA) scores plot of the plasma samples of 39 patients from the RS group, after 6 weeks of intake. Samples were coloured per gender, showing no distinction between men and women.

Decreased MFOI in RS (T6w)

While a number of resveratrol conjugates and other metabolites were increased after intake of resveratrol in dealcoholized wine for six weeks, two MFOI were significantly decreased in the plasma samples of the RS group after six weeks, as shown in **Fig. 7.6A and 7.6B**. These MFOI with $[M-H]^-$, 403.3054 and 417.3231, co-eluted at the same retention time (27.2 min). As presented in **Fig. 7.6A**, there is also a significant correlation between the abundance of these two MFOI in plasma from RS and control (NR) participants at all time points.

To identify these features, MS/MS experiments were performed using plasma samples that contained the highest amounts of these features. The MS/MS fragmentation showed that these MFOI correspond to two different compounds (**Fig. 7.6C**). When the data was searched against databases matches were found with dihydroxycholesterol and dihydroxycholestanone were obtained.

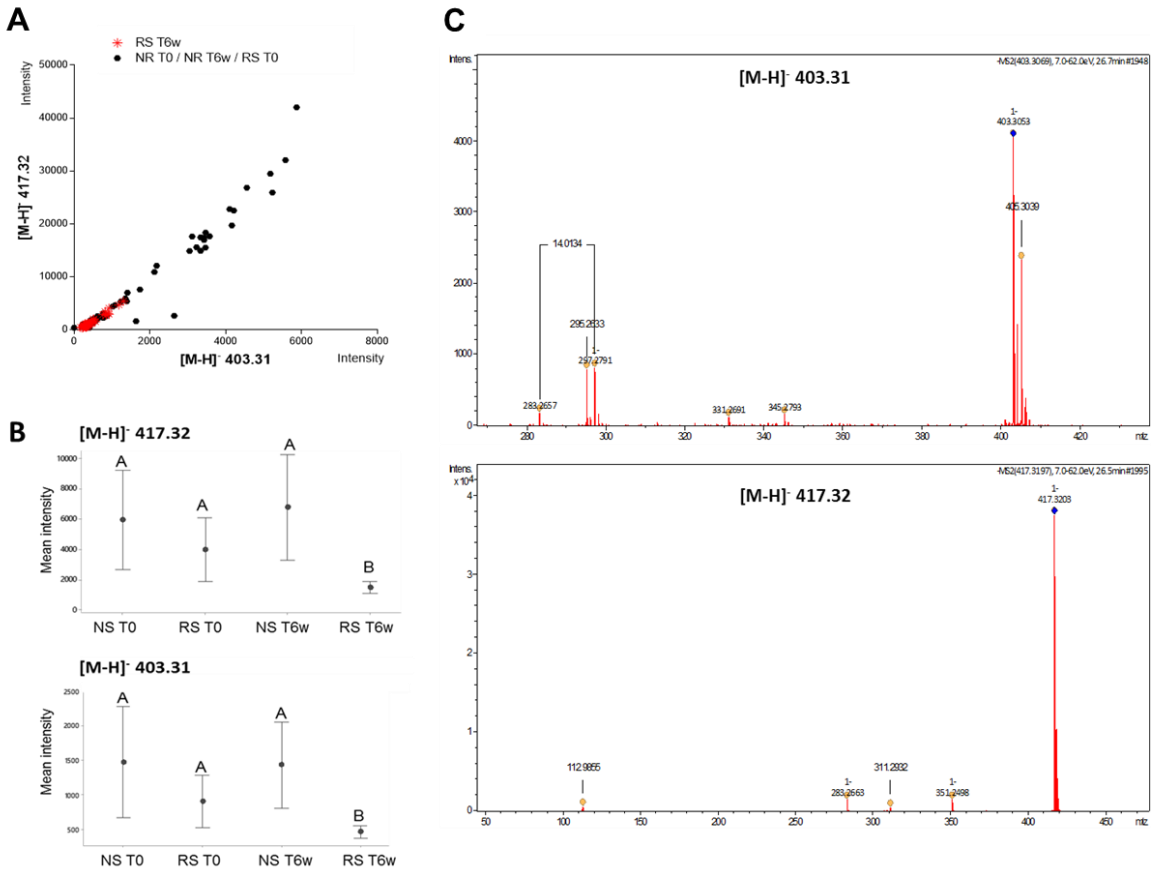

Fig. 7.6 - Details about the tentatively identified MFOI, decreased in RS T6w. A) Correlation between the mean intensities between the features. Samples from group RS, T6w are coloured in red. **B)** Interval plot IC 95% *Dunnet* test **C)** MS/MS spectra for each feature, presenting the parent ion and the respective fragments obtained. Collision energy of 7V.

7.4. Discussion

The main objective of this *in vivo* study was to characterise the effects of a daily consumption (over six weeks) of resveratrol in de-alcoholised red wine by analysing metabolites in human plasma with a non-targeted HPLC-MS/MS approach.

Changes in plasma concentrations of MFOIs which were likely caused by ingestion of resveratrol were identified by fold-change calculations, paired t-tests (p<0.05) and one-way ANOVA (p<0.05) for a comparison between treatment groups, RS and NS at t6w, and time points for RS, t0w and t6w.

In concordance with other authors we observed high levels of variability between individuals in relation to the concentration of MFOIs in the plasma (Vitaglione *et al*. 2005). Nonetheless, all the MFOIs were present at least in 50% of the plasma samples of the RS group, T6w. Notably, the increased levels of MFOIs after the six weeks of intervention were not significantly different between males and females, in-line with observations from a previous study. (Nunes *et al*. 2009)

The untargeted HPLC-MS approach confirmed that consumption of resveratrol resulted in traces of free *trans*-resveratrol in human plasma (Walle *et al*. 2004a; Vitaglione *et al*. 2005), together with substantial increases in phase-II conjugates, i.e. sulfates and glucuronides of resveratrol as described previously (Boocock *et al*. 2007). These observations corroborate the efficient uptake of resveratrol, its rapid metabolism, and the persistence of key metabolites in human plasma (Walle *et al*. 2004). In addition to metabolism by phase-II conjugation reactions, some resveratrol was reduced to dihydro-resveratrol and dihydro-resveratrol sulfate, confirming the results of Menet *et al*, 2014 (Menet *et al*. 2014).

From a mechanistic perspective, it remains to be established whether the phase-II conjugate dihydro-resveratrol sulfate is formed from dihydro-resveratrol, or indeed if the putative reductase reaction involves a resveratrol sulfates as substrate. In-line with earlier observations (Schroeter *et al*. 2015; Walle *et al*. 2004a), the results from this study emphasize the need for evaluating in greater detail the potential biological activities of all resveratrol conjugates.

The existence of resveratrol conjugates in plasma such as sulfates and glucuronides, was previously reported in other pharmacokinetic human studies, but only after single dose interventions that mainly focused on resveratrol absorption after 4 to 24 h of consumption (Boocock *et al*. 2007; Yiu *et al*. 2015). Additionally, the previous open-label trials performed for longer periods (more than one month) only described the clinical benefits associated with the intake of resveratrol, not focusing on the bioavailability of its molecules in plasma (Fujitaka *et al*. 2011; Tomé-Carneiro *et al*. 2012; Bhatt *et al*. 2012).

The results from the non-targeted metabolite analysis also showed that ingestion of resveratrol in de-alcoholised red wine resulted in significantly higher amounts of

additional MFOI in human plasma after the six weeks of ingestion (**Table 7.3**). These were present only at low abundance and a database search of these MFOIs led to the tentative identification of polyphenols, myricetin-arabinoside, pentahydroxy-methoxyflavone and myricetin-galloylglucoside. Myricetin has been described in red wine before (Gómez-Alonso *et al*. 2012) and it was herein detected on the based de-alcoholised wine through the targeted mass spectrometry analysis of polyphenols, hence the presence of these polyphenols in the RS-t6W plasma samples can be explained with resveratrol-induced changes to uptake and/or metabolism of other polyphenols in the de-alcoholised red wine. Alternatively, the resveratrol added to the de-alcoholised red wine might have contained traces of myricetin and other polyphenols.

Beyond polyphenol-related metabolites, two MFOIs could be observed in samples from RS and NS at all time points which were significantly reduced in the plasma samples of individuals from the RS group, after six weeks (**Fig. 7.6**). From MS/MS experiments and a database search, these MFOIs were found to be potential putative sterol derivatives. The reduction in plasma concentration of these putative sterols was only observed after six weeks of daily intake of a de-alcoholised red wine spiked with resveratrol, and this observation cannot be attributed to consumption typical red wine compounds such as alcohol or polyphenols. Hence this observation points towards a specific role of resveratrol ingestion in changing the profile of other human metabolites in plasma by a hitherto unknown mechanism. This provides further evidence of a likely biological activity of dietary resveratrol in humans, which should be characterised further together with the role of these MFOIs as biomarker candidates.

7.5. Conclusions

As far as we know, this is the first *in vivo* study describing the presence of resveratrol metabolites, and resveratrol-induced changes to other metabolites, in human plasma after a longer period of consumption of resveratrol (50 mg/100 mL per day for six weeks) in a de-alcoholised wine matrix.

Through the participation of a large number of individuals, and by using a non-targeted metabolomics experiment, it was possible to clearly differentiate between the resveratrol-related metabolic responses in RS and NS (control) groups, at different time points. This allowed identification of a large number of MFOIs that were significantly different between the groups. Further research is needed to understand if the phase-II resveratrol conjugates found in plasma are biologically active in target organs, and for obtaining evidence regarding the potential health impact of these metabolites in humans and the implications for our daily diet.

In addition, the observation of two putative sterol-related MFOIs which were decreased as specific response to resveratrol consumption points towards a specific role of resveratrol in changing the profile of other human metabolites in plasma by hitherto unknown mechanism(s). This provides further evidence of a likely biological activity of dietary resveratrol intake in humans.

Author Contributions

F.M. and C.S. designed the clinical study and are the chief investigators of the grant; V.B. is the clinical nurse who oversaw the clinical study including the collection of plasma samples.; N.L. provided lab supervision to J.F.; J.F, N.L., M.H. and C.S. designed the metabolomics experiments; J.F and N.L. performed the metabolomics experiments; J.F., N.L., M.H. and C.S. wrote the paper.

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General Discussion & Future work

S. cerevisiae protein extracts as endogenous alternatives for wine fining

Fining practices are frequently based in the application of oenological products from animal and mineral origins, which currently cause health and safety concerns among the wine sector. Consequently, their application in wine is becoming more and more restricted, according to the new health legislations defined by the International Organization of Vine and Wine (OIV) and the Codex Alimentarius International Code of Standards. For all these reasons, the development of cost-efficient, sustainable and safe fining alternatives is highly expected by the winemaking industry.

Herein, we have studied and benchmarked the fining potential of a wide range of currently available fining agents, in comparison with potential new fining alternatives, the oenological Yeast protein extracts (YPEs). In particular, we have demonstrated that the *S. cerevisiae* based formulations were not only able to efficiently improve the wine final color and brilliance of white and rosé wines, but also to reduce the polyphenolic content of red wines, when compared to the effects of reference fining agents. Indeed, we have validated the fining potential of YPEs for white wines clarification and stabilization, by indicating their higher efficiency to prevent and treat browning phenomena when compared to casein, an animal origin protein. Overall, these fining agents were found to be more efficient, sustainable and harmless, when compared to exogenous substances from mineral, animal or wheat origins.

The development of these oenological YPEs fulfils an increasing need among the wine sector to substitute animal proteins that might leave residues in wine and consequently cause allergies to susceptible individuals. Through the future production of these alternatives at large scale, we will support winemakers not only to produce less harmful wines to their consumers, but also to comply with the recent health and safety legislations. Such positive effects will enhance wine consumers' health and well-being and support the establishment of more trustful boundaries

between producers, regulation authorities and wine consumers. In fact, as a new worldwide ‘green trend’ has been raised across the marketplace, wine consumers will tend to acquire a broader variety of organic, eco-friendly products and to adopt healthier dietary and lifestyle habits. In fact, this consumers’ segment is currently more informed and demanding about the origin of the oenological additives applied into wine. Indeed, wines treated with the presented YPEs could be easily marked not only as safer, but also as a solution for vegetarian and vegan individuals that would previously exclude wines treated with exogenous fining agents based on animal proteins.

Considering the efficiency of these innovative fining agents, the production of YPEs at industrial scale denotes new prospects on the clarification and stabilization of *premium* quality wines and the possible creation of completely unique wine styles. Alongside to this, future investigations might be performed on the vast diversity of other oenological yeast strains and yeast-based components with post-fermentative applications in other types of wines. Accordingly, this study unveils other possible biotechnological potentialities of oenological yeasts among the sector, which go further beyond their primary function on conducting fermentation processes.

Alongside the introduction of innovative fining agents, we have also developed immunological-based assays that allow an accurate and simple detection of these haze-forming proteins. Consequently, these are useful tools to determine the minimum, essential amount of bentonite to prevent protein hazing, avoiding the common over-estimated application of bentonite clays. These immunological-based tools present an important upgrade to the conventional methods that are currently being used to estimate protein haze and which cause biased results.

In future, these novel tools will enable winemakers to test a wide-range of wine samples simultaneously at a winery environment. Therefore, this will reduce their usage in over-estimated dosages of bentonite at large scale, thus reducing the both fining costs and negative sensorial impact of bentonite clays on wine due to extensive aroma losses.

Wine as a functional beverage and the benefits of its polyphenols on consumers' health

Over the last decades, several health benefits have been associated with a moderate consumption of wine. Those effects have been mainly attributed to a complex variety of antioxidant polyphenols that are especially present in red wines. Despite these evidences, the potential synergic and/or antagonistic effects between bioactive molecules, such as individual polyphenols or between polyphenols and other wine constituents are still not fully understood. In fact, most of the studies have been focused on the effects of the specific antioxidant - resveratrol, due to its widely recognized bioactive potential, but knowledge is extremely scarce on the ultimate impact of the whole wine matrix, which comprises a complex variety of other bioactive molecules. In fact, while many studies highlight the beneficial health effects of resveratrol to wine consumers, evidences were obtained mostly from *in vitro* studies, both in animals and in humans.

In this thesis, we have demonstrated compelling evidences that the whole red wine matrix causes a higher stress reduction than its polyphenol components individually, by using an innovative *in vitro* ROS-generating environment, based on Progeria-SMCs, which stands as a cellular model for ageing. Additionally, we have observed that the average red wine content in (-)-epicatechin and procyanidin B2 tends to have a higher association with the cellular proliferation of these aging-model cells, when compared to resveratrol. Altogether, these findings stress out the importance to explore the potential bioactive impacts of the whole wine matrix and to study possible synergies between a wider range of its enclosed polyphenols. Moreover, this work emphasizes the importance to study wine as natural source of novel bioactive compounds, with potential applications for the nutraceutical and pharmaceutical industries.

Adding to this, the bioavailability of resveratrol and its conjugates is currently under an extensive debate, so it is crucial to comprehend to what extend the bioactive metabolites reach the target organs after their passage through long digestive

pathways in humans. Comprehensively, human trials are highly restricted due to ethical concerns, and restrained by the small number of available individuals and the short intervention periods. As result, the reduced number of *in vivo* studies performed so far lack in statistical power and frequently do not take individual variation into account.

To address this, herein we have combined a human intervention study with an untargeted metabolomics approach and confirmed the bioavailability of *trans*-resveratrol free from, resveratrol metabolites and other unexpected molecular features in plasma samples from patients with higher risk of having colon cancer, after daily consumption of de-alcoholised red wine for six weeks. Indeed, we have demonstrated the existence of the after consumption of de-alcoholised red wine enriched with *trans*-resveratrol. As far as we know, this was the first *in vivo* study that has demonstrated the existence of such resveratrol metabolites in plasma after a longer period of de-alcoholised red wine intake. Furthermore, this bioavailability study was conducted in a larger number of individuals, when compared to similar previous reports.

Overall, these findings underline the current need to further investigate the real cellular impact of resveratrol versus the impact of red-wine complex polyphenol matrices, as well as the bioavailability and activity of resveratrol-derived compounds and other important polyphenols in target organs, which altogether brings to the limelight the functional role of wine to the consumer's health.

General conclusion and future perspectives

The whole wine matrix encloses a complex range of bioactive metabolites, such as polyphenols, that may exert benefits to wine consumers' health. On the contrary, it might contain exogenous residues derived from winemaking practices that raise safety concerns. Overall, most of these potentially positive and negative effects remain unknown or under profound debate among the wine sector.

The innovative biotechnological solutions herein presented will improve the efficiency and safety of the current wine fining practices. In fact, the yeast-based alternatives developed are not only capable to fulfill the new demanding in the industry and in the markets, but also to support winemakers to comply with the current health regulations. Accordingly, the production and application of YPE should be implemented in future, at pilot and industrial scales, while certifying its quality along the production chain. Moreover, it will be important to perform a more detailed investigation on the sensorial impact of the YPEs in different wines and thus, guarantee the absence of any possible faults or off-flavours. As an important complement of the referred trials, the use of immunological-based methods to detect unstable proteins should be transferred to winery environments to efficiently minimize the current application of exogenous clays.

This work also highlights the potential antioxidant benefits of different wine matrices, complex polyphenolic mixtures and individual. According to our findings, and considering the vast array of possible wine metabolic compositions, arising from both the vast array of existing *Vitis vinifera* varieties, and their combination in blended wines, future work should explore the specific synergistic or antagonistic reactions occurring between different polyphenols and between polyphenols and other bioactive molecules from grape juice and wines. Additionally, the full potential of wine matrices should be further investigated as possible sources of a large range of other unknown bioactive molecules, with possible nutraceutical and pharmaceutical applications.

Considering our findings on the bioavailability of resveratrol, a widely recognized wine polyphenol, and its derived metabolites in the human body, underline the

urgent need to explore if resveratrol-derived compounds are biologically active in target organs through *in vivo* interventional studies. In future, clinical data should be acquired on the potential anti-carcinogenic effects of these metabolites and respective implication of implementing new dietary guidelines.

Globally, in this thesis, we have presented novel research evidences and have developed biotechnological tools that support the quality, safety and innovation of the global wine industry. Indeed, through the development of safer and more sustainable practices, as well as the valorization of important antioxidant and anti-carcinogenic wine constituents, our findings contribute to extend the current knowledge on Wine&Health that increases awareness from the winemaking stages until final consumers, thus fulfilling its ancient recognition as a functional beverage.

Annexes

Annex II - Chapter 2. Supplementary figures and tables.

Table S2.1 - Extensive description of the reference fining agents tested in this study.

Category	Code	Origin	Manufacture indications						Protein content (w/w %)
			Main protein	Other fining components	Form	Recommended wine type	Recommended dosage - Liquid form: ml/hL - Powder form: g/hL		
							Min	*Max*	
Bentonites	B1	Mineral	Not applicable	-	Powder	White/Rose	5	100	-
	B2	Mineral	Not applicable	-	Powder	White/Rose	40	120	-
	B3	Mineral	Not applicable	-	Powder	White/Rose	40	120	-
	B4	Mineral	Not applicable	-	Powder	White/Rose	20	100	-
	B5	Mineral	Not applicable	-	Powder	White/Rose	20	100	-
	B6	Mineral	Not applicable	-	Powder	White/Rose	10	60	-
	B7	Mineral	Not applicable	-	Powder	White/Rose	20	100	-
	B8	Mineral	Not applicable	-	Powder	White/Rose	20	100	-
Isinglasses	I1	Animal	Collagen (fish bladder)	-	Liquid	White/Rose	2	20	17,46
	I2	Animal	Collagen (fish bladder)	-	Liquid	White/Rose	2	3	23,86
	I3	Animal	Collagen (fish bladder)	-	Liquid	White/Rose	1	4	34,90
	I4	Animal	Collagen (fish bladder)	-	Liquid	White/Rose	100	120	35,51
	I5	Animal	Collagen (fish bladder)	-	Liquid	White/Rose	1,5	3	39,23
	I6	Animal	Collagen (fish bladder)	-	Liquid	White/Rose	0,5	1,5	10,41
	I7	Animal	Collagen (fish bladder)	-	Liquid	White/Rose	0,5	1,5	21,62
	I8	Animal	Collagen (fish bladder)	-	Liquid	Red	5	100	40,88
	I9	Animal	Collagen (fish skin)	-	Liquid	White/Rose	1	3	14,36
	I10	Animal	Collagen (fish skin)	-	Liquid	White/Rose	1	3	10,68
Caseins	C1	Animal	Casein	-	Powder	White/Rose	20	100	59,95
	C2	Animal	Casein	-	Powder	White/Rose	20	100	53,83
Vegetable proteins	VP1	Vegetable	Wheat gluten	-	Powder	White/Rose	10	30	60,00
	VP2	Vegetable	Wheat gluten	-	Powder	Red	5	25	50,31
	VP3	Vegetable	Wheat gluten	-	Powder	Red	5	30	57,71
Mixtures	M1	Mixed	Casein	PVPP	Powder	White/Rose	40	80	26,86
	M2	Mixed	Casein	PVPP; Bentonite	Powder	White/Rose	30	90	6,061
	M3	Mixed	Gluten	Bentonite	Powder	White/Rose	40	120	22,50
	M4	Animal	Casein	Gelatin	Powder	White/Rose	5	10	28,65
	M5	Mixed	Gelatin	Bentonite	Powder	White/Rose	10	15	39,71
	M6	Mixed	Casein	PVPP	Powder	White/Rose	15	70	2,580
	M7	Mixed	Gelatin	Bentonite; PVPP	Powder	White/Rose	15	100	1,641
	M8	Mixed	Gluten	Bentonite; PVPP	Powder	White/Rose	30	80	0,210
	M9	Mixed	Gluten	Cellulose; PVPP	Powder	Red	30	100	36,05
	M10	Mixed	Isinglass	Bentonite; PVPP	Powder	White/Rose	5	10	4,980
Gelatins	G1	Animal	Gelatin (pork source)	-	Liquid	Red	5	50	4,980
	G2	Animal	Gelatin (pork source)	-	Liquid	Red	5	100	11,44
	G3	Animal	Gelatin (pork source)	-	Liquid	Red	20	150	30,16
	G4	Animal	Gelatin (pork source)	-	Liquid	Red	20	100	32,37
	G5	Animal	Gelatin (pork source)	-	Powder	Red	20	40	16,40
	G6	Animal	Gelatin (pork source)	-	Liquid	Red	8	15	13,44
	G7	Animal	Gelatin (pork source)	-	Powder	Red	30	60	24,27
	G8	Animal	Gelatin (pork source)	-	Powder	Red	20	50	42,68
	G9	Animal	Gelatin (pork source)	-	Liquid	Red	7	10	24,22
	G10	Animal	Gelatin (pork source)	-	Liquid	Red	10	20	57,13
	G11	Animal	Gelatin (pork source)	-	Liquid	Red	5	100	32,63
	G12	Animal	Gelatin (pork source)	-	Powder	Red	40	100	39,18
	G13	Animal	Gelatin (pork source)	-	Powder	Red	30	60	52,50
	G14	Animal	Gelatin (pork source)	-	Powder	Red	20	80	27,62
	G15	Animal	Gelatin (pork source)	-	Liquid	Red	10	80	36,05
	G16	Animal	Gelatin (pork source)	-	Liquid	Red	10	60	24,26
	G17	Animal	Gelatin (pork source)	-	Liquid	Red	10	120	26,92
	G18	Animal	Gelatin (pork source)	-	Liquid	Red	5	20	29,75
Albumins	A1	Animal	Albumin	-	Powder	Red	5	10	61,30
	A2	Animal	Albumin	-	Powder	Red	8	12	63,84
	A3	Animal	Albumin	-	Powder	Red	6	10	73,31
	A4	Animal	Albumin	-	Powder	Red	5	10	71,52

Table S2.2 - Description of the Yeast Protein Extracts (YPEs) tested in this study.

Category	Code	Origin	Form	White/Rose Wine Tested dosage (g/hL)		Red Wine Tested dosage (g/hL)		Protein content (w/w %)
				Min	***Max***	***Min***	***Max***	
Yeast Protein Extracts	**YPE1**	Yeast	Liquid	10	20	30	40	53,02
	YPE2	Yeast	Liquid	10	20	30	40	58,19
	YPE3	Yeast	Liquid	10	20	30	40	55,23
	YPE4	Yeast	Liquid	10	20	30	40	50,35

Annex III - Chapter 3. Supplementary figures and tables

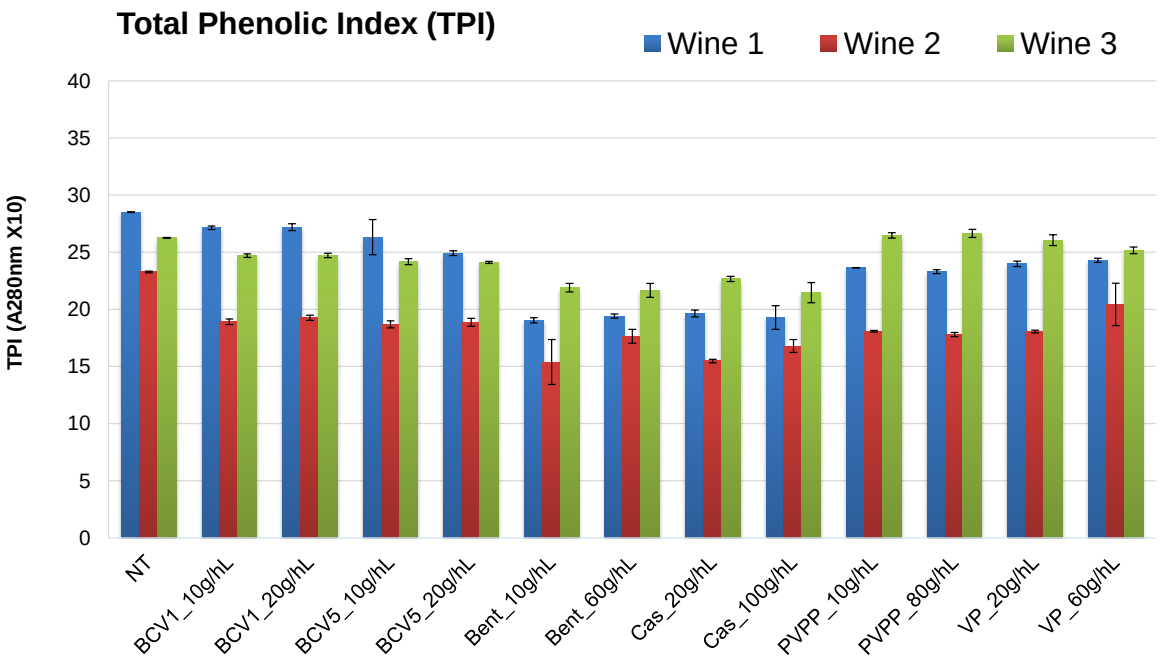

Fig. S3.1 - Total phenolic content before and after the fining experiments in 250ml bottles, for the three types of wine tested. Untreated wine: NT. Wines treated with: Yeast protein extracts: BCV1 and BCV5; Bent: Bentonite; Cas: Casein; PVPP: Polyvinylpolypyrrolidon; VP: Vegetable protein. Bars indicate mean ± SD (n = 3).

Table S3.2 - Conventional oenological parameters acquired by Infrared Fourier-transform spectrometer – Wine 2. Wines treated with: Yeast protein extracts: BCV1 and BCV5; Bent: Bentonite; Cas: Casein; PVPP: Polyvinylpolypyrrolidon; VP: Vegetable protein. Untreated wine: NT. Results are relative to fining experiments after fermentation, performed in triplicates. Values are presented as mean ± SD (n = 3).

Wine 2		Density (g/L)	Ethanol (% vol)	Volatile acidity (g/L H_2SO_4)	Tritable acidity (g/L H_2SO_4)	pH	Glycerol (g/L)	Malic Acid (g/L)	Lactic Acid (g/L)	Total SO_2 (mg/L)	Free SO_2 (mg/L)
BCV1	10g/hL	989,8 ± 0,1	13,32 ± 0,15	0,60 ± 0,04	6,53 ± 0,01	3,40 ± 0,01	5,81 ± 0,03	1,15 ± 0,01	0,52 ± 0,02	113,1 ± 0,1	34,9 ± 0,5
	20g/hL	989,8 ± 0,3	13,27 ± 0,17	0,60 ± 0,03	6,51 ± 0,02	3,40 ± 0,02	6,03 ± 0,02	1,19 ± 0,02	0,48 ± 0,04	114,3 ± 0,0	35,2 ± 0,4
BCV5	10g/hL	989,7 ± 0,1	13,30 ± 0,15	0,58 ± 0,00	6,58 ± 0,07	3,39 ± 0,01	6,22 ± 0,03	1,19 ± 0,01	0,51 ± 0,01	112,5 ± 0,4	34,0 ± 0,3
	20g/hL	989,6 ± 0,1	13,27 ± 0,11	0,54 ± 0,05	6,64 ± 0,03	3,38 ± 0,04	6,32 ± 0,05	1,27 ± 0,03	0,50 ± 0,02	115,0 ± 0,5	32,0 ± 0,5
Bent	10g/hL	989,1 ± 0,2	13,20 ± 0,17	0,60 ± 0,00	6,64 ± 0,05	3,35 ± 0,02	6,21 ± 0,01	0,98 ± 0,04	0,35 ± 0,03	112,7 ± 0,3	34,0 ± 0,4
	60g/hL	989,3 ± 0,4	13,02 ± 0,17	0,60 ± 0,06	6,44 ± 0,02	3,37 ± 0,04	6,05 ± 0,05	1,04 ± 0,02	0,41 ± 0,02	114,6 ± 0,3	37,0 ± 0,1
Cas	20g/hL	989,3 ± 0,2	13,18 ± 0,10	0,62 ± 0,05	6,65 ± 0,00	3,36 ± 0,02	6,01 ± 0,02	1,13 ± 0,03	0,29 ± 0,04	117,1 ± 0,3	35,0 ± 0,1
	100g/hL	989,4 ± 0,2	12,97 ± 0,17	0,57 ± 0,02	6,44 ± 0,02	3,38 ± 0,03	6,54 ± 0,05	1,10 ± 0,02	0,27 ± 0,04	112,4 ± 0,4	35,2 ± 0,2
PVPP	10g/hL	989,9 ± 0,0	13,29 ± 0,11	0,59 ± 0,06	6,64 ± 0,01	3,36 ± 0,02	6,80 ± 0,03	0,87 ± 0,02	0,53 ± 0,00	115,1 ± 0,0	36,0 ± 0,1
	80g/hL	990,0 ± 0,6	13,10 ± 0,10	0,58 ± 0,03	6,51 ± 0,03	3,36 ± 0,04	6,52 ± 0,02	0,90 ± 0,03	0,51 ± 0,00	116,4 ± 0,0	32,0 ± 0,0
VP	20g/hL	989,9 ± 0,2	13,26 ± 0,14	0,59 ± 0,03	6,60 ± 0,03	3,36 ± 0,02	6,69 ± 0,02	0,90 ± 0,04	0,52 ± 0,01	103,0 ± 0,3	36,0 ± 0,1
	60g/hL	990,0 ± 0,2	13,20 ± 0,15	0,59 ± 0,05	6,50 ± 0,05	3,37 ± 0,01	6,65 ± 0,05	0,92 ± 0,05	0,50 ± 0,01	103,3 ± 0,1	37,0 ± 0,4
NT		989,3 ± 0,0	12,96 ± 0,17	0,53 ± 0,04	6,49 ± 0,03	3,36 ± 0,00	7,02 ± 0,05	1,20 ± 0,05	0,27 ± 0,00	115,2 ± 0,0	35,3 ± 0,6

Table S3.3 - Conventional oenological parameters acquired by Infrared Fourier-transform spectrometer – Wine 3. Wines treated with: Yeast protein extracts: BCV1 and BCV5; Bent: Bentonite; Cas: Casein; PVPP: Polyvinylpolypyrrolidon; VP: Vegetable protein. Untreated wine: NT. Results are relative to fining experiments after fermentation, performed in triplicates. Values are presented as mean ± SD (n = 3).

Wine 3		Density (g/L)	Ethanol (% vol)	Volatile acidity (g/L H_2SO_4)	Tritable acidity (g/L H_2SO_4)	pH	Glycerol (g/L)	Malic Acid (g/L)	Lactic Acid (g/L)	Total SO_2 (mg/L)	Free SO_2 (mg/L)
BCV1	10g/hL	989,4 ± 0,0	15,60 ± 0,13	0,86 ± 0,02	7,69 ± 0,01	3,33 ± 0,00	7,21 ± 0,02	1,31 ± 0,01	0,75 ± 0,02	125,1 ± 0,1	52,0 ± 0,1
	20g/hL	989,5 ± 0,3	15,53 ± 0,12	0,85 ± 0,01	7,63 ± 0,04	3,34 ± 0,01	7,23 ± 0,05	1,34 ± 0,03	0,73 ± 0,08	124,3 ± 0,3	53,0 ± 0,5
BCV5	10g/hL	989,5 ± 0,4	15,58 ± 0,18	0,86 ± 0,00	7,66 ± 0,01	3,33 ± 0,00	7,22 ± 0,02	1,28 ± 0,00	0,76 ± 0,05	124,5 ± 0,1	54,0 ± 0,4
	20g/hL	989,7 ± 0,1	15,50 ± 0,16	0,86 ± 0,02	7,57 ± 0,05	3,33 ± 0,02	7,16 ± 0,00	1,23 ± 0,00	0,74 ± 0,00	126,0 ± 0,0	45,4 ± 0,3
Bent	10g/hL	989,7 ± 0,0	15,48 ± 0,20	0,95 ± 0,02	6,97 ± 0,03	3,32 ± 0,03	7,11 ± 0,06	1,12 ± 0,05	0,52 ± 0,05	128,7 ± 0,2	52,0 ± 0,2
	60g/hL	989,7 ± 0,0	15,23 ± 0,16	0,93 ± 0,01	6,68 ± 0,00	3,34 ± 0,06	7,10 ± 0,06	1,07 ± 0,02	0,47 ± 0,01	130,6 ± 0,0	52,0 ± 0,1
Cas	20g/hL	989,8 ± 0,1	15,42 ± 0,15	0,95 ± 0,02	6,94 ± 0,02	3,33 ± 0,02	7,05 ± 0,05	1,16 ± 0,03	0,52 ± 0,03	122,1 ± 0,1	50,0 ± 0,0
	100g/hL	989,8 ± 0,6	15,15 ± 0,11	0,91 ± 0,01	6,69 ± 0,04	3,35 ± 0,08	7,01 ± 0,03	0,99 ± 0,00	0,50 ± 0,06	123,4 ± 0,2	51,0 ± 0,2
PVPP	10g/hL	990,1 ± 0,0	15,43 ± 0,13	0,91 ± 0,03	7,58 ± 0,00	3,33 ± 0,05	7,13 ± 0,00	1,03 ± 0,01	0,78 ± 0,04	125,1 ± 0,0	52,0 ± 0,1
	80g/hL	990,1 ± 0,3	15,33 ± 0,10	0,91 ± 0,02	7,50 ± 0,01	3,35 ± 0,05	7,14 ± 0,01	0,99 ± 0,00	0,74 ± 0,04	123,4 ± 0,0	53,0 ± 0,8
VP	20g/hL	990,2 ± 0,1	15,39 ± 0,12	0,91 ± 0,01	7,64 ± 0,00	3,33 ± 0,01	7,05 ± 0,01	1,07 ± 0,02	0,77 ± 0,03	122,5 ± 0,5	54,0 ± 0,3
	60g/hL	990,3 ± 0,5	15,14 ± 0,10	0,90 ± 0,00	7,51 ± 0,01	3,33 ± 0,00	7,11 ± 0,00	1,05 ± 0,05	0,73 ± 0,01	130,2 ± 0,1	55,0 ± 0,1
NT		989,8 ± 0,0	15,49 ± 0,21	0,89 ± 0,02	7,64 ± 0,02	3,32 ± 0,02	7,56 ± 0,02	1,04 ± 0,03	0,78 ± 0,00	127,2 ± 0,2	51,0 ± 0,0

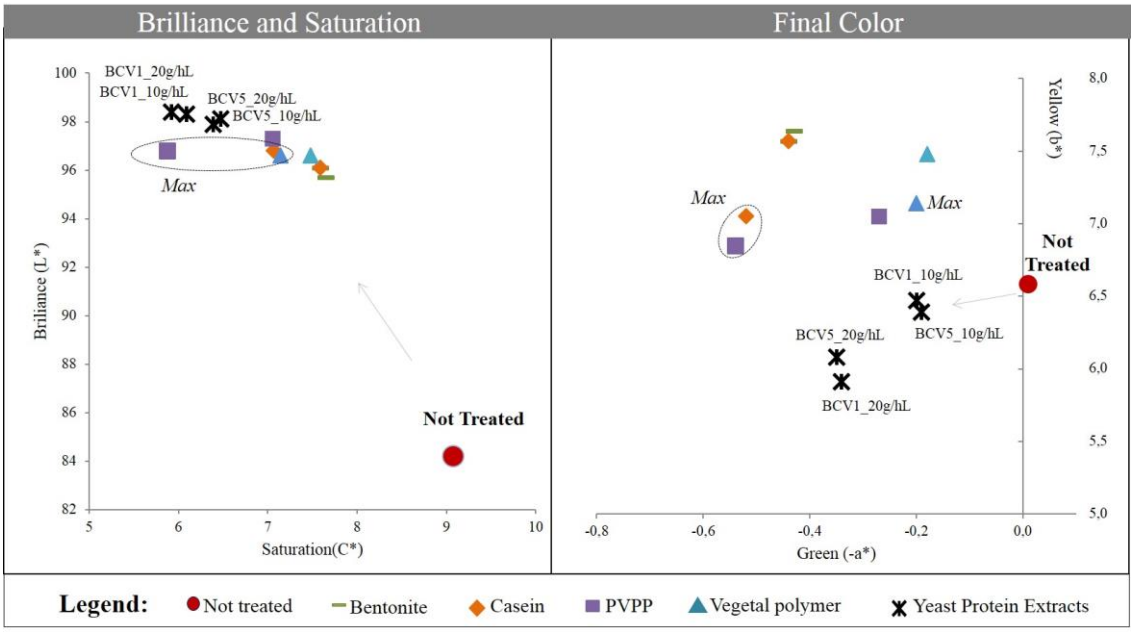

Fig. S3.2 - Chromatic characterization using CIELab system: Saturation (C*), Brilliance (L*), Green (-a*) and Yellow (b*) values. Results were obtained before and after treatment of wine 2 with YPE and different fining agents. Two dosages were tested by treatment (Min.-minimum; Max.-maximum).

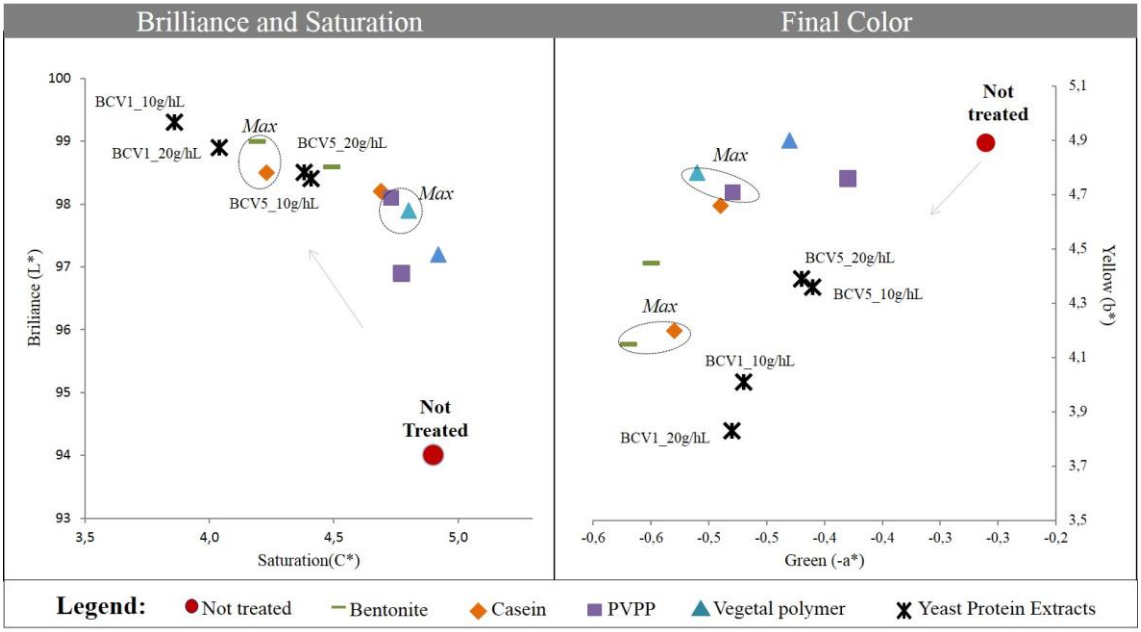

Fig. S3.3 - Chromatic characterization using CIELab system: Saturation (C*), Brilliance (L*), Green (-a*) and Yellow (b*) values. Results were obtained before and after treatment of wine 3 with YPE and different fining agents. Two dosages were tested by treatment (Min.-minimum; Max.-maximum).

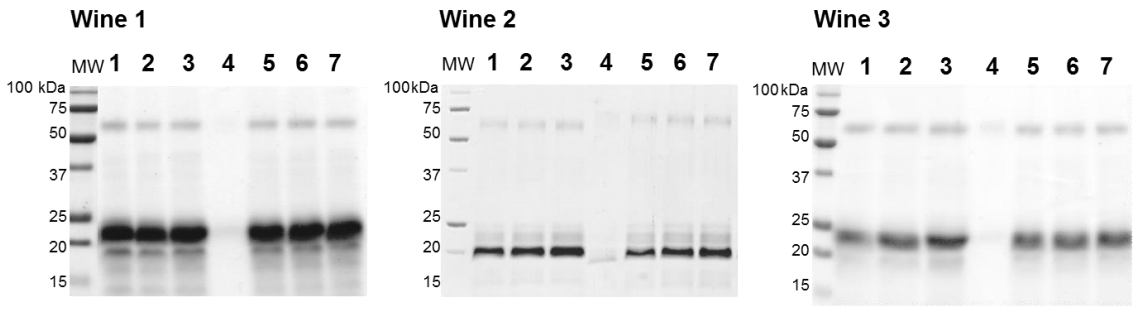

Fig. S3.4 - SDS-PAGE, 12,5%. Coomassie staining. Total protein precipitated from 1ml of each wine sample: 1) Not treated wine. Wines treated with fining agents (max. dosage): 2) BCV1; 3) BCV5; 4) Bentonite; 5) Casein; 6) PVPP; 7) Vegetable protein.

Table S3.1 - Conventional oenological parameters acquired by Infrared Fourier-transform spectrometer – Wine 1. Wines treated with: Yeast protein extracts: BCV1 and BCV5; Bent: Bentonite; Cas: Casein; PVPP: Polyvinylpolypyrrolidon; VP: Vegetable protein. Untreated wine: NT. Results are relative to fining experiments after fermentation, performed in triplicates. Values are presented as mean ± SD (n = 3).

Wine 1		Density (g/L)	Ethanol (% vol)	Volatile acidity (g/L H_2SO_4)	Tritable acidity (g/L H_2SO_4)	pH	Glycerol (g/L)	Malic Acid (g/L)	Lactic Acid (g/L)	Total SO_2 (mg/L)	Free SO_2 (mg/L)
BCV1	10g/hL	987,5 ± 0,2	13,56 ± 0,17	0,64 ± 0,05	7,47 ± 0,05	3,29 ± 0,01	6,81 ± 0,05	0,93 ± 0,02	1,09 ± 0,01	105,1 ± 0,0	44,9 ± 0,4
	20g/hL	987,6 ± 0,3	13,50 ± 0,15	0,63 ± 0,02	7,41 ± 0,01	3,30 ± 0,05	6,79 ± 0,01	0,91 ± 0,01	1,07 ± 0,00	104,3 ± 0,0	45,2 ± 0,4
BCV5	10g/hL	987,5 ± 0,3	13,54 ± 0,15	0,63 ± 0,01	7,47 ± 0,02	3,30 ± 0,04	6,75 ± 0,00	0,95 ± 0,02	1,08 ± 0,00	103,5 ± 0,2	45,0 ± 0,3
	20g/hL	987,6 ± 0,3	13,51 ± 0,15	0,63 ± 0,00	7,46 ± 0,00	3,29 ± 0,02	6,80 ± 0,00	1,02 ± 0,03	1,07 ± 0,00	105,0 ± 0,0	45,4 ± 0,5
Bent	10g/hL	987,7 ± 0,5	13,44 ± 0,15	0,68 ± 0,01	7,03 ± 0,10	3,27 ± 0,01	6,75 ± 0,02	0,96 ± 0,06	1,05 ± 0,01	104,7 ± 0,0	45,8 ± 0,2
	60g/hL	988,2 ± 0,2	13,19 ± 0,16	0,68 ± 0,02	7,08 ± 0,03	3,29 ± 0,01	6,82 ± 0,03	1,00 ± 0,01	1,07 ± 0,01	103,6 ± 0,1	45,1 ± 0,3
Cas	20g/hL	987,8 ± 0,4	13,34 ± 0,10	0,70 ± 0,06	6,83 ± 0,05	3,28 ± 0,02	6,93 ± 0,01	1,12 ± 0,00	1,12 ± 0,00	105,1 ± 0,5	44,9 ± 0,2
	100g/hL	988,1 ± 0,3	13,11 ± 0,11	0,69 ± 0,05	6,63 ± 0,07	3,31 ± 0,04	7,02 ± 0,00	1,17 ± 0,02	1,08 ± 0,00	103,4 ± 0,1	45,2 ± 0,1
PVPP	10g/hL	987,9 ± 0,3	13,47 ± 0,15	0,68 ± 0,05	7,43 ± 0,10	3,28 ± 0,00	6,87 ± 0,02	0,89 ± 0,01	1,12 ± 0,02	105,1 ± 0,1	44,8 ± 0,2
	80g/hL	988,0 ± 0,6	13,31 ± 0,15	0,68 ± 0,04	7,28 ± 0,04	3,28 ± 0,01	6,82 ± 0,03	0,90 ± 0,00	1,08 ± 0,02	105,4 ± 0,5	45,3 ± 0,0
VP	20g/hL	987,9 ± 0,2	13,47 ± 0,15	0,68 ± 0,00	7,39 ± 0,10	3,28 ± 0,05	7,01 ± 0,01	0,91 ± 0,03	1,10 ± 0,00	104,0 ± 0,8	45,2 ± 0,5
	60g/hL	988,0 ± 0,1	13,38 ± 0,15	0,68 ± 0,01	7,32 ± 0,02	3,30 ± 0,01	6,85 ± 0,00	0,91 ± 0,00	1,08 ± 0,00	103,3 ± 0,1	45,2 ± 0,6
NT		988,3 ± 0,2	13,04 ± 0,15	0,70 ± 0,10	6,69 ± 0,02	3,31 ± 0,00	7,21 ± 0,02	1,22 ± 0,05	1,05 ± 0,00	105,2 ± 0,1	45,3 ± 0,5

Annex VI - Chapter 6. Supplementary figures and tables

Table S6.1. Multiple reactions monitoring parameters for each polyphenol and internal standards; and analytical parameters for the calibration curves.

Polyphenol	Transition (m/z)[1]	Retention time (min)	CE (eV)	CXP (eV)	DP (eV)	Linear range (ng/µL)	r^2
trans-Caffeic Acid	181.2 → 163.3	3.8	15	14	56	0.018 - 0.901	0.999
(-) Epicatechin	291.2 → 139.3	4.5	23	12	101	0.029 - 2.032	0.998
(+) Catechin	291.2 → 139.3	3.6	23	8	106	0.029 - 2.032	0.999
(+) Gallocatechin	307.2 → 139.3	2.5	21	12	106	0.031 - 1.531	0.999
(-) Epigallocatechin	307.2 → 139.3	3.5	21	24	111	0.031 - 2.144	0.999
Procyanidin B2	579.3 → 127.2	3.8	45	10	111	0.058 - 4.050	0.995
Procyanidin C1	867.4 → 579.4	4.8	23	36	126	0.087 - 4.334	0.999
Ellagic Acid	300.9 → 228.9	6.4	-38	-1	-115	0.030 - 1.511	0.997
trans-Resveratrol	226.7 → 182.5	7.8	-28	-13	-65	0.029 - 2.032	0.999

[1] Transitions used for quantification.
Collision energy (CE), collision exit potential (CXP) and declustering potential (DP).

Table S6.2. List of the red wine tested in this study and respective details.

Red wines	Name	Year	DOC	Varieties	Variety main origin (>50%)
1	Carmim Reguengos Alentejo Doc	2014	Alentejo	(40%) Trincadeira; (40%) Aragonez; (20%) Castelão	Portuguese
2	Vale do Rico Homem	2014	Alentejo	Aragonez; Trincadeira; Syrah	Portuguese
3	Castelo de Arraiolos - Prestige	Lote	Alentejo	Syrah	Other
4	Herdade do Peso Trinca Bolotas	2014	Alentejo	(54%) Alicante Bouschet; (30%) Touriga Nacional; (16%) Aragonez	Other
5	Herdade do Peso Colheita Tinto 2013	2013	Alentejo	(55%) Syrah; (25%) Alicante Bouschet; (20%) Touriga Nacional	Other
6	Herdade do Peso Reserva Tinto 2013	2013	Alentejo	(60%) Syrah; (40%) Alicante Bouschet	Other
7	Marquês de Marialva Baga Reserva	2010	Bairrada	Touriga Nacional	Portuguese
8	Marquês de Marialva Tinto	2010	Bairrada	Touriga Nacional; Tinta Roriz; Baga	Portuguese
9	Colheita tinto Q. dos Carvalhais	2011	Dão	(93%) Touriga Nacional; (5%) Alfrocheiro; (2%) Tinta Roriz	Portuguese
10	Touriga Nacional Q. dos Carvalhais	2012	Dão	Touriga Nacional	Portuguese
11	Casa Ferreirinha Papa Figos Tinto 2013	2013	Douro	(35%) Tinta Roriz; (30%) Touriga Franca; (20%) Tinta Barroca; (15%) Touriga Nacional	Portuguese
12	Casa Ferreirinha Vinha Grande Tinto 2011	2011	Douro	(35%) Touriga Franca; (30%) Tinta Roriz; (25%) Touriga Nacional; (10%) Tinta Barroca	Portuguese
13	Casa Ferreirinha Quinta da Leda Tinto 2012	2012	Douro	(55%) Touriga Franca; (30%) Touriga Nacional; (10%) Tinta Roriz; (5%) Tinto Cão	Portuguese
14	Altano Douro Reserva Symington	2011	Douro	Touriga Nacional	Portuguese
15	Quinta do Poço do Lobo Vindima 1995	1995	Bairrada	Baga; Castelão Nacional; Moreto	Portuguese
16	Quinta da Laboeira Colheita 2011	2011	Bairrada	Touriga Nacional	Portuguese
17	Quinta das Bágeiras Vinho Tinto Reserva 2009	2009	Bairrada	Baga (60%); Touriga Nacional (40%)	Portuguese
18	FP Baga & Touriga Nacional Tinto 2009	2009	Bairrada	Baga; Touriga Nacional	Portuguese
19	Baga Campolargo	2010	Bairrada	Baga	Portuguese
20	Nelson Neves Merlot Reserva 2010	2010	Bairrada	Merlot	Other
21	Calda Bordaleza Campolargo Tinto 2009	2009	Bairrada	Merlot (20%); Petit Verdot (30%); Cabernet Sauvignon (50%)	Other
22	Luís Pato Red Wine 2011	2011	Bairrada	Baga; Touriga Nacional	Portuguese

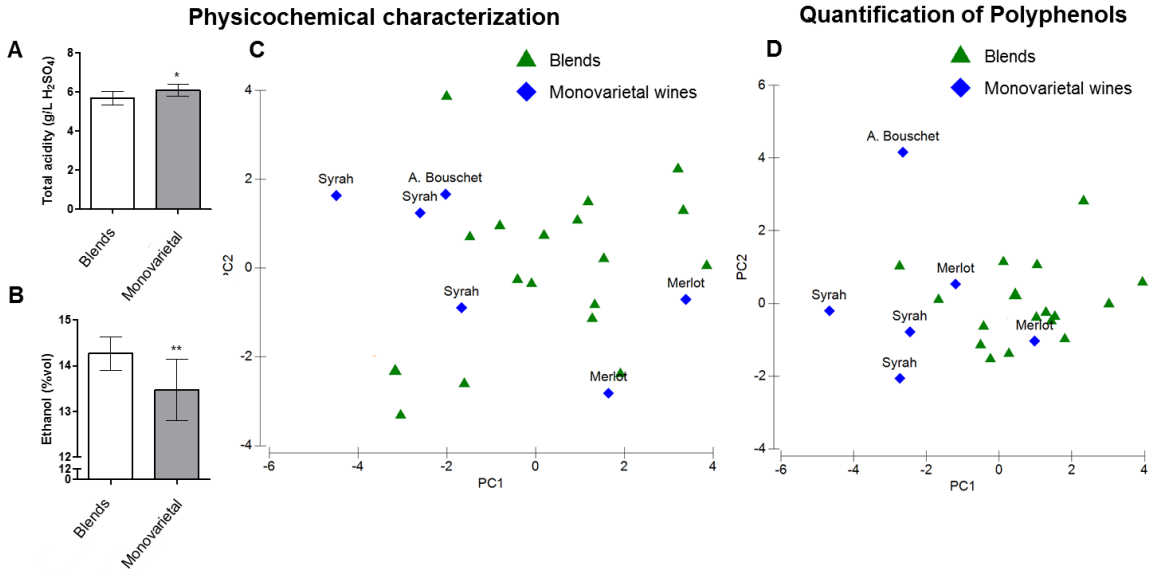

Fig. S6.1 - Physicochemical and polyphenolic comparison between different red wines (n=22). A-B). Comparison between blends and mono-varietal wines. Significant differences are presented as regards to Total acidity and Ethanol content. Asterisks represent: * p<0.05; ** p<0.005. Bars indicate mean ± SD. Blends (n = 17), monovarietal wines (n = 5). T-test shows significant differences (P<0.05); **C)** Principal component analysis based in all the enological parameters tested (PC1 variation 32.7%; PC2 variation 18.5%).

Table S.6.3. Results of the conventional oenological measurements performed on the red wines tested in this study.

Red wines	Total Polyphenols (g/L)	Total Catechins (mg/L)	Tartaric Acid (g/L)	Anthocyanins (mg/L)	Density (g/L)	Ethanol (% vol)	Reduc. sugars (g/L)	Volatile acidity (g/L H_2SO_4)	Total acidity (g/L H_2SO_4)	pH	Lactic acid (g/L)	Tartaric acid (g/L)	D280nm X100
1	2,35	1642,43	1,81	415	991,24	14,59	2,9	0,77	5,22	3,63	1,87	1,94	61,74
2	2,45	1215,69	1,40	401	990,75	14,7	3,1	0,75	5,35	3,58	1,79	1,46	52,05
3	2,19	1391,11	2,24	331	992,3	13,53	1,9	0,55	6,02	3,56	1,58	2,17	61,98
4	2,95	1614,89	2,09	549	991,66	14,37	2,7	0,7	5,78	3,63	1,93	2,2	75,38
5	2,77	1630,52	2,36	408	991,97	14,19	2,8	0,68	5,99	3,58	1,92	2,14	75,66
6	3,51	1629,10	2,33	503	992,31	14,57	3,3	0,63	6,36	3,56	1,97	2,13	86,07
7	2,36	893,266	1,97	103	992,06	13,72	4,8	0,62	6,10	3,43	1,77	1,68	59,43
8	2,02	877,531	2,04	257	993,78	13,17	6,2	0,63	6,24	3,40	1,78	1,77	56,98
9	2,15	1299,55	1,48	845	992,14	13,6	1,4	0,74	5,87	3,56	1,92	1,16	52,41
10	2,80	1793,64	1,47	439	991,72	13,46	1,5	0,74	5,57	3,57	1,87	1,02	61,22
11	2,58	1283,69	2,25	345	993,00	12,82	1,9	0,62	5,55	3,63	1,82	2,25	63,54
12	3,58	2841,91	1.93	306	993,13	13,4	2,1	0,69	5,90	3,64	1,83	1,72	81,67
13	3,06	1226,18	2.04	225	992,95	13,23	2,3	0,67	5,90	3,6	1,80	1,81	69,69
14	2,52	815,988	1.71	325	992,93	14,03	2,4	0,77	5,76	3,65	2,05	1,24	62,79
15	1,028	1026,61	2,08	214	992,03	13,08	1,3	1,02	5,89	3,59	1,91	1,14	60,6
16	1,856	1015,71	1,66	208	990,56	14,25	0,6	0,81	5,80	3,57	1,86	1,49	51,13
17	1,856	2137,94	1,76	215	991,73	13,13	0,4	0,87	5,61	3,70	2,14	1,04	52,66
18	1,134	1663,61	1,44	332	991,68	13,16	0,2	0,91	5,60	3,70	2,13	1,01	51,06
19	2,889	2052,77	1,71	186	991,54	12,54	1,0	0,73	4,75	3,70	1,53	1,25	54,22
20	2,022	1821,45	1,52	135	990,22	14,20	0,6	0,86	5,66	3,64	1,55	1,07	43,64
21	1,150	1366,29	1,60	253	991,10	14,59	0,7	0,98	6,39	3,65	1,82	1,17	58,93
22	2,303	1473,41	1,92	256	993,00	12,38	1,7	1,12	6,14	3,59	1,92	1,78	60,66