Universidade de Aveiro Departamento de Química
2012

FERNANDO JORGE ANDRADE GONÇALVES

CARATERIZAÇÃO DOS COMPOSTOS BIOATIVOS DO VINHO DESALCOOLIZADO

Valorização do Processo de Destilação

CHARACTERIZATION OF BIOACTIVE COMPOUNDS OF DEALCOHOLIZED WINE

Valuation of Distillation Process

Universidade de Aveiro Departamento de Química
2012

FERNANDO JORGE ANDRADE GONÇALVES

CARATERIZAÇÃO DOS COMPOSTOS BIOATIVOS DO VINHO DESALCOOLIZADO

Valorização do Processo de Destilação

CHARACTERIZATION OF BIOACTIVE COMPOUNDS OF DEALCOHOLIZED WINE

Valuation of Distillation Process

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Sílvia Maria da Rocha Simões Carriço, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

Apoio financeiro do POCTI no âmbito do III Quadro Comunitário de Apoio.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

Dedico este trabalho à Sandra, à Maria Inês e à Maria Leonor.

o júri

Presidente

Doutor Artur de Rosa Pires
professor catedrático da Universidade de Aveiro

Vogais

Doutor Victor Armando Pereira de Freitas
professor catedrático da Faculdade Ciências da Universidade do Porto

Doutor Jorge Manuel Ricardo da Silva
professor catedrático do Instituto Superior de Agronomia da Universidade Técnica de Lisboa

Doutora Ivonne Delgadillo Giraldo
professora associada com agregação da Universidade de Aveiro

Doutor Manuel António Coimbra Rodrigues da Silva
professor associado com agregação da Universidade de Aveiro (orientador)

Doutora Sílvia Maria da Rocha Simões Carriço
professora auxiliar da Universidade de Aveiro (coorientadora)

Doutora Ana Isabel Ramos Novo Amorim de Barros
professora auxiliar da Escola de Ciências da Vida e do Ambiente da Universidade de Trás-os-Montes e Alto Douro

agradecimentos

Aos meus orientadores, Doutor Manuel António Coimbra e Doutora Sílvia Rocha, pelos conhecimentos transmitidos, mas também o apoio incondicional. Para eles o meu profundo respeito e admiração.

À Doutora Luísa Helguero, do Departamento de Química da Universidade de Aveiro, pela ajuda prestada e pelos conhecimentos transmitidos.

Ao Professor Doutor Victor de Freitas pela colaboração neste trabalho, pela forma como me acolheu no CIQ do Departamento de Química e Bioquímica da Faculdade de Ciências do Porto. À Natércia Teixeira, pela ajuda prestada na realização experimental e na interpretação dos resultados.

À Professora Doutora Alice Santos-Silva pela colaboração neste trabalho, pela forma como me acolheu no IBMC da Universidade do Porto. Ao Doutor João Fernandes, por todo o apoio na realização experimental e na interpretação dos resultados.

Professora Doutora Maria Teresa Cruz Rosete pela forma como me acolheu no Centro de Estudos Farmacêuticos da Universidade de Coimbra. À Vera Francisco, pela ajuda prestada na realização experimental e na interpretação dos resultados.

Ao Departamento de Química da Universidade de Aveiro pelas condições proporcionadas, e todas a pessoas que contribuíram para a realização deste trabalho de diferentes formas.

A todos os meus colegas e amigos do laboratório, pelo ótimo ambiente de trabalho, pela interajuda e pelos momentos de alegria que foram proporcionados. Em particular, ao Vitor Martins, um amigo de 20 anos, e de uma forma muito especial à Cláudia Nunes, por todo apoio e pela amizade incondicional ao longo destes anos.

Aos meus colegas da ESAV que me ajudaram e apoiaram, em particular na fase da escrita.

À Sandra, pelo apoio, ajuda e sacrifício da sua vida pessoal, fundamentais para eu ter podido desenvolver este trabalho de investigação. À Maria Inês e Maria Leonor, pelos sorrisos que me motivavam a continuar nos momentos mais difíceis.

À FCT, pelo financiamento através de uma bolsa de doutoramento SFRH/BD/32268/2006.

palavras-chave

Vinho tinto, Compostos fenólicos, Antocianinas, Polissacarídeos, Interações, Condições de armazenamento, Atividade anti-inflamatória. Atividade anti-hemolítica.

resumo

O vinho tinto é uma importante fonte de compostos fenólicos com atividade antioxidante e que estão relacionados com a prevenção de doenças cardiovasculares e cancro. Estes compostos são um sub-produto do processo de destilação vínica utilizado para produzir aguardente necessária para a produção de Vinho do Porto. Esta tese tem como objetivo valorizar os compostos fenólicos resultantes das destilarias de vinho, através do estudo da sua composição, das interações com o material polimérico do vinho, da sua estabilidade durante o armazenamento e avaliação dos seus potenciais efeitos biológicos *in vitro*. Isto irá permitir definir aplicações para estes compostos como ingredientes alimentares com propriedades funcionais.

Dois vinhos tintos (RW1 e RW2) foram utilizados como fonte de compostos fenólicos. A fim de estudar estes compostos, cada vinho foi evaporado à pressão atmosférica, permitindo obter o respetivo vinho desalcolizado (DW1 e DW2). Os polissacarídeos e compostos fenólicos presentes nos vinhos desalcolizados foram fracionados por extração em fase sólida utilizando cartuchos C18 sep-pak. A fração hidrofóbica, rica em compostos fenólicos, foi separada em frações ricas em ácidos fenólicos, em procianidinas e em antocianinas, as quais foram usadas para avaliar a sua contribuição para a atividade antioxidante total e caracterização fenólica detalhada dos DW. Foram obtidas quantidades comparáveis de compostos fenólicos totais (1.3 g/L para RW1 e DW1, e 3.1 para RW2 e DW2), de taninos (1.2 g/L para RW1 e DW1 e 1.6 para RW2 e DW2) e de antocianinas (0.24 g/L para RW1 e DW1 e 0.41 para RW2 e DW2) para os vinhos e para os respetivos vinhos desalcolizados. A determinação da atividade antioxidante de RW e DW pelos métodos do DPPH e ABTS também originou valores semelhantes, permitindo inferir que o processo de destilação realizado não promoveu uma perda relevante de compostos fenólicos. A atividade antioxidante total de vinho deveu-se essencialmente à fração rica em antocianinas.

Os dois DW foram dialisados para se obter o material polimérico dos vinhos (WPM1 e WPM2). O WPM1 e WPM2 apresentavam 1.1 e 1.3 g/L de material sólido, respetivamente. O WPM (WPM1 e WPM2) era composto por polissacarídeos (31 e 36%), proteínas (10 e 12%) e também por compostos fenólicos (32 e 43%). A análise de açúcares mostrou que as manoproteínas e as arabinogalactanas eram os principais polissacarídeos presentes. A extração do WPM com metanol deu origem a um material insolúvel em metanol (PMi) e a uma fração solúvel em metanol, que continuava a conter hidratos de carbono e compostos fenólicos, mostrando uma forte interação entre estes compostos.

Para determinar a energia de ativação (E_a) da libertação dos compostos fenólicos de fracções de material polimérico do vinho, foram realizadas diálises do DW, WPM e PMi, utilizando-se quatro concentrações diferentes, a cinco temperaturas (5-40 °C). O valor da E_a foi 25 para o WPM e 61 kJ/mol para o PMi, mostrando que os compostos fenólicos do vinho podem estar associados de forma diferente à matriz polimérica e que uma fração pode estar, ainda, fortemente associada a esta matriz.

A fim de avaliar a possível existência de interações seletivas com os compostos fenólicos, o WPM foi fracionado, permitindo a obtenção de uma fração rica em manoproteínas (MP), através de uma cromatografia de afinidade com concanavalina A e 3 frações ricas em arabinogalactanas (AG0, AG1 e AG2) obtidas por cromatografia de troca aniónica.

Foi avaliada a difusão de nove antocianinas monoméricas através de uma membrana de diálise, em presença do WPM, e das frações ricas em MP e em AG. A diálise dos compostos fenólicos livres do vinho foi realizada como ensaio em branco. Todas as frações poliméricas mostraram capacidade para reter as antocianinas, embora em diferente extensão. Foi observada uma capacidade de retenção maior para as antocianinas acilglucosiladas do que para as antocianinas glucosiladas. A fração rica em AG teve uma maior contribuição para a capacidade de retenção das antocianinas pelo material polimérico vinho do que a fração rica em MP, principalmente quando as antocianinas estavam acetiladas.

Com o objetivo de estudar formas para preservar, a longo prazo, as propriedades antioxidantes dos compostos fenólicos, o extrato de compostos fenólicos (PCE), em pó, foi armazenado em diferentes condições de luz e atmosfera, à temperatura ambiente durante 1 ano. Observou-se que o PCE armazenado no escuro, dentro de um exsicador sob atmosfera de azoto, preservou 95% da atividade antioxidante inicial. Também foram avaliadas as melhores condições para preservar as antocianinas quando em solução, armazenadas a duas temperaturas (5 e 30 ºC) durante 3 meses. A adição de 0.5 g/L de uma fração rica em polissacarídeos a um vinho armazenado a 30 ºC promoveu a proteção das antocianinas, especialmente das antocianinas cumaroiladas.

Os potenciais efeitos biológicos dos compostos fenólicos foram avaliados em diferentes sistemas celulares *in vitro* utilizando as seguintes frações: WPM, WPS (polissacarídeos do vinho), WPC (compostos fenólicos do vinho), PA-E (fração rica em ácidos fenólicos), PR-E (fração rica em procianidinas) e APP-E (fração rica em antocianinas e procianidinas poliméricas). Foi observada uma maior viabilidade celular quando as células do carcinoma do cólon HT-29 foram expostas a dois agentes oxidantes (radiação UV e H_2O_2) em presença das frações PR-E e APP-E. Além disso, os extratos WPS, WPC, PR-E e APP-E mostraram propriedades anti-inflamatórias, avaliadas pela inibição da produção de NO por células de macrófagos RAW264.7, sendo o extrato APP-E (0.19 mg/mL) o que exibiu a maior capacidade anti-inflamatória. A fim de elucidar as propriedades antioxidantes dos extratos do vinho em células humanas, os glóbulos vermelhos (RBC) foram selecionados como um modelo metabolicamente simples. Os extratos WPM, WPS, WPC, PR-E, e APP-E mostraram efeito anti-hemolítico para a hemólise dos RBC provocada pelo peróxido de hidrogénio (H_2O_2) e pelo di-hidrocloreto de 2,2'-azo-bis(2-diaminopropano) (AAPH).

Os resultados obtidos permitem concluir que o processo de desalcoolização dos vinhos à pressão atmosférica, preservou as principais características antioxidantes dos compostos fenólicos. Estes compostos podem contribuir para a defesa das células contra agentes oxidantes, nomeadamente por terem um potencial de atividades anti-inflamatória e anti-hemolítica, promovendo a viabilidade celular. A interação dos compostos fenólicos do vinho com o material polimérico permite inferir uma dosagem contínua e gradual das antocianinas vinho tinto após a sua ingestão, contribuindo para um período mais longo da sua exposição e, como consequência, dos seus potenciais benefícios para a saúde.

keywords

Red wine, Phenolic compounds, Anthocyanins, Polysaccharides, Interactions, Storage conditions, Anti-inflammatory activity, Anti-hemolytic activity.

abstract

Red wine is an important source of dietary intake of phenolic compounds with antioxidant activity and that are related to the prevention of cardiovascular diseases and cancer. These compounds are a major by-product of the wine distillation process used to produce the spirits required for production of Port Wine. This thesis aims to add value to the phenolic compounds resultant from wine distilleries by analyzing their composition, studying their interactions with the wine polymeric components, evaluating their stability upon storage, and assaying potential in vitro biological effects. This will allow to define applications as food ingredients for these compounds.

Two red wines (RW1 and RW2) were used as source of phenolic compounds. In order to study these compounds, each wine was heat-evaporated at atmospheric pressure, allowing to obtain the respective dealcoholized wine (DW1 and DW2). The polysaccharides and phenolic compounds present in the DW were fractionated by solid phase extraction (SPE) using C-18 sep-pak cartridges, allowing to obtain the hydrophilic and hydrophobic fractions. The latter were further separated in phenolic acids-rich, procyanidins-rich and anthocyanins-rich fractions that were used to evaluate their contribution to the total antioxidant activity of DW and for the detailed phenolic characterization of DW by HPLC. Comparable amounts of total phenolic compounds (1.3 g/L for RW1 and DW1, and 3.1 for RW2 and DW2), tannins (1.2 g/L for RW1 and DW1 and 1.6 for RW2 and DW2), and anthocyanins (0.24 g/L for RW1 and DW1 and 0.41 for RW2 and DW2) were obtained. Also, the antioxidant activity of RW and DW, assayed by DPPH and ABTS, resulted in similar values, allowing to infer that the distillation process carried out did not promote relevant loss of phenolic compounds. The anthocyanins-rich fraction achieved the highest contribution to the total wine antioxidant activity.

Both DW were dialyzed to obtain the wines polymeric material (WPM1 and WPM2). The WPM1 and WPM2 accounted for 1.1 and 1.3 g/L of solid material. They were composed of polysaccharides (31 and 36%) and proteins (10 and 12%) associated with phenolic compounds (32 and 43%). Sugar analysis showed that mannoproteins and arabinogalactans were the main polysaccharides present. From the extraction of WPM with methanol resulted a methanol insoluble (PMi) and a methanol soluble fraction, which still contained both carbohydrate and phenolic compounds, showing a strong interaction between these compounds.

To determine the activation energy of phenolic release from the wine polymeric fractions, dialysis of DW, WPM and PMi, were performed using four different concentrations, and five temperatures (5-40 ºC) until a steady state was reached.

The E_a was 25 and 61 kJ/mol for WPM1 and PMi fraction, showing that wine phenolic compounds can be differently associated with the polymeric matrix and a fraction can be even strongly associated into this matrix.

In order to evaluate if a selective interaction exists between the phenolic compounds, the wine polymeric material was fractionated, allowing to obtain a mannoprotein-rich (MP) fraction using concanavalin A affinity chromatography and 3 arabinogalactan-rich fractions (AG0, AG1 and AG2) by anion-exchange chromatography. Using these compounds, the diffusion of nine monomeric anthocyanins through a dialysis membrane in presence of the WPM, MP and AG fractions was evaluated. In addition, the dialysis of free wine phenolic compounds was performed as a blank experiment. All polymeric fractions showed capacity for retaining the different anthocyanins, although in different extents. The higher retention capacity was observed for the acylglucosylated when compared with the glucosylated anthocyanins. The AG fraction had a higher contribution than MP fraction for retention capacity of wine polymeric material, especially when the anthocyanins were acetylated.

The phenolic compounds extract (PCE) as a powder, was stored in different conditions of light and atmosphere, at room temperature for 1 year in order to study forms to preserve the antioxidant properties of these phenolic compounds in long-term storage conditions. It was observed that the PCE stored in the dark, inside a desiccator under a nitrogen atmosphere, preserved 95% of the initial antioxidant activity. It was also evaluated the best conditions to preserve the anthocyanins when in solution, stored at two temperatures (5 and 30ºC) during 3 months. The presence of 0.5 g/L of a polysaccharide-rich fraction in a wine solution stored at 30 ºC promoted the protection to the anthocyanin content, especially of coumaroylated anthocyanins.

The potential biological effects of phenolic compounds were evaluated in different *in vitro* cellular systems using the fractions: WPM, WPS (wine polysaccharides), WPC (wine phenolic compounds), PA-E (phenolic acids fraction), PR-E (procyanidins-rich fraction), and APP-E (anthocyanins and polymeric procyanidins-rich fraction). A higher cell survival rate of HT-29 colon carcinoma cells was observed when they were exposed to two oxidative agents (UV radiation and H_2O_2) in presence of PR-E and APP-E extracts. Also, the WPS, WPC, PR-E and APP-E extracts showed anti-inflammatory properties using LPS-induced NO production in RAW 264.7 macrophage cells, where the APP-E extract (0.19 mg/mL) exhibited the highest capacity. In order to further elucidate the antioxidant properties of wine extracts in human cells, red blood cells (RBC) were selected as a metabolically simplified model system. The wine extracts (WPM, WPS, WPC, PR-E, and APP-E) showed anti-hemolytic effect of hydrogen peroxide (H_2O_2) and 2,2´-Azo-bis (2-amidinopropane) dihydrochloride (AAPH) induced RBC hemolysis.

The results obtained allowed to conclude that the dealcoholization process of wines at ambient pressure preserved the main antioxidant characteristics of phenolic compounds. These compounds may contribute for cell defenses against oxidative agents, namely having potential anti-inflammatory and anti-hemolytic activities, promoting cell viability. The interaction of wine phenolic compounds with the polymeric material allows to predict a continuous and gradual dosage of red wine anthocyanins upon ingestion, contributing for a longer period of their exposure and, as a consequence, of their potential health benefits.

General Index

List of abbreviations

fractions used

AG1	Acidic arabinogalactan fraction (eluted with 50 mM of phosphate buffer)
AG2	Acidic arabinogalactan fraction (eluted with 500 mM of phosphate buffer)
APP-E	Anthocyanins and polymeric procyanidins-rich extract
AG	Arabinogalactan-rich fraction
DAS	Samples stored in dark, in air atmosphere, sealed tubes
DNS	Samples stored in dark, in N_2 atmosphere, sealed tubes
DW1	Dealcoholized wine 1
DW2	Dealcoholized wine 1
DW	Dealcoholized wines
$DW_{Et}0$	fraction ethanol insoluble
$DW_{Et}30$	Fraction precipitated in solution of 30% of ethanol
$DW_{Et}50$	Fraction precipitated in solution of 50% of ethanol
$DW_{Et}80$	Fraction precipitated in solution of 80% of ethanol
$DW_{Et}SN$	Ethanol soluble fraction
F1	Phenolic acid fraction (eluted from SPE-C18 with phosphate buffer pH7)
F2	Procyanidins fraction (eluted from SPE-C18 with ethyl acetate)
F3	Anthocyanins and polymeric procyanidins fraction (eluted from SPE-C18 with acidic methanol)
LAO	Samples stored in Light, in air atmosphere, open tubes
LAS	Samples stored in Light, in air atmosphere, sealed tubes
LNS	Samples stored in Light, in N_2 atmosphere, sealed tubes
PM30	Fraction precipitated in solution of 30% chloroform:methanol
PM50	Fraction precipitated in solution of 50% chloroform:methanol
PM60	Fraction precipitated in solution of 60% chloroform:methanol
PM75	Fraction precipitated in solution of 75% chloroform:methanol
PMsn	Fraction soluble in solution of 75% chloroform:methanol
PM50M	Hydrophobic fraction of fraction precipitated in solution of 50% chloroform:methanol
PMiM	Hydrophobic fraction of polymeric material insoluble in methanol
WPMM	Hydrophobic fraction of wine polymeric material
PM50W	Hydrophilic fraction of fraction precipitated in solution of 50% chloroform:methanol
PMiW	Hydrophilic fraction of polymeric material insoluble in methanol
WPMW	Hydrophilic fraction of wine polymeric material
MP	Mannoproteins-rich fraction
AG0	Neutral arabinagalactan fraction
PA-E	Phenolic acids extract
PMi	Polymeric material insoluble in methanol

PR-E	Procyanidins-rich extract
RW1	Red wine 1
RW2	Red wine 2
RW	Red wines
WPC	Wine phenolic compounds
WPM	Wine polymeric material
WPS	Wine polysaccharides
W5	wine stored at 5ºC
W30	wine stored at 30ºC
W5P	wine stored at 5ºC with addition of 0.5 g/L of polysaccharides
W30P	wine stored at 5ºC with addition of 0.5 g/L of polysaccharides

Amino acids

Ala	Alanine
Ara	Arabinose
Asn	Asparagine
Asx	Aspartic acid–+ asparagine
Glx	Glutamic acid + glutamine
Gly	Glycine
Ile	Isoleucine
Leu	Leucine
Pro	Proline
Ser	Serine
Thr	Treonine
Val	Valine

Anthocyanins

3-AcGlc	3-(6-acetyl)glucosides
3-Glc	3-glucosides
Cy3AcGlc	cyanidin 3-(6-acetyl)glucoside
Cy3Glc	cyanidin 3-glucoside
Dp3AcGlc	delphinidin 3-(6-acetyl)glucoside
Dp3Glc	delphinidin 3-glucoside
Mv3AcGlc	malvidin 3-(6-acetyl)glucoside
Mv3CmGlc	malvidin 3-(6-coumaroyl)glucoside
Mv3Glc	malvidin 3-glucoside
Pn3AcGlc	peonidin 3-(6-acetyl)glucoside
Pn3Glc	peonidin 3-glucoside
Pt3AcGlc	petunidin 3-(6-acetyl)glucoside
Pt3Glc	petunidin 3-glucoside

Sugars residues

Ara	Arabinose
Gal	Galactose
GalA	Galacturonic acid
Glc	Glucose
Man	Mannose
Rha	Rhamnose
UA	Uronic acids

Other abbreviations used

AAPH	2,2´-Azo-bis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
EC_{50}	Amount of antioxidant needed to decrease in 50 % the initial radical concentration
AE	Antioxidant efficacy
CAE	Caffeic acid equivalents
C 0.25	Cells exposed to 0.25 mM of H_2O_2
C 0.5	Cells exposed to 0.5 mM of H_2O_2
C 1.0	Cells exposed to 1.0 mM of H_2O_2
C UV	Cells irradiated 10 min with UV light
CSR	Cell survival rate
K_c	kinetic constant
DPPH	1,1'-dihenyl-2-picrylhydrazyl
E_a	Activation energy
FPC	Free phenolic compounds
GAE	Gallic acid equivalents
GlcNAc	*N*-acetyl-D-glucosamine
H_2O_2	Hydrogen peroxide
HC11	Mammary epithelial cells
HPLC	High-performance liquid chromatography
HT-29	Human colon carcinoma cells
K	Kelvin
NO	Nitric oxide
PLS	Lipopolysaccharides
mol%	Molar percentage
R	Gas constant (8.314 J mol/K)
r^2	Correlation coefficient
RBC	Red blood cells
RC	Retention coefficient

SPE	Solid phase chromatography
T	Absolute temperature
TEC_{50}	Time needed to reach the steady state at EC50 concentration
TE	Trolox equivalents
TEAC	Trolox equivalentes antioxidante capacity

CHAPTER I - Introduction

1.1 Overview

Red wine is an important source of dietary intake of phenolic compounds with antioxidant activity which has been related to health benefits, such as the prevention of cardiovascular diseases and cancer. These compounds are a major by-product of the wine distillation process used to produce the spirits required for production of Port Wine. Concerning the add value of the phenolic compounds resultant from red wine distilleries, this chapter describes the state of the art of wine phenolic compounds structure and reactivity/stability, the knowledge achieved so far on the wine polymeric material composition, their interactions with phenolic compounds, and the potential use of these red wine compounds as a source of biologically active food ingredients.

The main wine phenolic compounds are phenolic acids (hydroxybenzoic and hydroxycinnamic acids), their derivatives, stilbenes, flavanols (catechins and procyanidins), and anthocyanins. The anthocyanins occur mainly as 3-*O*-monoglucosides of malvidin, delphinidin, cyanidin, petunidin, and peonidin, which can be acetylated or coumaroylated. These compounds are highly instable and undergo condensation reactions resulting into the formation of polymeric pigments. High temperature and pH, high oxygen concentrations, the light, and the presence of specific enzymes such as glucosidases are factors which contribute to the degradation of anthocyanins. In wine, the phenolic compounds can be found in free form or bounded with macromolecules, such as polysaccharides.

The present chapter also includes information about the main wine polysaccharides, namely, type II arabinogalactans, type II rhamnogalacturonans, both arising from grape pectic polysaccharides, and mannoproteins, released by yeasts during fermentation. Depending on the type and strength of the interactions between phenolic compounds and polymeric material, changes in their bioavailability and hence their biological properties are expected. Also, the knowledge of the release of phenolic compounds from the polymeric material is important to understand their behavior along the digestive tract and to modulate their bioavailability.

Some studies associated procyanidins with cardioprotective effects and with the prevention of induced colonic inflammation and colorectal cancer. Also, the anthocyanins have shown beneficial activity against cardiovascular diseases. For example, they can act as modulators of the immune response in activated macrophages, and might allow to prevent cancer. The data available in literature shows that the use of these red wine compounds as a source of biologically active food ingredients can and should be exploited.

1.2 Wine Molecules

The wine is constituted by phenolic compounds and polymeric material including macromolecules with a molecular mass of over 12 kDa, such as polysaccharides and proteins (Bravo *et al.*, 1994; Doco *et al.*, 2007; Ducasse *et al.*, 2010; Saura-Calixto & Díaz-Rubio, 2007). The main types of wine phenolic compounds and polysaccharides will be described in detail.

1.2.1 Phenolic Compounds

Phenolic compounds are important for quality of wines, since they are responsible for their sensorial and chemical properties. Wine phenolic compounds consist on both grape phenolic compounds, and new phenolic compounds derived from them during winemaking and ageing process (Katalinic *et al.*, 2004; Sun *et al.*, 2010). There have been numerous studies that have profiled the phenolic composition of wines. The phenolic profile of wines depends on grape viticulture (variety, ripening stage, climatic conditions), on winemaking techniques (crushing, pressing, sulphite addition, maceration and oak ageing) employed (Vinas *et al*., 2000) and also on numerous reactions that occur during aging and storage. The maceration phase favors the extraction of components from grape solids, been one of the most important processes that give red wine its characteristics taste and flavor profile, differentiating it from white wine. The use of macerating enzymes helps in phenolic extraction resulting in wines richest in phenolic compounds. The red wines are richer in phenolic compounds than white wines, in particular in anthocyanins and procyanidins, because the grape solids are in contact with the must during fermentation process.

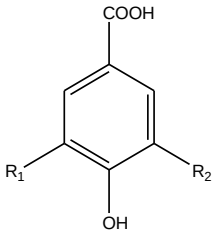
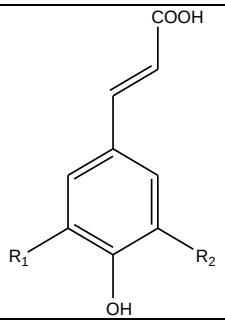
Despite all this diversity, red wines contain a total amount of phenolic compounds ranging from 1019 to 4059 mg GAE/L (Aguirre *et al.*, 2010; Fernandez-Pachon *et al.*, 2004; Frankel *et al.*, 1995; Goncalves & Jordao, 2009; Ivanova *et al.*, 2011; Katalinic *et al.*, 2004; Sanchez-Moreno *et al.*, 1999). The

phenolic content in white wines ranges from 87 to 407 mg/L (Fernandez-Pachon *et al*., 2004; Kallithraka *et al*., 2005; Recamales *et al*., 2006; Roussis *et al*., 2008). Usually the phenolic compounds of wine are classified as non-flavonoids or as flavonoids.

1.2.1.1 Non-flavonoids

Non-flavonoids comprise phenolic acids, their derivatives, and stilbenes. Phenolic acids can be divided in two subgroups according to their structure: the hydroxybenzoic (C_6-C_1) and the hydroxycinnamic acids (C_6-C_3). Table 1.1 shows a general structure of phenolic acids.

Table 1.1: Chemical structure and classification of phenolic acids (adapted from Sun *et al*., 2007).

		hydroxybenzoic acids	hydroxycinnamic acids
			
R_1	R_2		
H	H	*p*-hydroxybenzoic	*p*-coumaric
OH	H	protocatechuic	caffeic
OH	OH	gallic	---
OCH_3	H	vanillic	ferulic
OCH_3	OCH_3	syringic	sinapic

The most commonly found hydroxybenzoic acids are the gallic, *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids, while among the hydroxycinnamic acids are the caffeic, ferulic, *p*-coumaric, chlorogenic, and sinapic acids (Bravo, 1998). Hydroxycinnamic esters are the third most abundant group of phenolic compounds in grapes (Rodriguez-Diaz *et al.*, 2006).

The main stilbene (C_6-C_2-C_6) present in wine is the 3,5,4'-trihydroxystilbene, with the trivial name resveratrol, which exists as *trans*-resveratrol or *cis*-resveratrol (Figure 1.1), but only the first shows biological activities. In plant materials, the *trans*-isomer usually predominates. It is also possible to find its glucosilated form (*trans*-resveratrol glucoside).

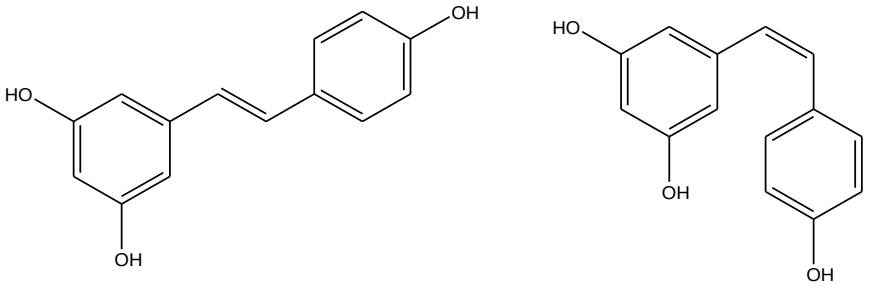


Figure 1.1: Chemical structure of *trans*-resveratrol and *cis*-resveratrol (adapted from Filip *et al*., (2003)).

Stilbenes are important in grapes due their antimicrobial effect against *Botrytis cinerea*, which causes significant losses for vineyard owners worldwide. The contents of resveratrol found in red wines are generally lower than 10 mg/L (Baptista *et al.*, 2001; Paulo *et al.*, 2011). however, Belisario-Sanchez *et al*., (2009) obtained higher amounts (17.6 mg/L) in red wines. Also, Moreno-Labanda *et al*. (2004) have found up to 30 mg/L in Monastrell wines made using macerative fermentations.

A different class of non-flavonoids is the hydrolysable tannins. Hydrolysable tannins are esters of gallic acid (gallotannins) and ellagic acid (ellagitannins) with glucose or related sugars. Quinn and Singleton (1985) described values of 250 mg/L of hydrolysable tannins for red wines aged during two years in oak barrels, and of 100 mg/L for white wines aged for 6 months.

1.2.1.2 Flavonoids

Flavonoids constitute the largest group of naturally occurring phenolic compounds, (Harborne *et al*., 1999). Their basic structures (Figure 1.2) are built upon a C_6-C_3-C_6 skeleton, consisting of two aromatic rings (A- and B-ring) linked by an oxygen containing pyran ring (C-ring).

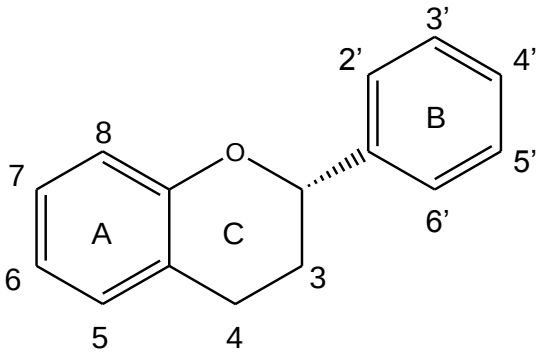


Figure 1.2: Basic structure of flavonoids showing A, B, and C rings and the numbers for the various positions in the flavan structure (Valls *et al.*, 2009)

They can be sub-divided in flavanols, anthocyanins, flavonols, isoflavones, and flavones (Alcalde-Eon *et al*., 2006; Peterson & Dwyer, 1998) according to the degree of unsaturation and degree of oxidation of the C-ring heterocycle and hydroxylation/methoxylation of the three rings.

a) Flavanols

One important group of flavonoids, the flavanols include monomers, oligomers, and polymers. The latter two forms are also known as proanthocyanidins or condensed tannins. They can have substituent groups in up to three positions in the B-ring (3', 4' and 5'). In flavanols structure, the C-ring is a saturated heterocycle with a hydroxyl group in position 3, that can be esterified with gallic acid. Catechin, epicatechin, catechin gallate, epicatechin gallate, gallocatechin, epigallocatechin, gallocatechin gallate, and epigallocatechin gallate

are the most common flavanol monomers. Most flavanols in nature are stereoisomers in *cis* or *trans* configuration with respect to carbons 2 and 3, like the (+)-catechin and (−)-epicatechin (Figure 1.3).

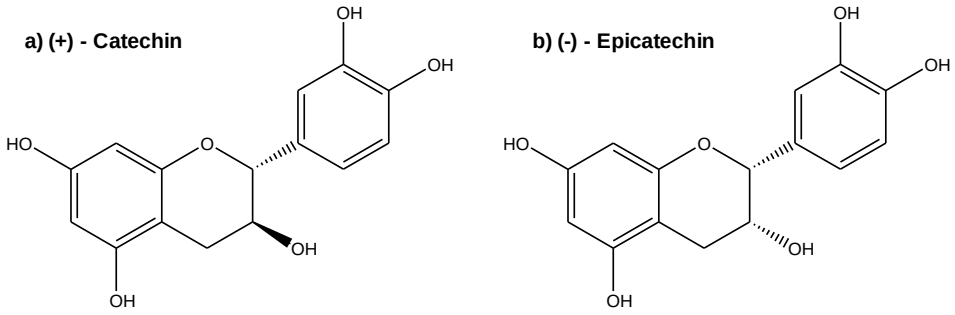


Figure 1.3: Chemical structure of (+) - catechin and (-) – epicatechin (adapted from Sun *et al*., 2007).

Proanthocyanidins are classified as procyanidins if they derived from catechin, epicatechin and their gallic esters. The procyanidins consisting exclusively of (epi)catechin units are the most widely in plants. Polymers composed by (epi)gallocatechin units are designed as prodelphinidin. The procyanidins differ in the position and configuration of their monomeric linkages. Type-B procyanidins are linked by C-C bonds, mainly through C4-C8, however C4-C6 linkages can also occur (Santos-Buelga *et al*., 1995). These structures may also be esterified through the (-)-epicatechin units forming 3-*O*-gallates (De Freitas *et al.*, 1998; Saucier *et al.*, 2001). In addition to the C4-C8 bond, the flavan-3-ol units can establish a second interflavonoid ether bond by a C2-O7 that result from an oxidative intramolecular reaction (Kondo *et al.*, 2000), giving origin to type-A structures (Gu *et al.*, 2003). Figure 1.4 shows the chemical structures of a B2-dimer and an A2-dimer of procyanidins. The structures of procyanidin dimers B1, B2, B3, and B4 are the best known.

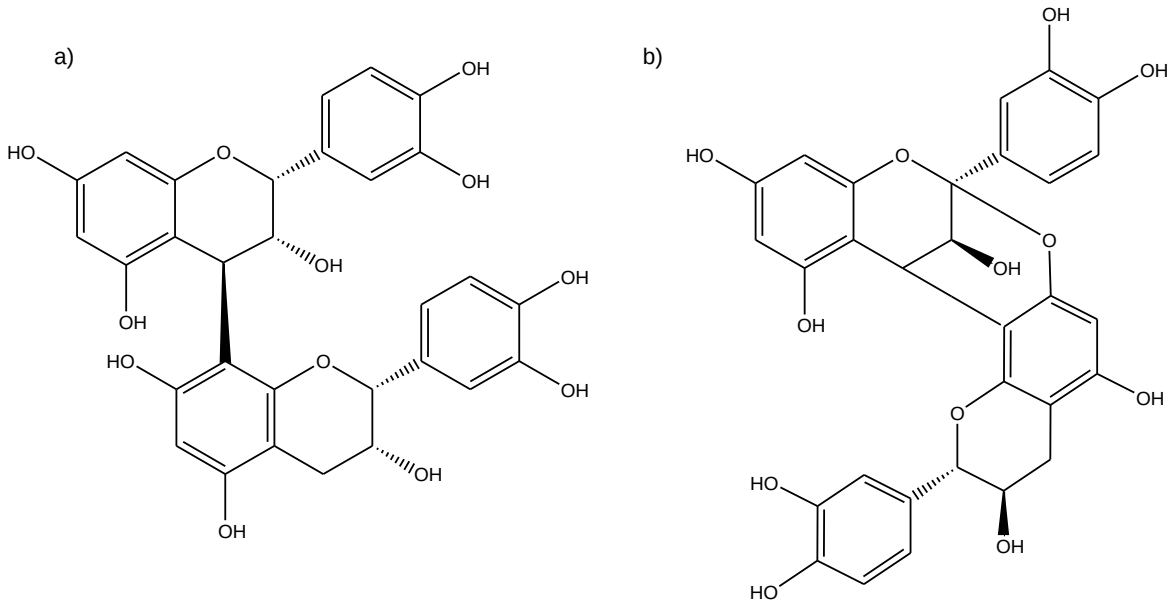


Figure 1.4: Chemical structures of dimeric procyanidins. a) B2-dimer and b) A2-dimer (adapted from Fraga *et al*., 2011).

In the grape, the catechins and proanthocyanidins are in the skins and seeds (Fuleki & Ricardo-da-Silva, 1997; Jordão et al., 2001). Seeds comprise only procyanidins (Souquet *et al.*, 1996), whereas skins comprise both prodelphinidins and procyanidins (Labarbe *et al.*, 1999; Souquet *et al.*, 1996). The molecular weight of proanthocyanidins expressed as degree of polymerization (DP) is one of their most important properties, since it influences wine astringency. Saucier *et al*., (2001) and Cosme *et al*. (2009) obtained for procyanidins of seeds a mean PD ranging from 3 to 12 units. Seeds proanthocyanidins have a lower DP and a higher percentage of galloylated subunits when compared to the skin (Cheynier, *et al*., 1997; Moutounet *et al*., 1996). Wine proanthocyanidins are procyanidins or prodelphinidins (Gonzalez-Manzano *et al.*, 2004) extracted from the solid parts of grape during winemaking (Sun *et al.*, 1999). The procyanidins B1 and B2 are the most abundant proanthocyanidins in wines. Cosme *et al*. (2009) reported values of total proanthocyanidins of 193-1230 mg/L for five portuguese wines. The data concerning the average DP values of polymeric proanthocyanidins reported in literature ranged from 6.9 to 15.6 (Monagas *et al.*, 2003; Sun *et al.*, 2001).

b) Anthocyanins

The anthocyanins are natural, water-soluble, non-toxic pigments responsible for the color of some fruits, vegetables, flowers, and other plant tissues (Mazza & Brouillard, 1990). They are constituted by basic structures of anthocyanidins bonded to a sugar moiety. Their chemical structure (Figure 1.5) consists on an aromatic ring (A) bonded to a heterocyclic ring (C) that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring (B) (Konczak & Zhang, 2004). From the chemical point of view, anthocyanins are glycosides of 2-phenylbenzopyrylium (flavylium) salts derivatives. The main differences between them are the number and position of hydroxylated/metoxylated groups located in the B-ring of the molecule, the nature and the number of bonded sugars to their structure, the aliphatic or aromatic carboxylates bonded to the sugar in the molecule and the position of these bonds (Kong *et al.*, 2003).

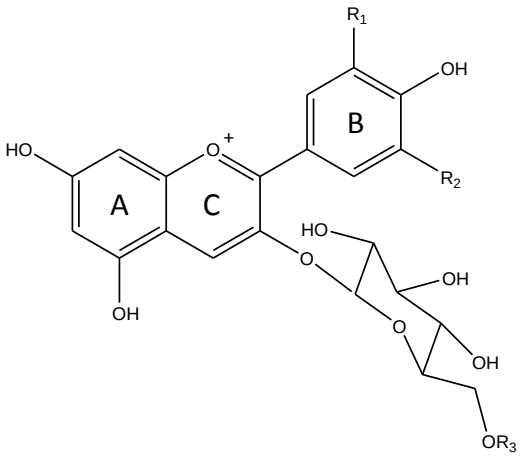


Figure 1.5: Chemical structure of anthocyanins (R_3 = H, anthocyanin 3-glucosides, R_3 = acetyl, anthocyanin 3-acetylglucosides; R_3 = coumaroyl, anthocyanin 3-coumaroylglucosides).

In the grape, the anthocyanins are mainly localised in the skin (Jordão *et al.* 1998), with the exception of the teinturier varieties that also contain anthocyanins in the pulp. The presence of glucose determines an increase in water solubility and allows anthocyanins to be extracted from the skins of grapes into the must and wine during crushing, pressing, and fermentation. The anthocyanins of wine from *Vitis vinifera* are 3-*O*-monoglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (Figure 1.5; Table 1.2).

Table 1.2: Position of the hydroxyl and methoxyl groups located in the B-ring of anthocyanidins (adapted from Castaneda-Ovando *et al*., 2009).

Anthocyanidin	R1	R2	color
Cyanidin	OH	H	Orange - red
Delphinidin	OH	OH	Blue - red
Peonidin	OCH_3	H	Orange - red
Petunidin	OCH_3	OH	Blue - red
Malvidin	OCH_3	OCH_3	Blue - red

These compounds may occur acylated with acetic (Bakker & Timberlake, 1985) and coumaric acids (Manfra *et al*., 2011) at the C6 of glucose. Also, caffeic (Baldi *et al.*, 1995; Monagas *et al.*, 2003) and lactic acids (Alcalde-Eon *et al*., 2006) were found. In wine, the malvidin 3-glucoside is the most abundant anthocyanin in wine. Its acetyl and coumaroyl derivatives are represented in Figure 1.6.

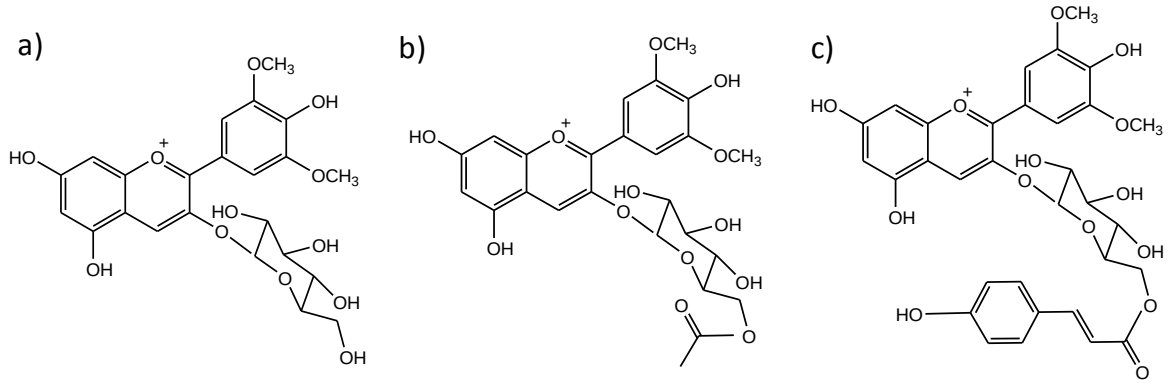


Figure 1.6: Struture of a) malvidin 3-glucoside, b) malvidin 3-acetylglucoside and c) malvidin 3-coumaroylglucoside.

Anthocyanins and their derivatives exist in the colored forms at wine pH. The color of the anthocyanins varies according to the number and position of the hydroxyl and methoxyl groups (Table 1.2). When wines are young, monomeric anthocyanins are the major components responsible for red wine color (Mazza *et al*., 1999) and for antioxidant activity (Ginjom *et al*., 2011). The changes in the color characteristics of red wine occur mainly during the first year of storage

(Somers & Evans, 1986). The color of red wine evolutes during maturation and ageing due to the interactions between anthocyanins and other compounds. Figure 1.7 shows a schematic representation of several reactions demonstrated to occur in red wine.

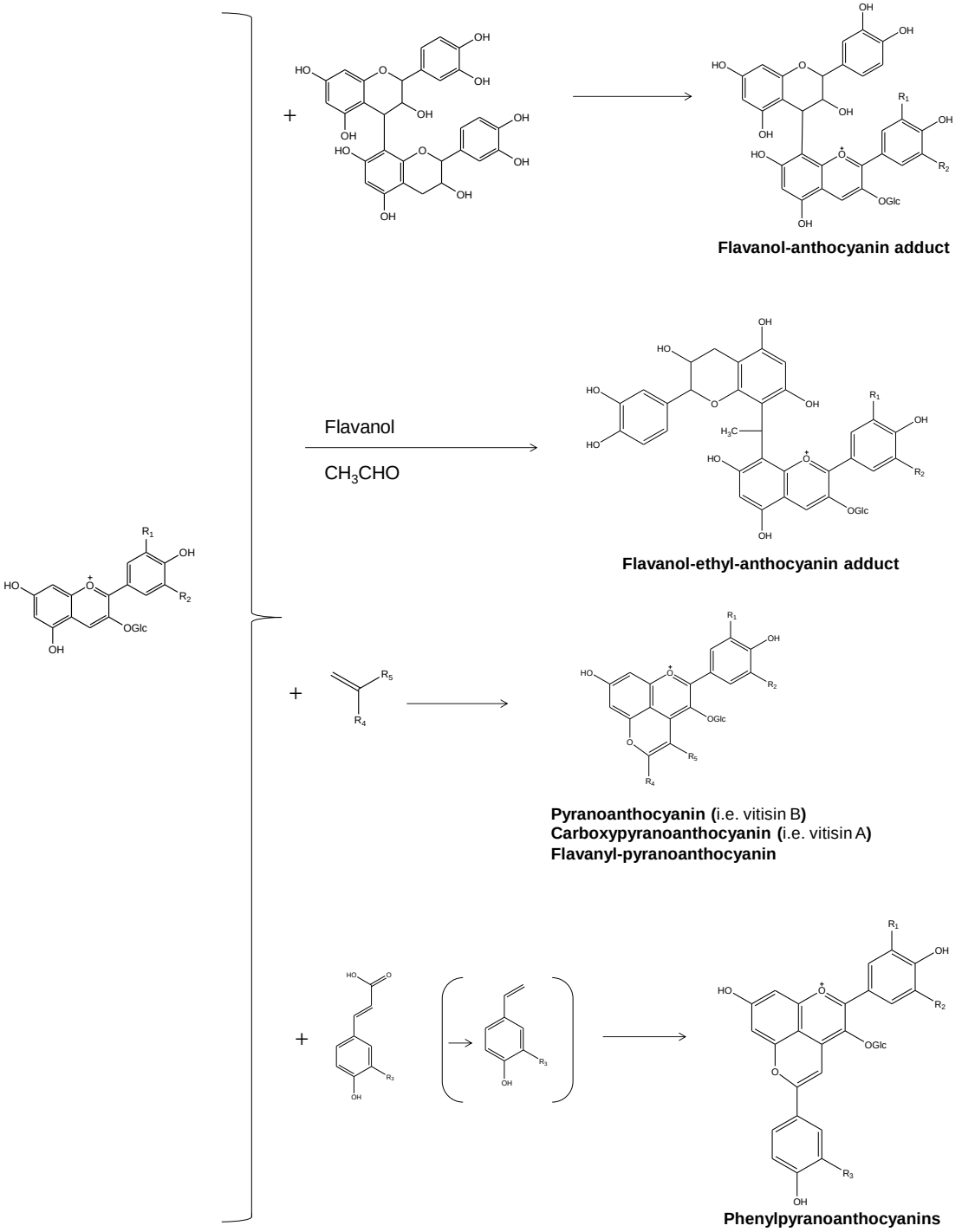


Figure 1.7: Schematic representation of anthocyanins reactions and the products formed (adapted from Wirth *et al*. 2012).

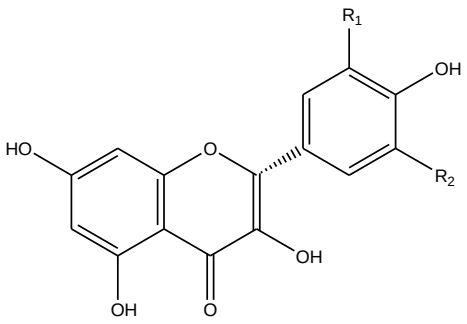
The anthocyanins are highly instable and they participate in reactions of degradation, oxidation, bleaching by SO_2, complexation with metals, polymerization with flavan-3-ols, and co-pigmentation (Liao *et al.*, 1992; Mirabel *et al.*, 1999). The wine anthocyanins undergo condensation reactions resulting into the formation of more stable polymeric pigments (Dallas *et al*., 1996; Mateus *et al*., 2002). The self-association, the copigmentation and the association with metals are reactions that contribute to anthocyanins stability (Liao *et al*., 1992; Malien-Aubert *et al*., 2001; Ribereau-gayon *et al*., 1998).

Self-association, a type of copigmentation, is the association of two or more anthocyanin molecules (Timberlake & Bridle, 1975), stabilized mainly through the hydrophobic interactions that take place between their aromatic core. Gonzalez-Manzano *et al.* (2008) demonstrated the existence of processes of self-association in wine model solutions of anthocyanins and its positive influence on their stability and color. In addition, The copigmentation of anthocyanins with other compounds, can be divided into intramolecular copigmentation with the aromatic groups of hydroxycinnamic acids and intermolecular copigmentation with uncolored substances such as flavonol or flavone glycosides (Cavalcanti *et al*., 2011). The compounds able to participate in the copigmentation are extremely diverse. The copigment may be a flavonoids, phenolic acids, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals, and anthocyanins themselves (Mazza & Brouillard, 1990). The contribution for wine color depends on the molecule. Strong copigments are the caffeic and ferulic acids (Eiro & Heinonen, 2002) and epicatechin and catechin are weak copigments (Mirabel *et al*., 1999). The association between an anthocyanin and the colorless co-pigments involves a physicochemical process without the formation of a covalent bond (Baranac *et al*., 1996; Mirabel *et al*., 1999). In addition, anthocyanins can react with small molecules, such as acetaldehyde (Bakker & Timberlake, 1997), pyruvic acid (Fulcrand *et al.*, 1996), vinylcatechol (Schwarz *et al.*, 2003), and vinylcatechin (Cruz *et al*., 2008), forming pyranoanthocyanins (Oliveira *et al.*, 2010), namely vitisin A or acetylvitisin A, formed from malvidin-3-glucoside (Degenhardt *et al*., 2000). These compounds are resistant to sulfite bleaching (Bakker & Timberlake, 1997). The pyranoanthocyanins in their diverse forms have been estimated to

constitute more than 50% of the total derived pigments present in red wines with two and five years old (Alcalde-Eon *et al.*, 2006; Boido *et al.*, 2006).

c) Flavonols

The C-ring of flavonols is characterized by a 2,3-double bond, a 4-keto group, and a 3-hydroxyl group (Figure 1.8). The most common wine flavonol compounds are kaempferol, quercetin, and myricetin. These molecules are present in red wine in concentrations order of 100 mg/L, while in white wine the concentration is between 1 and 3 mg/L (Ribéreau-Gayon *et al.*, 2006)



R_1	R_2	Name
H	H	Kaempferol
OH	H	Quercitin
OH	OH	Myrcitin

Figure 1.8: Some flavonols identified in wines (adapted from Sun *et al.*, 2007).

1.2.1.3 Stability of phenolic compounds

The stability of polyphenols has been studied by several researchers, since it is an important aspect to consider for use of phenolics in foods. The stability of antioxidant power during storage and usage is required for commercialization and application in foods, especially with a long shelf-life, such as beverages. The stability of phenolic compounds in foods may be improved using packaging films (Somboonkaew & Terry, 2010), controlled atmosphere (Deng *et al.*, 2005; Rocculi *et al.*, 2004), and microencapsulation technology (Ersus & Yurdagel, 2007).

In wine, phenolic compounds can undergo oxidation reactions due the presence of reactive oxygen species (ROS). ROS are oxygen radicals, including

superoxide anion ($O_2^{\bullet-}$), hydroperoxyl ($HOO^{\bullet}$), hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals. Figure 1.9 shows a squematic representation of oxygen reduction. At wine pH hydroperoxyl radical and hydrogen peroxide are dominants (Waterhouse & Laurie, 2006).

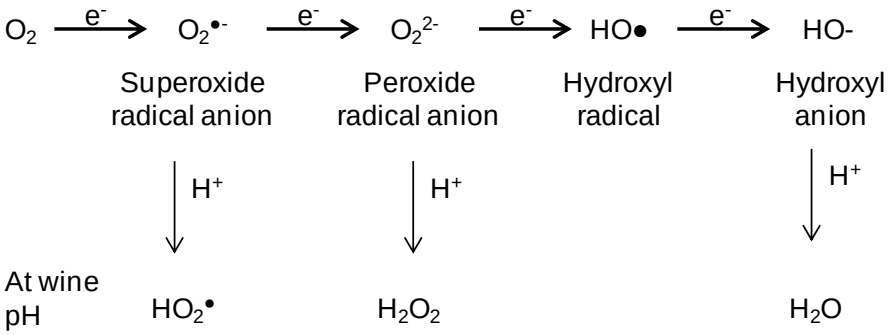


Figure 1.9: Intermediate oxygen species involved in its reduction to water. (Adapted from Waterhouse & Laurie, 2006).

Concerning flavonoids, the differences in the degree of oxidation of the heterocyclic ring and hydroxylation/methoxylation of the three rings result in structures with essential differences in physicochemical properties and stability. Anthocyanins are very instable and susceptible to degradation (Giusti & Wrolstad, 2003). High temperature and oxygen, pH above four, and the presence of specific enzymes such as glucosidases are the main factors which contribute to the degradation of anthocyanins (Queiroz *et al*., 2009; Shenoy, 1993). The chemical structure, concentration, light, solvents, flavonoids, proteins, sugars, sugar degradation products and metallic ions have also been reported to affect anthocyanins degradation or stability (Queiroz *et al.*, 2009; Rein *et al.*, 2005; Shenoy, 1993).

Thermal degradation of anthocyanins follows first order kinetic (Kirca & Cemeroglu, 2003) and can occur via two mechanisms: (1) hydrolysis of the 3-glycoside linkage to form the more labile aglycon; and (2) hydrolytic opening of the C-ring to form a substituted chalcone, which is degraded into the corresponding benzoic acid derivative derived from the B-ring and aldehyde compounds, that are more stable molecules (Castaneda-Ovando et al., 2009; Fleschhut *et al*., 2006)

(Figure 1.10). The syringic, vanillic, and protocatechuic acida were identified as the major degradation products of Mv3Glc, Pn3Glc, and Cy3Glc, respectively. Also, trihydroxybenzaldehyde has been identified as an end-product of the thermal degradation of anthocyanins (Furtado *et al*., 1993).

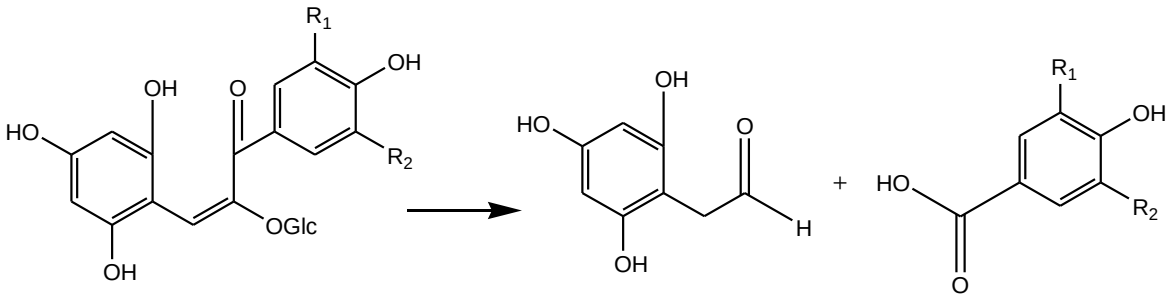


Figure 1.10: Degradation of chalcones to benzoic acids and aldeids (adapted from Castaneda-Ovando *et al*., 2009).

Tseng *et al*. (2006) reported the thermal degradation of 30% of an aqueous solution of malvidin 3-glucoside (50 mg/L) at wine pH when heated at 95 ºC during 1h. In addition, when the solution was prepared with 10% ethanol (v/v) it was verified an increase in the percentage of degradation of malvidin 3-glucoside of 40%.

Concerning phenolic acids and catechins, Palma *et al*. (2001) reported the degradation of seven phenolic acids, catechin and epicatechin when they were kept at 65 ºC during 45 min in contact with air. In the case of catechin, the concentration decreased 40%.

Anthocyanins in acidic solution consist of four molecular forms in equilibrium (Figure 1.11) presenting different colors depending on pH value (Iacobucci & Sweeny, 1983). At pH 1, the flavylium cation (FC) is the predominant specie and contributes to purple and red colors. Increasing pH values causes the deprotonation producing quinoidal base forms (QB), blue or violet. At the same time occurs hydration of flavylium cation (FC), generating the carbinol or pseudobase (PB) which reaches equilibrium with chalcone (CH), both colorless.

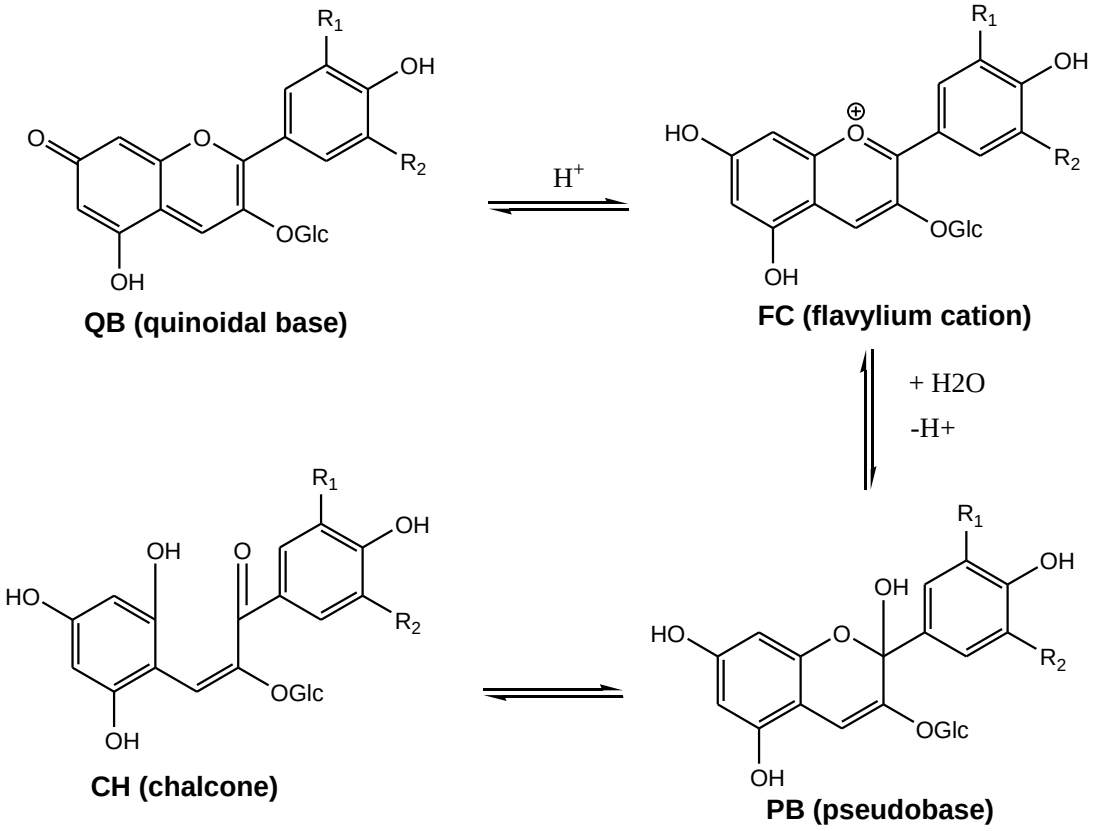


Figure 1.11: Main equilibrium anthocyanins species (Cavalcanti *et al.*, 2011).

Light accelerates the degradation of phenolic compounds, namely anthocyanins (Maier *et al.*, 2009). Bakowska *et al*. (2003) evaluated the anthocyanin stability of cyanidin 3-glucoside solution to UV radiation (λ = 253 nm, 1h) and light exposure (3 months), showing degradation effects. The sunlight exposure caused more 54% of degradation of cyanidin-3-glucoside when compared with the solution kept in the dark. However, a higher stability was verified to occur when the experiments were performed in presence of copigments (especially for flavones). Also, Maier *et al*. (2009), showed higher decreases on the amount of phenolic compounds and anthocyanins, when exposed to neon light during a 24 weeks storage period, compared with those keept in the dark.

Glycosidases are enzymes able to hydrolize the covalent bond between the glycosyl residue and the aglycone promoting the degradation and instability of anthocyanins. The glycosilation of anthocyanidins confers stability to the molecule because the sugar molecules avoid the degradation of instable intermediaries

(Fleschhut *et al*., 2006). Furthermore, the presence of the sugar residue prevents the dimerisation of the anthocyanidins by steric demanding. In addition, the acylation of the sugar residue of anthocyanins has a protective effect from the hydrophilic attack of water (Brouillard, 1981; Yoshida *et al*., 1991). Also, higher stability to heat and light has been described for acylated anthocyanins (Malien-Aubert *et al*., 2001). The phenol oxidases and polyphenol oxidases, both found naturally in fruits and berries, are also common anthocyanin degradating enzymes (Kader *et al.*, 1997).

1.2.1.4 Antioxidant activity of phenolic compounds

The antioxidant activity of a compound may be divided in direct antioxidant effects, (i.e., free radical scavenging and redox-active metal sequestration) and indirect antioxidant effects (i.e., regulation of protein synthesis and activities, signaling strategies). In general, higher concentrations are required to a direct antioxidant action comparing to an indirect action (Dinkova-Kostova & Talalay, 2008; Halliwell *et al*., 2005; Hollman *et al*., 2011).

a) Influence of chemical structure on antioxidant activity

The antioxidant capacity of phenolic compounds depends on their conformational chemical structure, namely on their ability to donate a hydrogen or an electron and their ability to delocalise the unpaired electron within the aromatic structure. The antioxidant efficiency depends not only on the antioxidant compound, but also on the model of oxidation used and of the method assayed. For instance, catechins are more efficient than procyanidins protecting liposomes from oxidation when ferrous iron is the oxidation promoter. In opposition, procyanidins are the most protectives when the oxidant agent is the thermo-labile free radical (2,2′-azobis(2,4-dimethylvaleronitrile)) (Lotito *et al*., 2000). Also, Tabart *et al*. (2009) reported higher antioxidant activity for gallic acid comparing with caffeic acid assayed by ABTS, while a similar activity was verified by DPPH assay.

The presence of 2,3-double bond in the C ring and the 4-oxo function of quercetin confers a high trolox equivalents antioxidant activity (TEAC) value to this compound. The central C ring of cyanidin allows conjugation, resulting in a value (4.4 mM) similar to quercetin (4.72 mM), while catechin, constituted by a saturated C ring possesses approximately half of the antioxidant activity of quercetin (2.4 mM) (Rice-Evans *et al*., 1996).

Some studies (Argyri *et al.*, 2006; Lee *et al.*, 2003) reported the higher antioxidant activity of free phenols when compared to their glycoside forms. The glycosilation of the 3-hydroxyl group in the C ring or removing it leads to a decrease of the antioxidant activity. A similar behavior was verified to occur when the C ring is saturated, suggesting that the 3-OH and the 2,3 double bond are both necessary for the the antioxidant activity. Accordingly to the results obtained by Rice-Evans *et al*. (1996), the antioxidant activity of flavonols in mainly due to the *O*-diphenolic structure in the B ring. The order of TEAC was quercetin (3,4-hydroxyl groups) > myrcetin (3,4,5-hydroxyl groups) > morin (3,5-hydroxyl groups) > kaempferol (4-hydroxyl group) (Frankel *et al*., 1995).

Concerning phenolic acids, the cinnamic acid derivatives possess a higher antioxidant capacity when compared to benzoic derivatives. The insertion of an ethylenic group between a phenyl ring carrying a *p*-hydroxyl group and the carboxylate group has a highly favourable effect on the reducing properties since contributes to the stabilization of the molecule by ressonance (Naczk & Shahidi, 2004). On average, the values of antioxidant activity obtained by Mudnic *et al*. (2010) for cinnamic acids were 62% higher than those obtained for the corresponding benzoic acids.

As a summary, the antioxidant capacity of phenolic compounds is related to their structure in terms of their capacity for electron delocalisation of the aromatic system, and is mainly due to three factors: the presence of two hydroxyl groups on position *ortho* or *para* of the aromatic ring; the 2,3 double bond in conjugation with a 4-oxo function in the C ring which is responsible for electron delocalization from the B ring; and the 3- and 5-OH groups with 4-oxo function in A and C rings (Heim *et al.*, 2002; Rice-Evans *et al.*, 1996).

b) Contribution of phenolic compounds to the antioxidant activity of wines

High correlation coefficients have been found between the total phenolic compounds and the antioxidant capacity of wines (Alén-Ruiz *et al*., 2009; Fernandez-Pachon *et al*., 2004). The contribution to the total antioxidant activity can be from specific compounds or from classes of compounds (Fernandez-Pachon *et al*., 2004; Katalinic *et al*., 2004). However, there are contradictory results about the relative contribution of each class for the antioxidant capacity of wines. According to Fernández-Pachón *et al*. (2004), anthocyanins and flavanols possess higher antioxidant capacity when compared with the phenolic acids and flavanones fractions. According to several studies (Beecher, 2003; Ghiselli *et al*., 1998; Simonetti *et al*., 1997; Wang et al., 1997), flavan-3-ols, flavonols and anthocyanins are the classes of phenolic compounds that exhibit higher contribution for the antioxidant capacity of red wines. More recent works (Radovanovic & Radovanovic, 2010; Rivero-Pérez *et al*., 2008) showed that free anthocyanins are the main fraction responsible for total antioxidant capacity of red wines. In contrast, the results obtained by Alén-Ruiz *et al*. (2009) suggested that the polymerized compounds were those that most strongly contributed to antioxidant capacity of red wines. Otherwise, Burns *et al*. (2000) reported that there are no correlation between anthocyanin content and antioxidant activity of wines. Katalinic *et al*. (2004) reported that the low molecular weight catechins, especially monomers and dimers, are the main class of phenolic compounds responsible by wine antioxidant activity.

In summary, the data available in literature suggests that the antiradical activity should be attributed to the different classes of phenolic compounds and also to the molecular structures of the individual phenolic compounds that each fraction contains. Futhermore, the antioxidant efficiency depends on the antioxidant compound and on the model of oxidation used and of method assayed.

The antioxidant activity of wines has been assessed applying different methodologies (Fernandez-Pachon *et al*., 2004; Teissedre & Landrault, 2000). In a chemical point of view, these methods can be divided in assays based on

scavenging by hydrogen- or by single electron-donation reactions. The latter includes the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay (Miller *et al*., 1993), the 1,1'-dihenyl-2-picrylhydrazyl (DPPH) assay (Brand-Williams *et al*., 1995), the N,N-dimethyl-p-phenylendiamine (DMPD) assay (Fogliano *et al*., 1999) and the ferric ion reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996). However, different experimental conditions performing the assays, such as radical generator, time of measure or expression of results, make it difficult to compare reported data (Fernández-Pachón *et al*., 2004). For Huang *et al*. (2005) $ABTS^{\cdot+\bullet}$ and $DPPH^{\bullet}$ radicals have a different stereochemical structure and another method of genesis and thus they lend, after the reaction with the antioxidants, a qualitatively different response to the inactivation of their radical. Nevertheless, in general, the results obtained showed similar conclusions.

These are spectrophotometric methods and the results can be obtained as function of the percentage of inhibition, and measures the sample antioxidant potential with an equivalent antioxidant potential of a standard (usually trolox, the watersoluble vitamin E analog) in a well defined time reaction (for example 2 or 15 min for ABTS and 30 min for DPPH assay). The concentration of antioxidant needed to decrease by 50% the initial substrate concentration (EC_{50}) is another parameter widely used to measure the antioxidant power (Robak & Gryglewski, 1988). The lower the EC_{50}, the higher the antioxidant power. Sánchez-Moreno *et al*. (1998) proposed antiradical efficiency (AE) as a new parameter to express the antioxidant power. The AE combines the concentration and the time (T_{EC50}) needed to the reaction reach the steady state to EC_{50} concentration. The antiradical efficiency value is obtained by the expression: $AE = 1 / (EC_{50} \times T_{EC50})$. In addition to concentration, the reaction time is also important to define the antioxidant power of a compound. Larrauri *et al.* (1999) reported values of T_{EC50} ranging from 26.0 to 39.9 min for 12 red wines. Also, a large range of T_{EC50} values was obtained by Villano *et al.* (2006) for 20 phenolic compounds varying from 11 min to caftaric acid until 180 min for catechin.

1.2.2 Polysaccharides

Polysaccharides have been thoroughly studied because of their importance to the technological and sensorial properties in wines (Ducasse *et al*., 2010). The extent that they affect the properties of the wine depends on its composition, structure and concentration. Their presence in wine contributes to the physical and chemical stability (Feuillat, 1998; Moine-Ledoux & Dubourdieu, 2002) and to the organoleptic properties, acting as stabilizers of the aroma, flavor, color and foam (Chalier *et al.*, 2007; Coelho *et al.*, 2011; Escot *et al.*, 2001). They improve the organoleptic quality of wines (Guadalupe & Ayestarán, 2007; Jimenez *et al.*, 2010) increasing the roundness and body of red wines, and reducing astringency and bitterness (Feuillat, 2003). Moreover, polysaccharides are important for biological properties, since they can interact with phenolic compounds modulating their bioavailability.

The structure and amount of polysaccharides present in wines depend on the winemaking process and can be modified by the temperature, degree of clarification of the must, enzyme treatment, maceration time and on the population of yeasts (Doco *et al.*, 2007; Guadalupe & Ayestarán, 2007). The fine structure of the pectic polysaccharides governs the biological roles of these molecules within the cell wall. The amount of polysaccharides may be present in the range between 0.2 and 2 g/L (Ayestarán *et al*., 2004; Boulet *et al*., 2007; Doco *et al*., 1999). Wines from red grapes are usually richer in polysaccharides when compared with those from white grapes (Vernhet *et al*., 1999), since they are produced by maceration.

Wine polysaccharides arise from both grape and microorganisms. Pectic polysaccharides are originated from grape berry after degradation and solubilization by pectic enzymes during grape maturation and during the first steps of winemaking. Yeasts produce mannans and mannoproteins during and after fermentation (Waters *et al*., 1994).

1.2.2.1 Pectic Polysaccharides

Pectic polysaccharides are constituted by different associated polysaccharides, such as homogalacturonans (Vincken *et al*., 2003), xylogalacturonans, arabinans (Belleville et al., 1993), galactans, type I and type II arabinogalactans (Pellerin *et al*., 1995), and type I and type II rhamnogalacturonans (Doco *et al*., 2007; Pellerin *et al*., 1996). These polysaccharides are believed to be covalently linked to one another because they are all solubilized by endo-polygalacturonase treatment of primary cell walls (Doco *et al.*, 1995; O´Neill *et al.*, 1996; Puvanesarajah *et al.*, 1991).

Different models were proposed for the macromolecular structure of pectic polysaccharides. According to Pérez *et al.* (2003), pectic polysaccharides were constituted by one continuous backbone formed by D-galacturonic acid residues interrupted by the insertion of L-rhamnose (1→2)-linked in adjacent or alternate positions (Coimbra & Delgadillo, 1997). This structure (Figure 1.12) was composed by linear areas composed of homogalacturonans or xylogalacturonan and by branched areas formed by type I rhamnogalacturonans (RG-I) and type II rhamnogalacturonans (RG-II). Neutral sugars, mainly L-arabinose and D-galactose, but also L-rhamnose and D-xylose were often linked to the backbone forming side chains of variable length. Other residues, such as D-glucose, D-mannose, L-fucose and glucuronic acid can also be found. The galactose and arabinose are in more complex chains with a structure similar to arabinans and arabinogalactan.

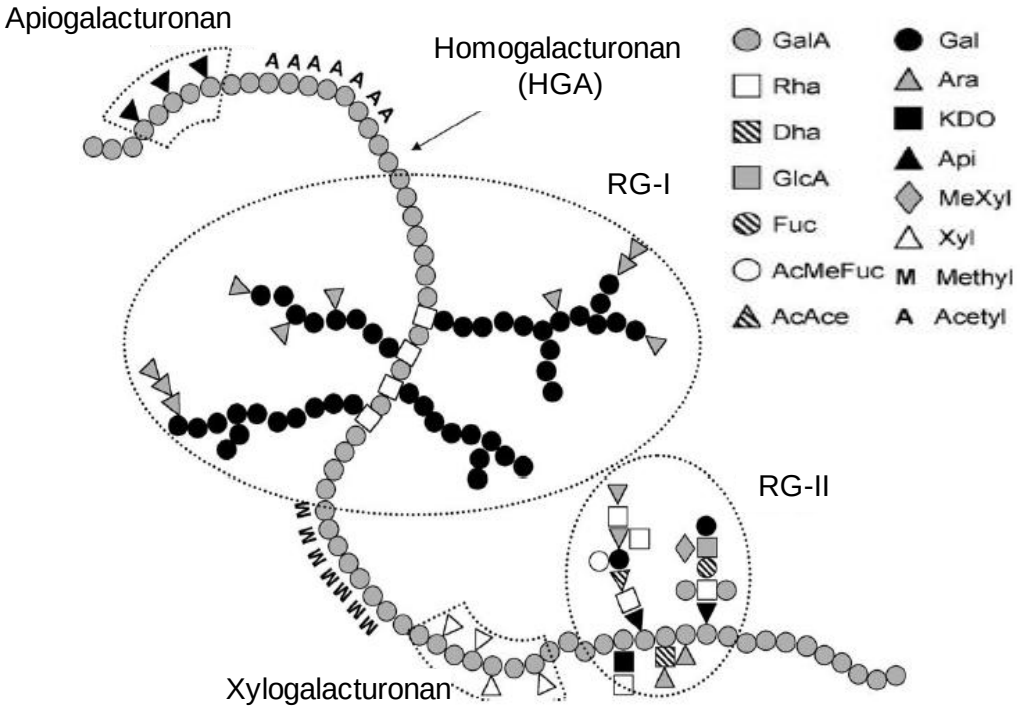


Figure 1.12: Schematic representation of pectic polysaccharides (Pérez *et al.*, 2003).

Vincken *et al.* (2003) proposed a different model for structure of pectic polysaccharides. The main difference of this model is that the homogalacturonans (HG) constituents are depicted as side chains of RG-I. Xylogalacturonan (XGA), type I arabinogalactanas (AG-I), type II arabinogalactanas (AG-II), and RG-II also appear as side chains of RG-I. This distribution results in a hairy structure of pectic polysaccharides.

a) Galacturonans

Galacturonans are structural elements of pectic polysaccharides that have a backbone of α-1,4-linked galacturonic acid (Gal*p*A). Three types of galacturonan can be distinguished in the plant cell wall: the unsubstituted homogalacturonan, xylogalacturonan, and type II rhamnogalacturonan.

Homogalacturonans, comprise 60–70% of total pectic polysaccharides amount. HG can be partially methylesterified at the C-6 carboxyl group and/or O-

acetylated at O-2 and/or O-3. The proportion of galacturonic acid residues that are methylesterified or acetylesterified is important to determine the characteristics and functional properties of pectic polysaccharides (Dumitriu, 1998).

The xylogalacturonan has a galacturonan backbone, but 25–75% of Gal*p*A residues are branched at C-3 with xylose (β-D-Xyl*p*). Xylose is mainly present as single residues but occasionally an additional β -2-linked or β-4-linked Xyl*p* to form a disaccharide have been identified (Le Goff *et al.*, 2001; Zandleven *et al.*, 2006). Furthermore, the Gal*p*A residues comprising the XGA backbone can be methylesterified and the methyl esters seem to be equally distributed among the substituted and unsubstituted Gal*p*A residues.

b) Type II arabinogalactans

The type II arabinogalactanas (AG-II) are a group of polysaccharides that are found in various plants (Pellerin *et al*., 1995). They represent an important part of the total soluble polysaccharides of wine, 100-200 mg/L in red wines and 50-150 mg/L in white wines (Doco et al., 1995).

The AG-II (Figure 1.14) are mainly composed of arabinose (Ara) and galactose (Gal) in different proportions (Doco *et al*., 1995). These polysaccharides are composed of a β-(1→3)-linked Gal*p* backbone branched at C-6 by side chains of Gal β-(1→6) highly substituted by Ara*f* and in small amount by Ara*p,* Rha, Xyl, and glucuronic acid (Pellerin *et al*., 1995). Individual units or short chains of α-L-Ara that are sometimes attached directly to the backbone in the carbons C-4 or C-6 (Ribéreau-Gayon, 1998). Glucuronic acid is also found as terminal non-reducing and 4-linked in amounts that can range between 3 and 20% (Pellerin *et al*., 1995). The different content in uronic acids confers on them characteristics of weak acidic or even acidic polysaccharides.

AG-II are mainly associated with proteins (2-10%), so called arabinogalactan proteins (AGP). The hydroxyproline, serine, alanine, and glycine are the main amino acids constituents of the protein part of AGP (Gaspar *et al.*, 2001). Vidal *et al*. (2003) described six different fractions of AGP isolated from wine. The

heterogeneity of AGP is attributed to the percentage of the protein, uronic acid content (3-20%) (Pellerin *et al*. 1995; Vernhet *et al*., 1999), type of linkage and spacial conformation (Vidal *et al*., 2003) in the different fractions.

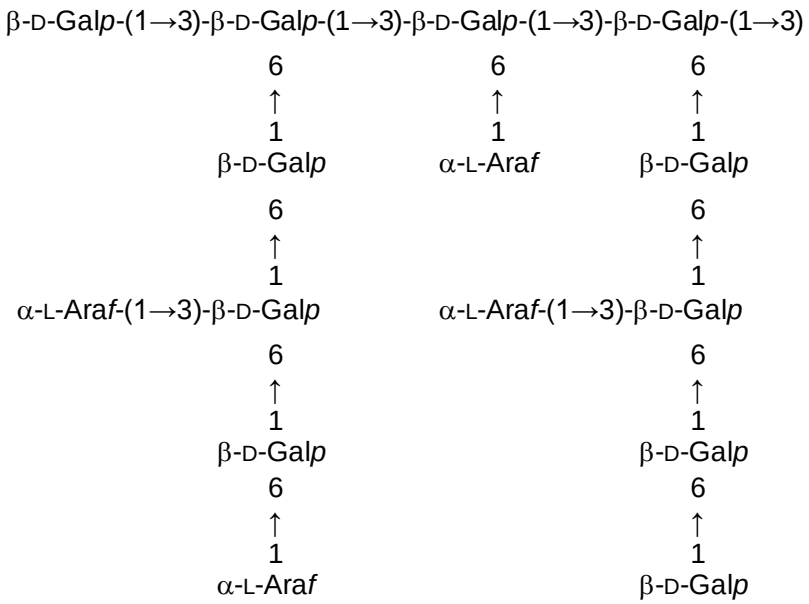


Figure 1.13: Structure of type II arabinogalactans (Ribéreau-Gayon *et al.,* 1998).

c) Type I arabinogalactans

The type I arabinagalactans (AG-I) may appear as neutral polysaccharides or as individual subunits covalently linked to the chains of rhamnogalacturonans (Coimbra & Delgadillo, 1997). These polysaccharides are composed of a β-(1→4)-linked D-Gal*p* backbone, where α-L-Ara*f* residues can be attached to the *O*-3 of galactosyl residues.

d) Type II rhamnogalacturonans

The type II rhamnogalacturonans (RG-II) represent about 20% of soluble polysaccharides (Doco & Brillouet, 1993; Pellerin *et al*., 1996). The levels of RG-II in red wines are between 100-150 mg/L and between 20-50 mg/L in white wines

(Doco *et al*., 2000). These polysaccharides can be present either as a monomer (mRG-II) (Figure 1.14). or as a dimer (dRG-II), with a mass approximately 5 and 10 kDa, respectively (O´Neill *et al.*, 1996). In the primary cell wall they are predominantly as a dimer complexed in a 1:2 borate diol diester (Ishii & Matsunaga, 1996; O' Neill *et al.*, 1996). Dimers can form stable complexes with cationic di- and trivalent species, particularly with Pb^{2+} ions (Pellerin *et al*., 1996).

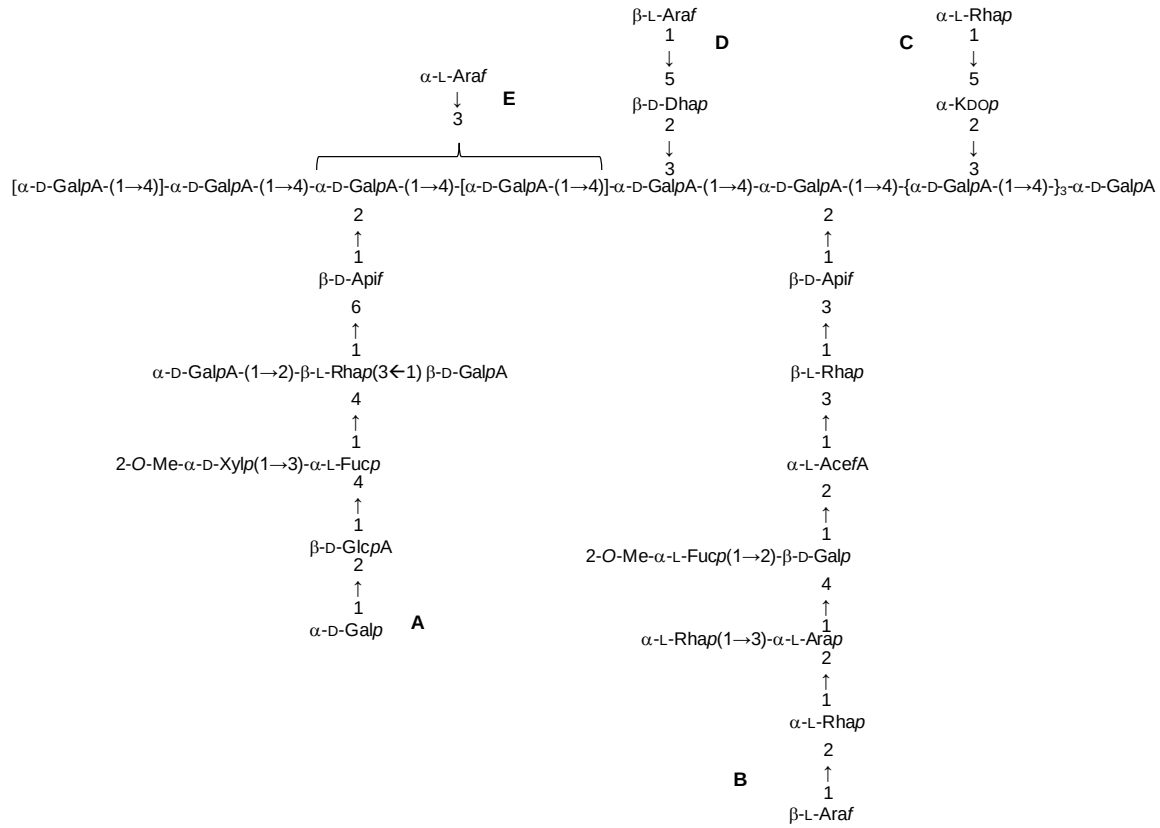


Figure 1.14: Schematic representation of the primary structure of RG-II monomer (mRG) (Pérez *et al.,* 2003).

The RG-II are a pectic polysaccharide with a complex structure (Pellerin *et al*., 1996; Puvanesarajah, 1991), containing twelve different sugar residues in over 20 different linkages, where Rha and Gal are the sugars present in higher amount (Pérez *et al*., 2003). The backbone of RG-II consists of seven to nine α-(1→4)-linked D- galacturonic acid (Gal*p*A) residues, partially methylesterified. The RG-II have five oligosaccharide side chains (A-E) with well-defined structures (Pellerin *et*

al., 1996; Pérez *et al*., 2003) (Figure 1.14). The side chains A and B, bind themselves to the *O*-2 of the GalA residue by apiose. The B-side chain is located at the fifth GalA residue from the reducing end. The aceric acid and 2-*O*-Me-Fuc*p* residues are acetylated. The C-side chain is linked to *O*-3 of residue of GalA of the main chain through KDO, while the D-chain is linked to *O*-3 of the sixth residue of GalA from the reducing end through DHA (Vidal *et* al., 2000). The side E-chain is constituted by Ara*f* residues.

e) Type I rhamnogalacturonans

The type I rhamnogalacturonans (RG-I) are polysaccharides consisting of repeating units of the disaccharide [→2)-α-L-Rha*p*-(1-4)- α–D-Gal*p*A-(1→]. The presence of residues of Rha induces a deviation of 90º in the backbone, called the "pectic tail" (Doco *et al*., 1995). The presence of 2,4- and 2,3-linked rhamnose indicates that these residues are branching points of short side chains (Vidal *et al*. 2003), composed by arabinose and/or galactose distributed on a non-regular way (Coimbra, 1993). The RG-I are relatively abundant in the cell walls of grape but are in small amounts in red wines (Doco *et al*., 2000).

f) Arabinan

The arabinan isolated from red wine by Belleville *et al*. (1993) is constituted by a backbone containing a small number of L-arabinose residues (1→5) linked. This backbone is weakly branched by individual units of arabinose at C-3. Arabinans come from the side chains of RG-I, and are released by the degradative action of pectinolytic enzymes during ripening of the grape. The insolubilization of polysaccharides by the action of an arabinofuranosidase explains the low level of arabinans found in wine (Doco *et al*., 2000).

1.2.2.2 Polysaccharides released by microorganisms

The yeasts are the second largest source of wine polysaccharides. The yeasts cell walls are mainly composed of mannoproteins and also of glucans. Mannans and mannoproteins are produced by yeasts, such as *Saccaromices cerevisiae* (Feuillat, 2003; Waters *et al*., 1994). Glucans are produced by *Botrytis cinerea*, which infects grape berries (Dubourdieu *et al.*, 1981; Vidal *et al.*, 2003)

a) Mannoproteins

The mannoproteins (MP) are composed, on average, by 70% of carbohydrates and 30% of proteins (Waters *et al*., 1994) whereas mannose (Man) represents around 98% and glucose 2% of sugar residues. However, Vidal *et al*. (2003) isolated six different fraction of mannoproteins from the red wine with a rather low content of protein, ranging from 1.4 to 9.3% and with a Man content that represents between 88 and 97% of sugar residues. The molecular weight is highly variable, ranging from 5 to 800 kDa (Doco *et al*., 2003; Saulnier *et al*., 1991). The protein fraction is mainly composed by serine, glycine, threonine and alanine. The net charge of mannoproteins, which may be responsible for their properties, results from the presence of phosphate groups and amino acids possessing carboxyl or amine groups (Vernhet *et al*., 1996).

The mannoproteins are composed by small chains of mannose with one to four D-Man residues in α-(1→ 2) or α-(1→ 3), and by chains with multiple residues of Man α-(1→ 6)-linked, branched by side chains of Man α-(1→ 2) or of Man α-(1→ 3) (Feuillat, 2003). The small chains are N-linked or O-linked to the polypeptide chains on serine or threonine residues (Figure 1.15).

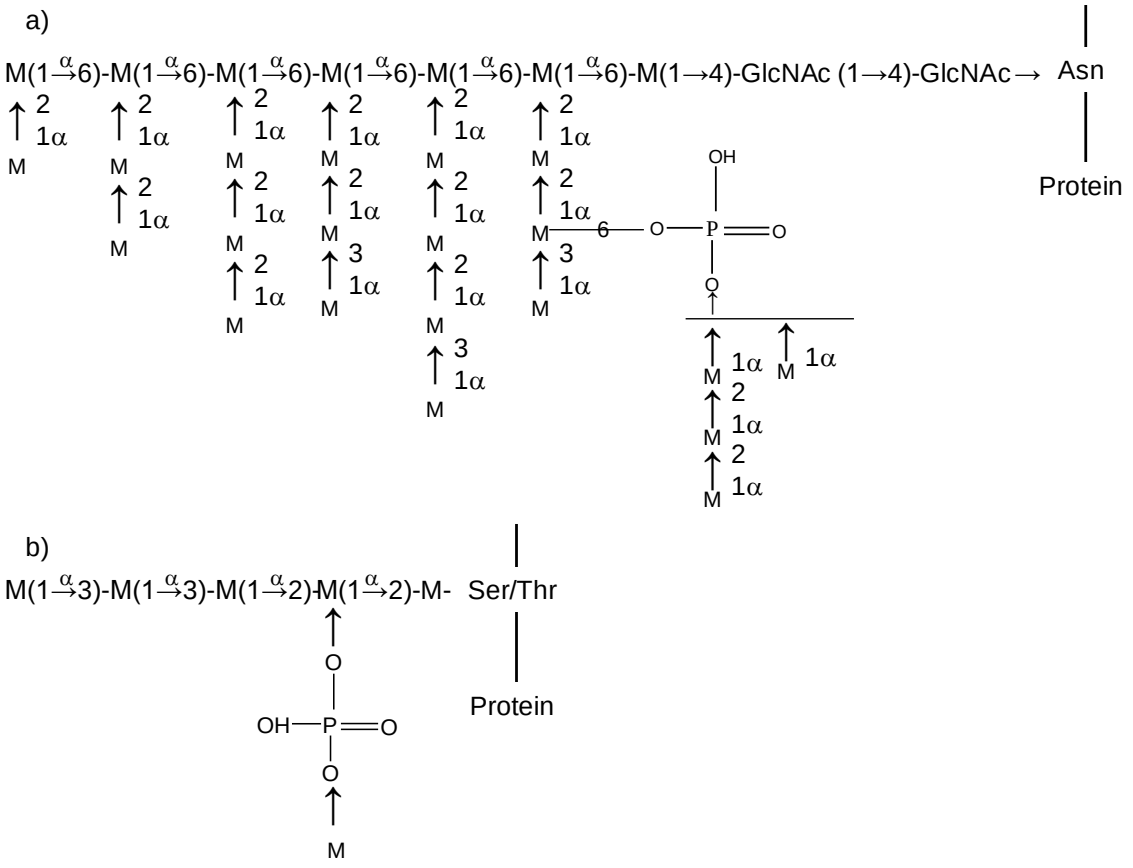


Figure 1.15: Structural model of mannoproteins. a) N-Asn protein linkage; b) O-Ser/Thr protein linkage (Jigami & Odani, 1999). M- mannose, GlcNAc- *N*-acetyl-D-glucosamine, Ser-serine, Thr-threonine, Asn- Asparagine .

MP are mainly located in the outermost layer of the yeast cell wall, where they act as structural components and are partially responsible for their permeability (Klis *et al*., 2002). The release of mannoproteins occurs in the early stages of alcoholic fermentation (Waters *et al*., 1994) or when cell death leads to its release by autolysis (Feuillat, 2003). They can be hydrolyzed by α-mannosidases and proteases, releasing small peptidomannans into wine (Doco *et al*., 2003). Mannoproteins represent around 35% of the total polysaccharides in wine (Vidal *et al*., 2003), accounting from 100 to 150 mg/L.

b) Glucans

The fungus *Botrytis cinerea* produces a β-glucan of high molecular weight (10^5-10^6 Da) and a heteropolysaccharide (10^5-5×10^5 Da). The β-glucan is a polymer of β-(1→3)-glucose being two of five residues branched by β-(1→6)-glucose units. The release of these polysaccharides also depends on the action of β-(1,3)-endoglucanase and of the β-(1,6)-exoglucanase (Ribéreau-Gayon, 1998).

1.2.2.3 Antioxidant activity of polysaccharides

Polysaccharides have also been associated to antioxidant properties (Hensel & Meier, 1999; Kogan *et al.*, 2005; Y.-H. Tseng *et al.*, 2008) with potential health benefits such as lowering cholesterol, reducing the disease symptoms of constipation, and reducing the risk of diabetes (Plaami, 1997). In addition, tea polysaccharides conjugates have been reported to have immunological, antiradiation, anti-cancer, and anti-HIV activities (Chen *et al.*, 2008). However, studies reporting antioxidant activity of wine polysaccharides are scarse. Aguirre *et al*. (2009) isolated a neutral fraction from wine polisaccharides consisting mainly of an arabinogalactan and also Man and Glc in minor amounts, which possessed antioxidant activity determined by hydroxyl radical scavenging.

1.2.3 Interactions between wine polymeric material and phenolic compounds

The wine polymeric compounds have been shown to interact with long chain esters, such as ethyl decanoate (Rocha *et al*., 2007) as well as with phenolic compounds, namely the anthocyanins and flavonols (Saura-Calixto & Díaz-Rubio, 2007). Non-covalent and covalent associations of polyphenols with food macromolecules are two of the most fundamental factors affecting the quality of polyphenol-rich food products.

Proteins can interact with phenolic compounds at wine conditions. In addition to proteins, the nitrogenous fraction of wines contains peptides, and amino acids. Wine proteins are originated predominantly from the grapes and in minor amounts from yeasts. These nitrogenous compounds are important in wines because they may affect the aroma complexity and taste of wines (Monteiro & Bisson, 1991). The phenolic nucleous has a favorable structure to interact with proteins. It contains a benzenic ring that can interact with the non-polar regions of proteins. Phenolic compounds also possess hydrophilic parts such as hydroxyl groups that can establish hydrogen bonds with the carbonyl and amino groups of proteins. The major interactions must be hydrogen bonding and hydrophobic interactions, since the electrostatic and ionic forces are not determinant to their reactivity at wine pH (Vernhet *et al*., 1996). Proteins might interact with tannins differently. The type of interactions between tannins and proteins is influenced by the medium composition, such as the ethanol percentage, ionic strength, the type of tannin (Vidal *et al*., 2004) and the type of protein (Carvalho *et al*., 2006). The affinity of tannins toward proteins in simple solutions increases *in vitro* with increasing DP (Arnold *et al*., 1980).

Some polysaccharides have the ability to form gel-like structures or to develop secondary structures in solution forming hydrophobic pockets able to encapsulate and complex phenolic compounds (Le Bourvellec *et al*., 2005). On the other hand, dextran gels are able to encapsulate polyphenols inside their pores via hydrogen bonds between the hydroxyls groups of phenols and the oxygen atoms of the cross-linking ether bonds, as in the apolar cavity of cyclodextrins (Ozawa *et al*., 1987). Polysaccharides must have a suitable structure, composition, as well as a sufficient size and flexibility to be able to complex polyphenols (De Freitas *et al.*, 2003; Luck *et al.*, 1994). Conformational flexibility and hydrophobicity can strongly influence the retention of phenolic compounds by polysaccharides (Le Bourvellec *et al*., 2005). Simonsen *et al*., (2009) in their study about interaction between barley and oat β-glucans and phenolic compounds showed that the more retained compounds shared the presence of a hydroxyl group at position 4 and a CHO group at position 1. However, different binding properties are important for the retention of different small phenolics by β-glucan fiber matrix from oak.

The polysaccharides have the ability to establish electrostatic and ionic interactions with other compounds such as proteins (Vernhet *et al*., 1996) and to interact with phenolic compounds (Riou *et al*., 2002). The association of tannins with mannoproteins, (Escot *et al*., 2001) and with RGII dimer (Riou *et al*., 2002) has been suggested at wine concentrations. The interactions of mannoproteins with color compounds and tannins can influence color stability (Escot *et al*., 2001; Poncet-Legrand *et al*., 2007; Riou *et al*., 2002; Vasserot *et al*., 1997) and sensory qualities, namely the reduction of wine astringency (Fornairon-Bonnefond *et al.*, 2002; Vidal *et al.*, 2004) and improving sweetness and roundness (Guadalupe *et al*., 2007). These interactions result in a decrease of wine tannin content, suggesting the precipitation of tannin and the formation of mannoprotein aggregates (Guadalupe *et al*., 2010).

1.2.4 Effect of polysaccharides on protein-tannin interactions

Several salivary proteins, such as α-amylase and proline-rich proteins (PRP), have been reported to interact with tannins (Bacon & Rhodes, 2000; Carvalho *et al.*, 2006; Kandra *et al.*, 2004). The polysaccharides can inhibit protein-tannin interactions (Doco et al., 2001) by two different mechanisms in alternative or in consonance (De Freitas *et al.*, 2003; Luck *et al.*, 1994; Ozawa *et al.*, 1987). On one hand, polysaccharides can encapsulate polyphenols inhibiting their capacity to bind proteins. On the other hand, some polysaccharides can form protein-tannin-polysaccharide ternary complexes, enhancing their solubility in aqueous medium due to the polyelectrolyte character of polysaccharides. This prevention of protein-tannin aggregation can be related to their ionic characteristics. The capacity of ionic polysaccharides, such as pectin, gum arabic, and polygalacturonic acid to prevent protein-tannin aggregation has been described (De Freitas *et al.*, 2003; Luck *et al.*, 1994). Acidic fractions of AGP and mannoproteins have been shown ability to inhibit the aggregation between condensed tannins and salivary proteins at wine concentrations (Carvalho *et al*., 2006). RG-II has been relatively effective in preventing aggregate formation

between α-amylase and tannin but favoring the formation of aggregates between IB8c and tannin. The α-amylase is a globular protein, while IB8c is a PRP with an elongated structure (type II helix). A possibility to explain the action of RG-II is their capacity to bind α-amylase preventing the tannins to access to α-amylase. Moreover, RG-II enhance tannins particle size, suggesting co-aggregation between RG-II and tannins (Riou *et al*., 2002).

1.3 Biological Effects of Phenolic Compounds

Several studies have associated the antioxidant properties of phenolic compounds with the potential health benefits of wine consumption, such as prevention of heart diseases and cancer (Katalinic *et al*., 2004; Virgili & Contestabile, 2000). However, the effectiveness of potential health impact of bioactive compounds is strongly influenciated by their bioavailability. Some bioactive compounds remain poorly available after ingestion. Bioavailability depends of their interactions with other compounds or transporters during intestinal digestion, permeability in the gut, cellular uptake, metabolism and further transport in the circulatory system.

1.3.1 Bioavailability of phenolic compounds

Functional foods enriched in bioactive compounds such as phenolic compounds have been developed and are object of interest since they may positively contribute to human health. However, the effectiveness of the physiological benefits of such products depends on preserving the bioavailability of the active ingredients. Bioavailability is defined as the rate and extent to which the active substances or therapeutic moieties contained in a drug are absorbed and become available at the site of action (Shi & Le Maguer, 2000). This definition also applies to nutrients present in foods. This concept is associated with the amount of an ingested compound that is available for absorption in the gastrointestinal tract (Stahl *et al*., 2002), as end result of absorption distribution, metabolic conversion to bioactive compounds in the body and elimination via urine and faeces (Brouns, 2002). A relate concept to bioavailability is the bioaccessibility. Bioaccessibility is defined as the amount of a food constituent that is present in the gut, as a consequence of the release of this constituent from the solid food matrix, and may be able to pass through the intestinal barrier.

Bioavailability of phenolic compounds depends of their stability during food processing and storage conditions. The incorporating of anthocyanins into dietary

supplements, their storage and their passage through the human digestive system usually lead to a fast degradation (Hager *et al*., 2008; Kirca *et al*., 2007), resulting in a small amounts of anthocyanins in the human bloodstream (He *et al*., 2010; Koli *et al*., 2010; Manach *et al*., 2005). After ingestion, to an active ingredient became bioaccessible is necessary its release from the matrix during gastrointestinal digestion. This release is dependent of tissue maturity, cooking, mastication and susceptibility of the food matrix to the digestion (Sanz & Luyten, 2006). Only what is released can be bio-accessible for absorption (Tedeschi *et al*., 2009), predominantly in the small intestine. Otherwise the absorption phenomenon cannot take place and the non-released compounds will be excreted in the faeces (Bohn *et al.*, 2007). Some of the constituents, such as phenolic compounds, associated with dietary fibre, are not totally susceptible for absorption (Kulkarni *et al.*, 2007; Saura-Calixto & Díaz-Rubio, 2007). The fiber matrix can carry functional components, such as antioxidant compounds, embedded in them, which are gradually released in the intestinal lumen and partly absorbed into gut epithelial cells (Vitaglione *et al.*, 2008). Pérez-Jiménez *et al*. (2009) observed that dietary fiber delays the absorption of the phenolic compounds associated with it. Accordingly, the bioavailability of ferulic acid is lower when it is bounded to the indigestible polysaccharides of the cell walls of cereals (Anson *et al.*, 2009). The bioavailability of ferulic acid depends largely of their source; for instance, it presents a low bioavailability by consumption of cereal products (2.5–5%) and is highly bioavailable by beer consumption (19–98%) where is predominantly present in its free form (Adam *et al.*, 2002; Bourne *et al.*, 2000).

Digestion is a physiological process that allows the extraction of macronutrients such as carbohydrates and proteins, or their basic units, micronutrients and phytochemicals such as polyphenols from the food matrix, for sub-sequent absorption. The first step of the human digestive process occurs in the mouth, whereas the food is acted by salivary enzymes, such as α-amylase and lingual lipase (Pedersen *et al.*, 2002). This stage is characterized by the initial degradation of polysaccharides and triacylglycerides during mastication, and by the release of components related to food taste and flavour (Malone *et al.*, 2003). In the second step (stomach), the food bolus is subjected to a highly acidic

environment (pH 1–3), and the food constituints are subjected to enzyme hydrolysis (e.g., proteases and gastric lipase), and to the action of various surface active substances (e.g., phospholipids, mucins and proteins) (Armand *et al.*, 1994). During gastric digestion, chemical and structural changes may occur in the food constituents becoming available for uptake in the intestine. The last phase occurs in the small intestine and later in the large intestine (Biehler & Bohn, 2010). In the small intestine, the constituents are mixed with digestive juices containing bile salts, phospholipids, pancreatic lipase, proteases, salts, and bicarbonate (Fave *et al.*, 2004; Pafumi *et al.*, 2002). The pH in the small intestine is close to neutral due to the mixing of the chymo with the alkali digestive juices. The phenolic compounds may be released from the food matrix by the action of digestive enzymes in small intestine and also colonic bacterial fermentation in the large intestine (Biehler & Bohn, 2010) and therefore potentially bioavailable.
Phenolic acids are able to be released from bound complexes by the metabolization of intestinal microflora (Manach *et al.*, 2004).

During digestion, phenolic compounds may be metabolized by deglycosylation glucuronidation, sulfonation and methylation or cleavage by esterases (Argyri *et al.*, 2006; Cilla *et al.*, 2009). The metabolization of catechin and epicatechin was described to occur in the small intestine (Kuhnle *et al.*, 2000). Also, has been reported the glucuronidation of trans-resveratrol (Vitaglione *et al*., 2005) and of pelargonidin 3-glucoside (Felgines *et al.*, 2003). In addition the presence of trimers in plasma after the intake of a grape-seed phenolic extract was reported by Serra *et al*. (2010). Ou *et al*. (2012) demonstrated that these oligomers can pass very slowly through the Caco-2 cell membranes, compromising their bioavailability.

The *In vitro* methods used to study bioaccessibility of nutrients are based on the simulation of gastrointestinal digestion, followed by determination of the soluble fraction of the compound capable of dialyzing through a semi permeable membrane with a specified pore size (Van Campen & Glahn, 1999). Despite their limitations, such as typically constituting only a static model of digestion, the *in vitro* GI models have been well correlated with *in vivo* methods (Biehler & Bohn, 2010). In addition, plasma antioxidant capacity was used to determined the

bioavailability of phenolic compounds present in foods (Cao *et al*., 1998; Perez-Jimenez *et al*., 2009). For evaluation of bioavailability of individual phenols is necessary to test the compounds isolated from foods matrix.

The knowledge of the factors that influences the release of phenolic compounds during all gastrointestinal tract and therefore in their bioavailability would enable food industry designs more efficient functional foods.

1.3.2 Interactions of Phenolic Compounds with Cell Membrane

In biological systems, an antioxidant is any substance that, when present in low concentration relative to those of oxidizable substrates (lipids, proteins, DNA or carbohydrates) can delay, retard, or prevent the oxidation of these substracts (Frankel & Meyer, 2000). Moreover, the resulting radical formed after scavenging must be stable (Shahidi *et al.*, 1992).

The capacity of phenolic compounds to interact with cell membranes is of great importance to their beneficial health effects (Hendrich, 2006). The induction of changes in membrane physical properties by phenolic compounds can affect the biological mechanisms that occur at the cellular membrane level, such as the enzymes activities, the transport of metabolites, the signal transduction, the membrane constituents interactions and also the oxidation of membrane constituents (lipids and proteins) (Hashimoto *et al.*, 2001).

The protection by phenolic compounds of membrane oxidation can be attributed to their capacity to scavenge free radicals or to the decreases of membrane fluidity. As membranes become more fluid, the motion of fatty acids increases as also the probability of lipid radical interactions leading to lipid oxidation (Verstraeten & Oteiza, 2000).

Both, biological and model membranes have been used to study the interactions between the flavonoids and the bilayer of the membrane. The interactions of flavonoids with bilayers depend on their chemical properties, namely their polarity. Figure 1.16 shows the hypothetical location of interaction of flavonoids with membrane. In Figure 1.16 is also possible to observe the initiation

of a liquid ordered phase (Lo) caused by non-polar flavonoid and a superliquid ordered phase (SLo) caused by polar flavonoid. The rest of the lipid molecules is in a liquid disordered state (Ld). The more non-polar polyphenols interact with the hydrophobic interior of the membrane (Figure 1.16a), while the more hydrophilic flavonoids can establish hydrogen bonds with the polar head groups of lipids at the membrane interface (Figure 1.16b).

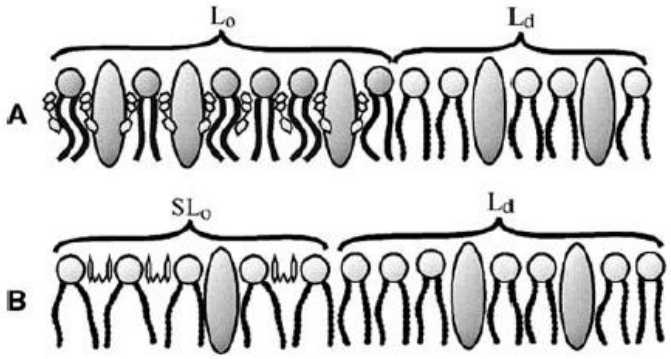


Figure 1.16: Hypothetical location of interaction of flavonoids with membrane (for simplicity, only one lipid monolayer is shown). (a) non-polar flavonoid molecules intercalate in the hydrophobic region of the membrane; (b) polar flavonoid molecules intercalate the polar heads of lipid (adapted from Tarahosvsky *et al*.(2008)).

The presence of hydroxyl groups in the flavonoids structure is responsible for their polarity and expression of weak acidic properties. The more hydrophobic compounds can interact with the hydrophobic core of the membrane and affect transmembrane potential. The cinnamic acid showed capacity to promote liposome permeability, while its hydroxylated derivative (4-hydroxy-cinnamic acid) had no effect. The partition of certain phenolic compounds in the hydrophobic core can results in a antioxidant activity.

The interaction of phenolic compounds with the polar interface of the bilayer can protect it from the deleterious effects of oxidant agents. The number and distribution of hydroxyl groups of phenolic compounds are important to their capacity to binding at the water–lipid interface through the formation of hydrogen bonds (Ollila *et al.*, 2002). Also, the presence of the 2,3 double bond at the C ring is a important factor to the capacity of these compounds to form hydrogen bonds, otherwise the methoxy group at position 4 of the C ring has no effect (Oteiza *et al.*,

2005). The glycosilation of flavonoids is a factor important to rule their interaction with membrane, since that enhances their hydrophilicity. For example, Tsuchiya *et al*. (2002) showed that rutin glycoside had the ability to interact only with the surface of the phospholipid bilayer while their influence on the hydrocarbon region was negligible.

1.3.3 Health benefits of phenolic compounds

The health benefits associated to the moderate wine consumption. They include effects against circulatory (Sabolovic *et al*., 2006), neurodegenerative diseases (Kim *et al*., 2009), cancer (Tapiero *et al*., 2002), and cardiovascular diseases (Virgili & Contestabile, 2000), decrease of inflammatory processes, inhibition of platelet aggregation (Gryglewski *et al.,* 1987), and prevention of oxidation of human low-density lipoproteins (Frankel *et al.,* 1993). These benefits have been associated to biological active compounds with strong antioxidant properties such as phenolic compounds (Katalinic *et al.,* 2004).

Epidemiological studies have shown that polyphenols isolated from red wine inhibited the growth of some tumor cells *in vitro* (Gomez-Cordoves *et al.*, 2001; Iijima *et al.*, 2000). In addition, *in vivo* studies described anti-tumor activity of wine phenolic compounds in rats induced with colon cancer (Dolara *et al.*, 2005) or breast cancer (Hakimuddin *et al.*, 2008). The phenolic properties contribute to protection by inhibiting cell proliferation and inducing detoxification enzymes as well as apoptosis (Birt *et al*., 2001). However, at high concentrations, they may induce cells to death by a direct toxic effect (Saleem *et al.*, 2002). Thus, the effect of phenolic compounds is dependent of their concentration and also vary according to the physiology of the tested cells. For example, the delphinidin 3-glucoside (Df3Glc), petunidin 3-glucoside (Pt3Glc), peonidin 3-glucoside (Pn3Glc), and Malvidin 3-glucoside (Mv3Glc) shown anti-proliferative activity on human colon carcinoma cells (HT-29), but only in concentrations upper to 100 mg/L (Yun *et al.*, 2010). In addition, Glavas-Obrovac *et al.* (2006) obtained different growth inhibitory effects on HT-29 and Caco-2 cells testing four red wines. The inhibition

of the proliferation of HT-29 carcinoma cells by red wine phenolic compounds has been related to the modulation of mitogen-activated protein kinases intracellular signal transduction pathways (Briviba *et al.*, 2002).

Some studies associated procyanidins with cardioprotective affects and with the prevention of induced colonic inflammation and colorectal cancer. The anthocyanins can act as modulators of the immune response in activated macrophages (J. Wang & Mazza, 2002), and might allow to prevent cancer (Hou, 2003). Also, decrease in the development of intestinal adenomas due to ingestion of an extract obtained from grapes, containing 22% (w/w) of anthocyanins was reported (Cai *et al.*, 2010). Due to the above described effects, phenolic compounds are considered potentially important constituents of the human diet for the chemoprevention of cancer.

Red wine has been shown to exert anti-inflammatory activity (Xanthopoulou *et al.*, 2010). In particular, red wine was described to prevents nuclear factor kappa B (NF-kB) activation in peripheral blood mononuclear cells, a process that activates genes involved in immune and inflammatory responses (Blanco-Colio *et al.*, 2000) and to reducing the expression of adhesion molecules that participate in the passage of monocytes and T lymphocytes into the arterial wall (Estruch *et al.*, 2004). Also, grape phenolic compounds have shown significant anti-inflammatory effects on mice and humans that has been mainly ascribed to immunomodulatory and antioxidant activity (Bralley *et al.*, 2007; Chacón *et al.*, 2009; Panico *et al.*, 2006). The inhibition of the production of inflammatory stimulus by macrophage-like cell lines, of C-reactive protein in rat plasma, (Bralley *et al*., 2007), and modulation of cytokine gene expression as basic pathway to anti-inflammatory process (Chacón *et al*, 2009) was attributed to grape procyanidins. Additionally, proanthocyanidins could prevent the inducible nitric oxide synthase (NOS) activity and the N-acetyl-β-D-glucosaminidase (Li *et al.*, 2001), preventing inflammatory activity. Also, extract of anthocyanins presented anti-inflammatory effects decreasing cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) expression by inactivating NF-kB in rats induced with liver fibrosis (Hwang *et al.*, 2011).

The protective effect of wine phenolic extracts on red blood cells (RBC) oxidative hemolysis was reported (Frankel *et al.*, 1993; Renaud & Delorgeril, 1992; Tedesco *et al.*, 2000). In particular the anthocyanin fraction of a red wine was suggested to be the main responsible to anti-hemolytic properties of red wines (Tedesco *et al.*, 2001). Some standard solutions of phenolic compounds usually present in wine, such as anthocyanins (Cy3Glc and Df3Glc), flavanols (catechin, epicatechin epigallocatechin and gallocatechin) have been showed to delay AAPH-induced hemolysis of red blood cells (Tabart *et al*., 2009).

In particular, in this thesis, it was evaluated the effects of wine phenolic fractions on different *in vitro* model systems: the cell viability of carcinoma HT-29 cells, the anti-inflamatory capacity using lipopolysaccharide (LPS)-stimulated Raw 264.7 macrophages, and the anti-hemolytic efficacy against human red blood cell lisys.

1.4 Objectives

In order to add value to the dealcoholized wine as source of bioactive compounds, the general objective of this thesis is to study the composition of the phenolic compounds resultant from wine distillation process, their interactions with the polymeric material, and their potential *in vitro* biological effects.

To accomplish the general objective, the following specific objectives were defined:

1. Characterization the total phenolic compounds, tannins and anthocyanins of wines, and respective dealcoholized wines.
2. Evaluation the effect of distillation process on the antioxidant activity.
3. Evaluation of the interactions between the phenolic compounds and wine polymeric material.
4. Evaluation of possible selectivity of the retention of anthocyanins by different wine polysaccharide fractions.
5. Determination of the activation energy necessary for the release of phenolic compounds from wine polymeric material.
6. Definition of storage conditions for the preservation of wine and wine extracts antioxidant activity.
7. Evaluation of potential biological effects of the wine extracts using *in vitro* assays.

The specific objectives 1 and 2 were fulfilled in section 3.1 of the Results and Discussion Chapter; section 3.2 answered the specific objectives 3-5; the specific objective 6 was accomplished in section 3.3; and section 3.4 was devoted to respond to the specific objective 7.

CHAPTER II - Material and Methods

2.1 Samples

The red wines (RW) used in this work were obtained from Dão Appellation, in Portugal, from 2006 and 2009 vintage. RW1 was produced by classic vinification from grapes of *Vitis vinifera* L. varieties (Touriga Nacional, Tinta Roriz, and Jaen; ~2:1:1) with a 8 days maceration period, whereas RW2 was a produced only from grapes of *Vitis vinifera* L. Touriga Nacional, with a 12 days maceration period.

Each wine was heat-evaporated until 75% of its initial volume allowing to obtain the dealcoholized wines (DW1 and DW2). The process was carried-out during 1 h at atmospheric pressure. At the end of distillation process, distilled water was added in the same volume of the alcoholic fraction that was removed. With this process, the concentration of the wine components understudy remained equal to the departing wine.

In addition, a red wine from Dão Appellation, in Portugal, from 2011 vintage was used for evaluate the effect of polysaccharides-rich material addition (Chapter III – section 3.3.3)

2.2 Fractionation of Wine Constituents

2.2.1 Preparation of wine polymeric material

The dealcoholized wines were dialyzed (MW cutoff 12-14 kDa, Visking size 8, Medicell International Ltd, London, UK) at 4 °C with several water renewals. The volume of retentate represented 5% of dialysate. The retentate obtained was frozen and freeze-dried, giving the wine polymeric material (WPM1 and WPM2).

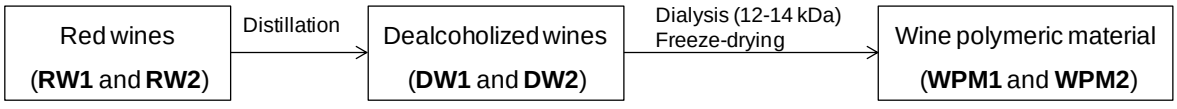


Figure 2.1: Preparation of wine polymeric material.

2.2.2 Selective precipitation of polysaccharides in ethanol solutions

Prior to the ethanol precipitation, the DW2 was cooled at 5ºC, and the insoluble material was recovered by centrifugation (fraction $DW_{Et}0$). The soluble material was fractionated according to its solubility in ethanol. Absolute ethanol was added in order to perform 30% of ethanol, assuming additive volumes. This solution was stirred for 1 h at 4 ºC. This solution was then centrifuged (20 min, 4ºC, 15000 rpm) and the residue obtained was removed (fraction $DW_{Et}30$). To the supernatant, absolute ethanol was added successively in order to perform 50%, and 80% of ethanol, and the procedure repeated as above obtaining the fractions $DW_{Et}50$, $DW_{Et}80$. The material that remained soluble in ethanol was assigned as $DW_{Et}sn$. Each fraction was dissolved in water, rotary evaporated, frozen, and freeze-dried (Coimbra *et al*., 1996).

2.2.3 Selective precipitation of phenolic compounds in methanol/chloroform solutions

A aqueous solution of 10 g/L of polymeric material was submitted to a liquid-liquid extraction with ethyl acetate (1:1, v/v). The organic phase containing mainly oligomers, was recovered and the aqueous phase, containing the polymers, was evaporated to dryness, and extracted with acidic MeOH (10 g/L). The methanol insoluble material (PMi) was obtained by centrifugation (15000 rpm; 10 min; 4ºC). The methanol soluble material was fractionated according to the methanol/chloroform graded precipitations proposed by Saucier *et al*. (2001). To the supernatant chloroform was added successively in order to perform 30%, 60%, 70%, and 80% of the total volume, allowing to obtain four precipitates (PM30, PM50, PM60 and PM75). The material soluble in chloroform after the last precipitation was assigned PMsn. All fractions were rotary evaporated at 35ºC, re-dissolved in water, frozen, and freeze dried. The process in summarized in Figure 2.2.

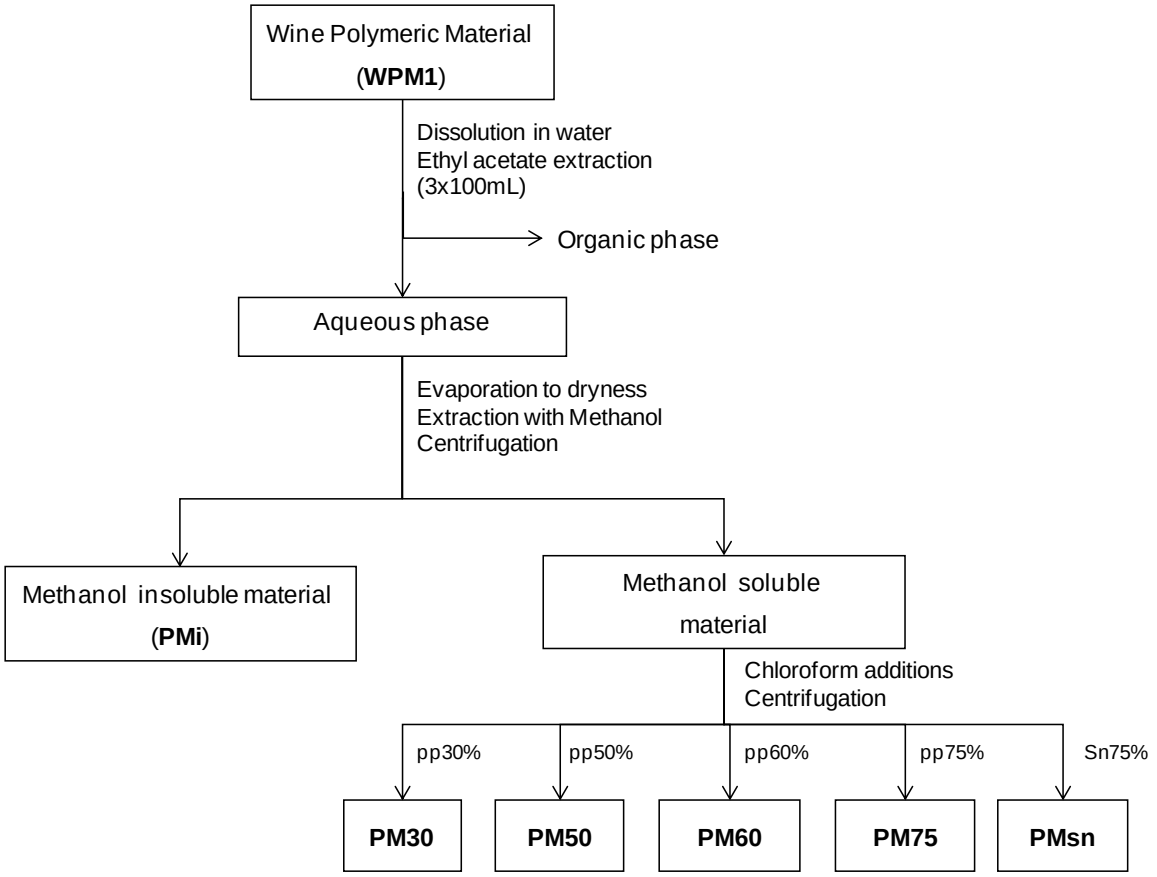


Figure 2.2: Fractionation of phenolic compounds according to their solubility in chloroform/methanol solutions.

2.2.4 Separation of polysaccharides from phenolic compounds by solid-phase extraction

The C18 sep-pak cartridges (SPE-C18, Supelco-Discovery, 20 g) was used to separate the hydrophilic (sugars-rich fraction) from the hydrophobic (phenolic-rich fraction) material. SPE-C18 column was preconditioned with 20 mL of methanol followed by 20 mL of water and 20 mL of 2% acetic acid. The sample was loaded into column at pH ± 3.4 and eluted with water in order to obtain the sugar-rich fraction. The wine phenolic compounds were eluted with methanol acidified by 0.1% of HCl. This solution was rotary evaporated to remove MeOH and re-dissolved in water.

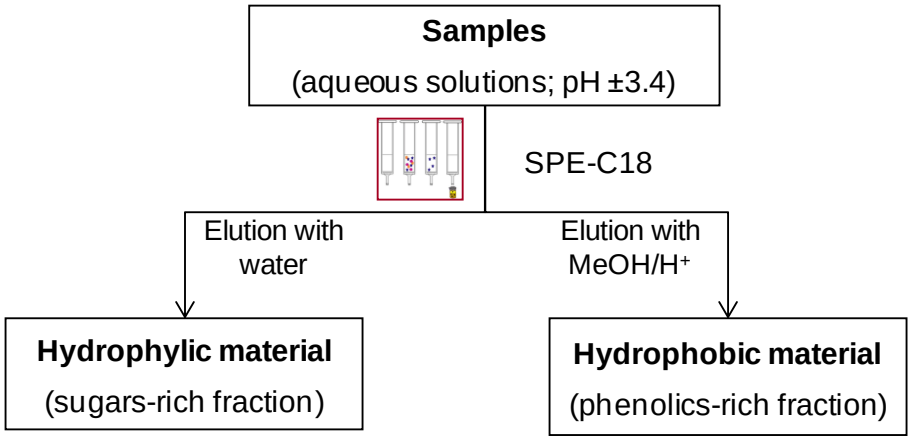


Figure 2.3: Separation of polysaccharides from phenolic compounds by SPE C-18 (fractionation 1).

This procedure was aplayed to DW2, whereas the resulting hydrophilic material and hydrophobic material were, respectively, wine polysaccharides WPS) and wine phenolic compounds (WPC). This procedure was also aplayed to WPM1, PMi and PM50 solutions (10 g/L).

2.2.5 Separation of DW2 polysaccharides fraction

The hydrophilic fraction of DW2 obtained from SPE-C18 column was dialyzed (12 kDa cutoff membrane, Medicell) in order to remove tartaric acid and other small molecules. The retentate was concentrated, frozen, and freeze dried, to give the wine polysaccharides (WPS) as a powder. The WPS were separate according to Coelho *et al*. (2011). An affinity medium of concanavalin A (Con A) Sepharose 4B (GE Healthcare, Uppsala, Sweden) in a column with 30 cm length and 2 cm diameter, operated at 5ºC with a constant flow of 1 mL/min was used to obtain mannoproteins. Prior to elution, the column was pre-washed with a solution of 1M of NaCl, 5mM of $MgCl_2$, 5mM of MnCl2, and 5 mM of $CaCl_2$, and equilibrated with a buffer solution of Tris-HCl 20mM and 0.5M of NaCl at pH 7.4. The non-retained fraction was eluted with Tris-HCl buffer, and the retained fraction, containing the mannoproteins (MP) was desorbed with two bed volumes of the same buffer containing 100 mM methyl-α-D-mannopyranoside. Due to the large amount of material handled, successive batches were done, always after

regeneration of Con A resin with 0.1 M Tris buffer, 0.5 M NaCl at pH 8.5 followed by 0.1 M sodium acetate, pH 4.5, containing 1 M NaCl.

Anion-exchange chromatography was performed for the non-retained fraction rich in arabinogalactans using a HyperSep SAX 10 g (Thermo Fisher Scientific, U.K.). Prior to elution, the column was conditioned with methanol followed by water and MeOH:water (5:95 v/v). The neutral fraction (AG0) was obtained by elution with water. Two acidic fractions (AG1 and AG2) were obtained by sequential elution with 50 mM and 500 mM phosphate buffer pH 6.5. All fractions were dialysed against water, and freeze-dried.

2.2.6 Separation of DW1 and DW2 phenolic compounds

The fractionation of wine phenolic compounds (WPC) was based on the methodology proposed by Sun *et al*. (2006). The column was activated with 25 mL of methanol and than washed with 50 mL of distilled water. The column was pre-conditioned by 25 mL of commercial pH 7.0 phosphate buffer before the fractionation of phenolic compounds. The phenolic solution was neutralized by addition of NaOH 0.1 M solution until pH≈7.0 followed by addition of commercial pH 7.0 phosphate buffer to adjust the wine pH to be exactly 7.0. This solution was loaded into the column in a flow of less than 2 mL/min. Phenolic acids fractions were eluted with 50 mL of diluted pH 7.0 phosphate buffer (1/8, v/v). The column was than washed with 30 mL of distilled water and dried under enhanced vacuum. The fraction containing monomer flavanols and oligomer procyanidins, was eluted with 50 mL of ethyl acetate. The fraction composed of anthocyanins, polymeric proanthocyanidins and other pigmented complexes was removed from the column by elution with 30 mL of methanol acidified by 0.1% of HCl. The pH of phosphate buffer fraction was adjusted to pH 2.0, and reloaded into another C18 cartridge. The salts were removed with distilled water and the fraction containing the phenolic acids was recovered by elution with methanol. The ethyl acetate fraction was further separated into monomer flavanol fraction and oligomer procyanidin fraction using another C18 column previously pre-conditioned, by sequential

elution with 50 mL of diethyl ether and 30 ml of methanol, respectively. The process is summarized in Figure 2.4

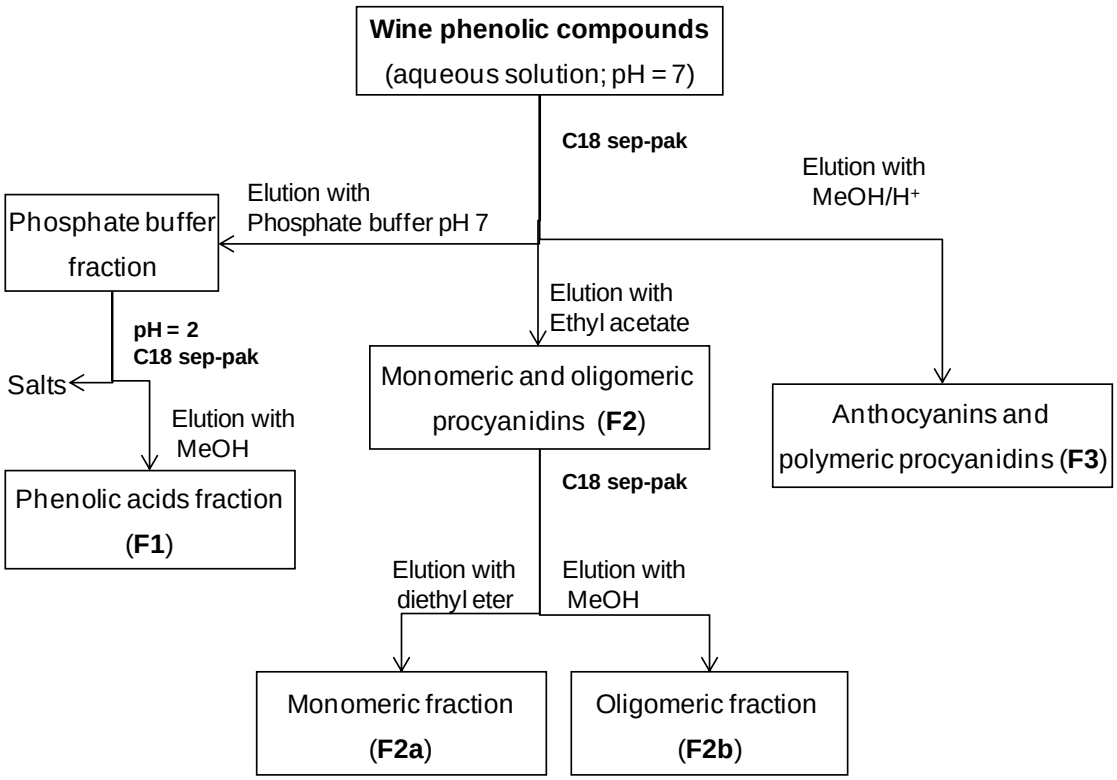


Figure 2.4: Fractionation of phenolic compounds by SPE C-18 (fractionation 2).

The fractions F1, F2, and F3 resultant from DW2 were evaporated to remove MeOH and re-dissolved in water and freeze dried, allowing to obtain the PA-E, PR-E and APP-E extracts as podwers.

2.3 Effect of Storage Conditions on Antioxidant Activity and Phenolic Content

2.3.1 Storage conditions of wine phenolic compounds

The wine phenolic compounds as powder (3 mg) were stored in plastic tubes (5 mL of head-space) with stopper, for 342 days under 5 different conditions

of light exposure and atmosphere composition, at room temperature. The following conditions were assayed: DNS: samples stored in dark, under N_2 atmosphere in a desiccator containing P_2O_5 in solid state, in sealed tubes; LNS: samples stored exposed to day light, under N_2 atmosphere in a desiccator containing P_2O_5 in solid state, in sealed tubes; DAS: samples stored in dark, under air atmosphere in a desiccator containing P_2O_5 in solid state, in sealed tubes; LAS: samples stored exposed to day light, under air atmosphere in a desiccator containing P_2O_5 in solid state, in sealed tubes; LAO: samples stored exposed to day light in open tubes. For each storage condition, 25 individual tubes were prepared. The antioxidant activity of samples, stored in different conditions, was evaluated at day 0, 12, 19, 26, 40, 47, 57, 71, 92, 106, 142, 162, 252 and 342 of storage period. For each time point, two tubes were used to evaluate the antioxidant activity.

2.3.2 Storage conditions of wine solutions

The wines were bottled in 15 mL flask tubes with screw caps were stored for 3 months at two temperatures (5 and 30 ºC) and with and without addition of 7.5 mg of a polysaccharide-rich material resulting in four storage conditions: W5: wine stored at 5 ºC; W5P: wine stored at 5 ºC, with addition of polysaccharide-rich material; W30: wine stored at 30 ºC; W30P: wine stored at 30 ºC, with addition of polysaccharide-rich material. The results obtained after the 3 months period were compared with the control wine (WT0) analyzed at day 0.

2.4 General Phenolic Composition Analysis

2.4.1 Total phenolic content

The phenolic content of the different fractions was determined by Folin-Ciocalteu reagent (Singleton & Rossi, 1965). Each sample (0.125 mL) was added

to 0.5 mL of deionized water and 0.125 mL of Folin-Ciocalteu reagent (Sigma). After 6 min, 1.25 mL of 7.5% solution of sodium carbonate and 1.0 mL of deionized water were added. The mixture was left 90 min at room temperature in the dark and the absorbance at 760 nm was measured. A calibration curve was made with standard solutions of gallic acid (range 50 to 500 mg/L), and the results were expressed in equivalents of standard used. All analyses were done in triplicate. This procedure was performed in samples RW1, RW2, DW1,DW2, WPM1, WPM2, PMi, PM30, PM50, PM60, PM75, PMsn, WPC, PA-E, PR-E, APP-E, WT0, W5, W5P, W30, W30P, $W01_{WPM}$–$W11_{WPM}$, and $W1_{FPC}$-$W5_{FPC}$.

2.4.2 Total tannins

Total tannins (TT) were estimated according to Ribereau-Gayon and Stonestreet (1966). Briefly, the sample was diluted to 1/50 in water. 2.0 mL of the previous solution was added to 1.0 mL of water and 3.0 mL of 12 M HCl. The content was divided into two tubes. One of them was heated for 30 min in boiled water and cooled (tube A), while the last one stayed at room temperature (tube B). A 0.5 mL amount of 95% ethyl alcohol was added. The absorbance was read at 550 nm for each tube, Abs_{tA} and Abs_{tB}. Total tannins was calculated using the equation $TT = 19.33 \times (Abs_{tA} - Abs_{tB})$. Analyses were realized in triplicate. This procedure was performed in samples RW1, RW2, DW1,DW2, WT0, W5, W5P, W30, and W30P.

2.4.3 Total anthocyanins

Total anthocyanins (TA) were determined using the SO_2 bleaching method, (Boulton, 2001; Cheynier *et al*., 1989). Each sample (1 mL) was added to 1 mL of ethanol acidified by 0.1% HCl and 20 mL of 2% HCl solution. In one tube, 2 mL of previous solution was added to 0.8 mL of water (t1). In another tube (t2) were

mixed 2 mL of previous solution and 0.4 mL of $HNaSO_3$ solution (15% w/v). After 20 min at dark room temperature, the absorbance at 520 nm was measured. The TA were calculated using the equation TA = 875 × (abs_{t1} - abs_{t2}), and results expressed as malvidin equivalents. Analyses were realized in triplicate. This procedure was performed in samples RW1, RW2, DW1,DW2, WT0, W5, W5P, W30, W30P, $W01_{WPM}$–$W11_{WPM}$, $W1_{FPC}$-$W5_{FPC}$, $W1_{B}$-$W5_{B}$, $W1_{MP}$-$W5_{MP}$, and $W1_{AG}$-$W5_{AG}$.

2.5 Antioxidant Activity Analysis

2.5.1 ABTS assay

The 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was performed according to Miller *et al*. (1993). The ABTS was dissolved in potassium persulfate water solution to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h reaching a stable oxidative state. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.700 at 734 nm. Each fraction, properly diluted, (0.1 mL) was added to 1.7 mL of ABTS solution and stirred. The decrease in absorbance was determined at 734 nm at end of 15 min of reaction in the dark.

2.5.2 DPPH assay

The 1,1'-dihenyl-2-picrylhydrazyl (DPPH) was performed according to Brand-Williams *et al*. (1995). A methanolic solution (6×10^{-5} mol/L) of the radical DPPH• was prepared daily and protected from light. Absorbance was recorded to check the stability of the radical throughout the time of analysis. The initial

absorbance was close to 0.700 in all cases. Briefly, 0.1 mL of sample (properly diluted) were added to 3.9 mL of $DPPH^{\bullet}$ solution and stirred. The absorbance at 515 nm was recorded at end of 30 min of reaction in the dark.

For all ABTS and DPPH assays used, the determinations were performed in triplicate. The percentage of inhibition was calculated according to the equation: % Inibhition = $(1-A_f/A_0)\times100$, where A_0 is the value of absorbance of the blank at 0 min and A_f is the absorbance measured of the antioxidant samples at the end of reaction. Standard curves were prepared using different concentrations (0-0.5 mM) of trolox in each assay. The results of ABTS and DPPH were express as mM of trolox equivalents. This procedure was performed in samples RW1, RW2, DW1,DW2, F1, 2a, 2b, 3, WT0, W5, W5P, W30, and W30P.

2.5.3 Antiradical efficiency by DPPH assay

The antiradical efficiency (AE) was estimated by using the DPPH assay. In this case, the absorbance at 515 nm was recorded at different time intervals until the reaction reached equilibrium. Five different concentrations of each sample have been assayed in order to check the linearity of response and to establish the antioxidant activity values in the adequate linear range. All measurements were performed in triplicate.

The AE was determined using the equation $AE = 1/(EC_{50} \times t_{EC50})$, were EC_{50} is the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration and t_{EC50} is the time needed to reach the steady state to the concentration corresponding at EC_{50}. This procedure was performed in samples WPM1, PMi, PM30, PM50, PM60, PM75, PMsn.

2.6 Determination of Retention Coefficient of Anthocyanins by Wine Polymeric Material

A dialysis membrane (MW cut-off 12-14 kDa, Visking size 8, Medicell International Ltd, London, UK") was filled with 10 mL of DW solution and immersed into a flask containing 190 mL of distilled water containing a few drops of chloroform and toluene to prevent microbial growth. After 6 h at 4 ºC with continuous stirring with a magnetic stirrer, the dialysis membrane was transferred into a new flask containing 190 mL of distilled water with few drops of chloroform and toluene for a new dialysis using the same conditions. Each dialysate was concentrated and stored at -20 ºC. This material was used for analysis of total phenolic compounds and anthocyanins. This procedure was repeated every 6 h, during 66 h, allowing to obtain 11 dialysate solutions, defined as $W01_{WPM}$ to $W11_{WPM}$, according to the serial number of the water changes performed.

In order to evaluate the extent of the retention of phenolic compounds by the polymeric material, 80% of the material recovered from each one of the 11 dialysate solutions obtained from the dealcoholized wine were combined and concentrated under vacuum at less than 40 ºC. The resultant solution, containing the wine phenolic compounds that diffused previously through the dialysis membrane, was dialysed during 30 h using the dialysis procedure previously described, with water renewals every 6 h. In this dialysis, performed in the absence of the wine polymeric material, 5 dialysate solutions were obtained ($W1_{FPC}$ to $W5_{FPC}$, in accordance with the serial number of the water changes performed). This was defined as the blank experiment for the release of phenolic compounds in these dialysis conditions. Each one of the five dialysate solutions obtained was concentrated and stored at -20 ºC for posterior analysis of total phenolic compounds and anthocyanins.

For determination of retention capacity of MP and AG, the dialyses membrane were filled with 10 mL of phenolic compounds in presence of 1.50 mg of MP and AG. The dialyses were performed during 15 h,allowing to obtain 5 dialysate solutions for each experiment: $W1_{MP}$-$W5_{MP}$ for MP trial, and $W1_{AG}$-$W5_{AG}$ for AG trial. A dialysis of phenolic compounds was performed as blank experiment, also resulting 5 dialysate solutions $W1_{B}$-$W5_{B}$.

In order to quantify the retention capacity of WPM, MP, and AG fractions towards the anthocyanins, for each anthocyanin a retention coefficient (RC) was

calculated based on the percentage of its release to the first dialysate soluition in the presence of WPM, MP, and AG and in its absence. The retention coefficient was calculated for each compound using the equation RC = [1 – ($\%W1_{PM}$ / $\%W1_{FPC}$)], where $\%W1_{PM}$ is the relative amount of each anthocyanin released to the first dialysate solution in the presence of WPM, MP, and AG and $\%W1_{FPC}$ is the relative amount of anthocyanin released to the to the first dialysate solution in the absence of polymeric material. A RC=0 means that the polymeric material did not have any capacity of retention while RC=1 represents 100% of retention.

2.7 Determination of Kinetic Constant and Activation Energy of Phenolic Compounds Adsorption to Polymeric Material

For each experiment, a dialysis bag was filled with sample solutions (DW1, WPM1, and PMi) and was immersed in distilled water. The kinetic constant of release of phenolic compounds from the polymeric material, for each temperature (5, 25, 30, 35 and 40 ºC) was determined by monitorization of the phenolic compounds present in the dialysis water along the time for different concentrations of material inserted in a dialysis bag of 12-14 kDa cut-off. The amount of phenolic compounds presented in the dialysis water was plotted against the release time until reaching a steady state. The slope of the linear region corresponds to the velocity of release of phenolic compounds expressed as gram of equivalents of gallic acid/second. Each sample was assayed using 4 concentration levels, allowing the determination of 4 velocities. The kinetic constant was determined plotting velocities of release against the initial amount of phenolic compounds inside the dialysis bag.

The activation energy was obtained from the slope of the logarithmic form of the Arrhenius equation, $\ln k = -(E_a/RT) + \ln(k_0)$, where E_a is the activation energy of the reaction (kJ/mol), k is the kinetic constant; and k_0 is the pre-exponential constant; R is the gas constant (8.314 J mol/K); T is the mean absolute temperature (Kelvin, K) of the considered dialysis temperature range. This procedure was performed in samples DW1, WPM1 and PMi.

2.8 Individual Phenolic Compounds Analysis

The individual phenolic acids and monomeric anthocyanins were analyzed using a HPLC Dionex Ultimate 3000 Chromatographic System (Sunnyvale, California, USA) equipped with a quaternary pump Model LPG-3400 A, a ACC-3000 auto sampler, having a thermostatted column compartment (adjusted to 30 ºC) and a multiple Wavelength Detector MWD-300. The column (250 x 4.6 mm, particle size 5 µm) was a C_{18} Acclaim® 120 (Dionex, Sunnyvale, California, USA) protected by a guard column of the same material.

2.8.1 Phenolic acids

The phosphate buffer fraction obtained by C18 sep-pak was used for chromatographic analysis of the phenolic acids present in the wines. The solvents were (A) water/formic acid (95:5 v/v), and solvent (B) methanol. Analysis conditions were as follow: a linear gradient analysis for a total run time of 80 min was used as follows: starting from 5% solvent B during 2 min, increase to 80% solvent B over 68 min and then isocratic for 8 min, decreasing to 5% solvent B over 2 min, and finally isocratic for 5 min. The sample volume injected was 40 µL, the flow rate was 1.0 mL/min, and the column temperature was maintained at 30 ºC during the run.

The quantification of the individual benzoic and cinnamic acids was made by a calibration curve obtained with standard solutions of gallic and caffeic acids (Extrasynthese, Genay, France), respectively. The results for each target phenolic compound were expressed in equivalents of standard used. The chromatographic peaks of all anthocyanins were identified by comparing their retention times with the retention time of standard compounds. All analyses were done in triplicate. This procedure was performed in sample DW2.

2.8.2 Monomeric anthocyanins

The monomeric anthocyanins present in the sample solutions were analyzed by HPLC. The solvents were (A) 40% formic acid, (B) pure acetonitrile and (C) bidistilled water. The initial conditions were 25% A, 10% B, and 65% C, followed by a linear gradient from 10 to 30% B, and 65 to 45% C for 40 min, with a flow rate of 0.7 mL/min. The injection volume was 20 µL. The detection was made at 520 nm and a Chromeleon (version 6.8) software program (Sunnyvale, California, USA) was used.

The quantification of the individual anthocyanins was made by a calibration curve obtained with standard solutions of malvidin-3-glucoside (Extrasynthese, Genay, France), and the results for each target phenolic component were expressed in equivalents of standard used. The anthocyanin structures were isolated and identified according to their UV-Vis spectrum (Dallas & Laureano, 1994). The chromatographic peaks of all anthocyanins were identified by comparing their retention times with the retention time of isolated standard anthocyanins. All analyses were done in triplicate. This procedure was performed in samples RW2, DW2, $W01_{WPM}$–$W11_{WPM}$, $W1_{FPC}$-$W5_{FPC}$, $W1_{B}$-$W5_{B}$, $W1_{MP}$-$W5_{MP}$, and $W1_{AG}$-$W5_{AG}$.

2.8.3 Procyanidins

The ethyl acetate fraction obtained by C18 sep-pak was used for chromatographic analysis of the catechins and procyanidins (dimers and trimers) present in the wines. The solvents used were (A) water/acetic acid, 2.5% (v/v) and (B) solvent A/acetonitrile, 20:80 (v/v). Analysis conditions were as follow: the initial conditions were 7% B in isocratic for 5 min; gradient elution from 7 to 20% B in 85 min; 20 to 100% B in 90 to 95 min; 100% B isocratic in 5 min, followed by washing (100% B, 10 min) and reconditioning of the column (100 to7% B in 5min). The analysis was carried out at room temperature at 1 mL/min. Two columns Merkc LiChrospher reverse phase C18 ODS (4.6 x 250mm). placed in line and protected

with a guard column packed with the same packing were used for all analysis as described by Freitas and Glories (1999). The sample volume injected was 20 µL, the detection was performed at 280 nm using a UV detector (Merck L-7420A). The column temperature was 25 ºC, and the flow rate was fixed at 1 mL/min.

Calibration curves were established with corresponding catechins (catechin and epicatechin) and procyanidins (procyanidin dimers B1, B2, B3, B4, B1-3-*O*-gallate, B2-3-*O*-gallate, and trimers C1) standards. This procedure was performed in sample DW1. These experiments were carried out in the "Centro de Investigação em Química (CIQ) do Departamento de Química e Bioquímica da Faculdade de Ciências do Porto".

2.9 Carbohydrate Analysis

2.9.1 Neutral sugars

Neutral sugars present in the wine polymeric material were determined by gas chromatography (GC) as alditol acetates using 2-deoxyglucose as internal standard (Coimbra *et al.*, 1996) and GC analysis as described by Nunes et al. (2008). Monosaccharides were released from samples by a pre-hydrolysis in 0.2 mL of 12 M H_2SO_4 for 3 h at room temperature, followed by 2.5 h hydrolysis in 1 M H_2SO_4 at 100 ºC (Selvendran *et al.*, 1979). Monosaccharides were reduced with $NaBH_4$ (15% in 3 M NH_3) during 1 h at 30 ºC. The excess of $NaBH_4$ was destroyed by the addition of 0.100 mL of glacial acetic acid and the alditols formed were subsequently acetylated with acetic anhydride (3 mL) in the presence of 1-methylimidazole (0.450 mL) during 30 min at 30 ºC. Alditol acetate derivatives were extracted with dichloromethane and analyzed by GC with a flame ionization detector and equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA, USA) with i.d. and film thickness of 0.25 mm and 0.15 µm, respectively. The oven temperature program used was: initial temperature 200 ºC, a rise in temperature at

a rate of 40 ºC/min until 220 ºC, standing for 7 min, followed by a rate of 20 ºC/min until 230 ºC maintaining this temperature for 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min. This procedure was performed in samples WPM1, WPM2, WPS, MP, AG0, AG1, AG2, WPC, PR-E, and APP-E.

2.9.2 Uronic acids

Uronic acids of wine polymeric material were quantified by a modification (Coimbra *et al.*, 1996) of the 3-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen, 1973). Samples were prepared by pre-hydrolysis in 0.2 mL of 12 M H_2SO_4 for 3 h at room temperature followed by hydrolysis for 1 h in 1 M H_2SO_4 at 100 ºC. The samples were diluted 1:10 previously to analysis in order to prevent the appearance of the characteristic pink absorbance of anthocyanidins and proanthocyanidins formed upon acid hydrolysis. A calibration curve was made with D-galacturonic acid. All neutral sugars and uronic acid analyses were done in triplicate. This procedure was performed in samples WPM1, WPM2, WPS, MP, AG0, AG1, AG2, WPC, PR-E, and APP-E.

2.9.3 Glycosidic-linkage composition of polysaccharides

Glycosidic-linkage composition was determined by gas chromatography quadrupole mass spectrometry (GC-qMS) of the partially methylated alditol acetates. The sample (1-2 mg) was weighed into glass tubes and placed in a vacuum oven, at 40 ºC, overnight in the presence of P_2O_5 (s). Afterward, it was dispersed in 1 mL of anhydrous dimethyl sulfoxide (DMSO) and stirred overnight for total solubilization. NaOH pellets (30 mg) were powdered under argon, added to the solution and kept stirring during 30 min. The polysaccharides were methylated with 80 µL of methyl iodide, added with a syringe into the closed tube

with a cap with a silicone septum. The mixture was allowed to react for 20 min under stirring. Two mL of water was added, and the solution was neutralized with HCl 1 M. The methylated material was then extracted with 3 mL of CH_2Cl_2 and the aqueous phase was removed after centrifugation. The dichloromethane phase was then washed three times with 2 mL of water until the dichloromethane phase became limpid. The organic phase was transferred to a clean tube and dried by centrifugal evaporation (Univapo 100 ECH, UniEquip, Germany). This methylation procedure was repeated. The permethylated polysaccharides were hydrolyzed with 0.5 mL of 2 M trifluoroacetic acid (TFA) (1 h at 121 ºC) and dried by centrifugal evaporation. The reduction of monosaccharides was performed during 1 h at 30 ºC with 20 mg of sodium borodeuteride (Isotec, Switzerland) in 300 µL of 2M NH_3. The reaction was terminated by the addition of 0.1 mL of glacial acetic acid. The acetylation was performed with 3 mL of acetic anhydride using 450 µL of 1-methylimidazole as catalyst, during 30 min at 30 ºC. Then, 3 mL of distilled water was added to decompose the acetic anhydride, and the acetylated sugars were extracted with 5 mL of CH_2Cl_2. The organic phase was washed three times with water and then dried by centrifugal evaporation. The partially methylated alditol acetates were dissolved in 70 µL of acetone, and 0.2 µL were injected and analyzed by GC-qMS on an Agilent Technologies 6890N Network gas chromatograph, equipped with a 30 m×0.25 mm (i.d.), 0.1 µm film thickness DB-1 fused silica capillary column (J&W Scientific Inc., CA, USA), connected to an Agilent 5973 quadrupole mass selective detector.

The oven temperature was programmed as follows: hold 5 min at 45 ºC, to 140 at 10 ºC/min (hold 5 min at 140 ºC), to 170 at 0.5 ºC/min (hold 1 min at 170 ºC) and then to 280 at 15 ºC/min (hold 5 min at 280 ºC). Helium carrier gas had a flow of 1.7 mL/min and a column head pressure of 2.8 psi. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 40-500 m/z, in a full scan acquisition mode. Identification was achieved comparing the standard mass spectra and other spectra with a laboratory made database.

For the permethylated fractions, prior to acid hydrolysis, the dichloromethane solutions were split in two portions and a carboxyl reduction was performed. The permethylated polysaccharides were dried and dissolved in 1 mL of anhydrous

tetrahydrofuran, and 20 mg of lithium aluminum deuteride (Aldrich, WI, USA) was added under argon. The suspension was kept at 65 ºC during 4 h under stirring.28 The reagent in excess was eliminated by adding 2-3 drops of ethanol and 2-3 drops of distilled water. The solution was neutralized by addition of 1 M of HCl. Two mL of chloroform/methanol 2:1 (v/v) mixture was then added. The reduced polymers were removed from the white precipitate by centrifugation and washed thoroughly with the chloroform/methanol solution. The supernatant was collected and evaporated, and the carboxyl-reduced material was submitted to hydrolysis with TFA, reduction, and acetylation, as described above.

2.10 Nitrogen Compounds Analysis

2.10.1 Protein analysis

Protein quantification was based on the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as standard, using the Bicinchoninic Acid Protein Assay Kit from Sigma (Aldrich-Chemie, Steinheim, Germany). The samples were incubated in a water bath at 60 ºC during 15 min. The absorbance was measured at 562 nm) against a blank in the reference cell. The data were correlated with the calibration curve of BSA standard (concentration range of 0.05–0.40 mg/mL), also analyzed in the same conditions of the samples. At least three replicates of each concentration were carried out for all experiments. This procedure was performed in samples, WPS, MP, AG0, AG1, AG2, WPC, PR-E, and APP-E.

2.10.2 Amino acids analysis

The methodology for acid hydrolysis of protein material was adapted from Zumwalt *et al.* (1987) To a test tube with a screw cap with PTFE coating was rigorously weighed 10 mg of freeze-dried material. To each sample, 2 mL

hydrochloric acid (HCl) 6 M was addedand the hydrolysis took place during 24 h at 110 ºC using a heating block. After cooling to room temperature, 500 μL of the internal standard solution (norleucine 5.0 mM in HCl 0.1 M) was added and the tubes content was evaporated to dryness under vacuum in a centrifugal evaporator. The resulting material was dissolved in 1 mL HCl 0.1 M and filtered with 0.45 μm filters. For the analysis of free amino acids, 10 mg of each sample was suspended in 2 mL of a solution of HCl 0.1 M and spiked with 500 μL of the internal standard solution. The suspension was left stirring for several hours and then was filtered with 0.45 lm filters. The solutions containing the released amino acids were dried under vacuum using a centrifugal evaporator. The derivatization of amino acids for GC analysis was performed according to the methodology described by MacKenzie *et al.* (1974). The resultant solid residue was dissolved in 200 μL of a solution of 3 M HCl in isobutanol. This solution was prepared by adding 270 μL of acetyl chloride per mL of dry isobutanol; the isobutanol was dried with calcium hydride, distilled and stored with molecular sieves. The mixture was heated to 120 ºC for 10 min and, after shaking in a vortex, was heated for further 30 min. After cooling to ambient temperature, the excess of reagent was evaporated under vacuum using a centrifugal evaporator. Then, 200 μL of a solution of 0.2 mg/mL BHT prepared in ethyl acetate was added and the solvent was removed under vacuum in a centrifugal evaporator. Afterwards, 100 μL of heptafluorobutyric anhydride was added and the mixture was heated during 10 min at 150 ºC. After cooling to room temperature, the excess of solvent was removed under vacuum and the material obtained was dissolved in 50 μL of ethyl acetate and analysed immediately or frozen at -20 ºC until analysis.

Separation of amino acids was achieved by gas chromatography, carried out in a PerkinElmer Clarus 400 instrument (PerkinElmer, Massachusetts, USA) equipped with a flame ionisation detector (FID). The injector was kept at 250 ºC and the detector at 260 ºC. Hydrogen was used as carrier gas. A DB-1 (30 m, 0.25 mm i.d. and 0.15 μm thickness) fused-silica capillary column (J & W Scientific) was used with the following temperature programme: 1 min hold at 70 ºC, increase to 170 ºC at 2.0 ºC/min and then to 250 ºC (5 min hold) at 16 ºC/min. The compounds were identified by their retention times and chromatographic

comparison with authentic standards. Quantification was based on the internal standard method using L-norleucine, and the calibration curves were built for 18 amino acids. For asparagine (Asn) and aspartic acid (Asp), as well as for glutamine (Gln) and glutamic acid (Glu), the methodology does not allow the distinction between the amide and carboxylic acid functions. As such, those amino acids were quantified together as Asx and Glx, respectively. Also, the methodology used did not allow the detection of His. The limit of quantification of analysed amino acids was determined to be ten times the value of the residual signal peaks. This procedure was performed in samples WPM1, and WPM2, PMi.

2.11 Study of Potencial Biological Effects

2.11.1 Evaluation of stability of phenolic compounds under *In vitro* digestive tract model

The *in vitro* digestion procedure was carried out in dealcoholized wines and in phenolic fractions. The procedure was adapted from McDougall *et al*. (2005). The method consists of three sequencial steps: mouth, stomach and small intestine. To simulate the mouth conditions, 5 mL of sample solutions was added to 5 mL of α-amilase solution (1% w/v) during 2 min at pH 6.5. The pH of sample solutions was adjusted to 2.0 with HCl 5 N was added the solution was incubated at 37 ºC in a shaking bath for 2 h in presence of pepsin (1% w/v), to simulate the gastric conditions. At the end of the gastric digestion, the pH was brought to 7.0 with $NaHCO_3$, and than it were added 4 mg/mL of pancreatin and 25 mg/mL of bile salts to simulating small intestine conditions. The solution was then incubated at 37 ºC in a shaking bath for further 2 h. Solutions of pepsin, pancreatin and bile salts was prepared in 0.1% of NaCl. At end of each digestion phase, the samples solutions were centrifuged and the supernatants were withdrawn for the analysis of phenolic compounds by the Folin-Ciolcateau method and of the antioxidant activity by DPPH assay. For each step, blank experiments were prepared in the

same simulating conditions but without samples. This procedure was performed in samples DW1, DW2, F1, F2 and F3.

2.11.2 Determination of cell viability by WST-1 Assay

The assay is based on the reduction of WST-1 reagent (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate) by the mitochondrial dehydrogenases in viable cells to formazan dye (Ngamwongsatit *et al*., 2008). Briefly, 200 µl of cells (HC11 or HT-29) and freshly isolated peripheral blood mononuclear cell (PBMC) were independently seeded in 96-well, flat bottom microplates at a concentration of 5×10^4 and 5×10^5 cells/mL, respectively. the cells were seeded in the following medium (HC11 cells: 10% of fetal bovine serum (FBS), 5 µg/mL insulin, 10 ng/mL epidermal growth factor (EGF) in phenol red free RPMi 1640 medium, with addition of antibiotic gentamicin; HT-29: 10% of FBS in phenol red free RPMI 1640 medium with addition of antibiotic penicilin/streptomicin) and allowed to adhere for 24 h and were then treated with different concentrations of the wine extracts prepared in medium (1% steroid stripped FBS, 5 µg/mL insulin, 10 ng/mL of EGF in phenol red free RPMI 1640 medium, with adition of antibiotic gentamicin) cultured for 48 h in a humidified atmosphere of 37 °C, 5% CO_2. Thereafter, 20 µL of cell proliferation reagent WST-1 were added with further incubation for variable time periods (0.5 h to 4 h) at 37 °C. The microplates were then shaken thoroughly for 1 min and the optical density was measured (30, 60, 90 and 120 min) in an ELISA plate reader (Molecular Devices, Spectra Max 190 with soft max pro software) at 450 and 690 nm. The experiments were conducted twice with four replicates. The results are expressed as the optical density ratio of the treatment to control (Konczak-Islam *et al*., 2003). This procedure was performed in samples F1, F2 and F3 of DW2.

2.11.3 Determination of cell viability by MTT assay

Assessment of metabolically active cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction colorimetric assay, as previously reported (Mosmann, 1983). The mouse macrophage cell line, Raw 264.7 (ATCC number: TIB-71), were plated (3×10^5 cells/well) and allowed to stabilize for 12 h. Following this period, cells were either maintained in culture medium (control) or pre-incubated with sample extract, or with inhibitors, for 1 h, and later activated with 1 g/mL of lipopolysaccharides (LPS) for 24 h. Previous addition, extract samples were filtered through 0.45 μm filters. After treatments, the MTT solution (5 mg/mL in phosphate buffered saline) was added and cells were further incubated at 37 ºC for 15 min, in a humidified atmosphere of 95% air and 5% CO_2. Supernatants were then removed and dark blue crystals of formazan were solubilized with acidic isopropanol (0.04 M HCl in isopropanol). Quantification of formazan was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm. This procedure was performed in samples WPM2, WPC, PA-E, PR-E, and APP-E.

2.11.4 Measurement of nitrite production by the Griess reagent

The production of nitric oxide (NO) was measured through nitrite accumulation in the culture supernatants, using a colorimetric reaction with the Griess reagent (Green *et al.*, 1982) Briefly, 170 μL of culture supernatants were diluted with equal volumes of the Griess reagent [0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H_3PO_4] and maintained during 30 min, in the dark. The absorbance at 550 nm was measured in an automated plate reader (SLT,Austria). Culture medium was used as blank and nitrite concentration was determined from a regression analysis using serial dilutions of sodium nitrite as standard (2.5–250 μM). This procedure was performed in samples WPM2, WPC, PA-E, PR-E, and APP-E, and it was carried

out in the “Centro de Estudos Farmacêuticos - Faculdade de Farmácia, Universidade de Coimbra”.

2.11.5 Assay system for hemolysis

Blood was obtained from healthy, non-smoker volunteers by venipuncture and collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Samples were immediately centrifuged at 400g for 10 min; plasma and buffy coat were carefully removed and discarded. Red blood cells (RBC) were washed three times with phosphate buffered saline (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) at 4 ºC and finally resuspended in PBS, to obtain an RBC suspension at 2 % (v/v) hematocrit. RBC suspensions were used in the day they were prepared.

The hemolysis assays were performed by using H_2O_2 or 2,2’-azobis (2-amidinopropane) hydrochloride (AAPH) as the oxidant agent at a final concentration of 7.5 μM (or 60 μM). In all sets of experiments (n = 4), a negative control (RBC in PBS, with no oxidant) was used, as well as extract compound controls (RBC in PBS, with each compound at each concentration). We calculated the hemolysis induced by the compounds at each concentration, and used this as a baseline result. These values were below 3% hemolysis for all concentrations and compounds tested (Fernandes *et al*., 2008). The results are presented as percentages relative to the control hemolysis values.

All control and sample tests were run in duplicate. Incubations of RBC suspensions were carried out at 37 ºC for 3 h, under gentle shaking, in the presence of each individual compound or in the presence of the phenolic compound plus H_2O_2 or AAPH. Extracts were incubated for 15 min with RBC before addition of oxidant agent and they were tested at concentrations of 0.05-0.50 mg/mL.

Hemolysis was determined spectrophotometrically, according to the protocol described in Ko *et al.* (1997). After the incubation period, an aliquot of the RBC suspension was taken out, diluted with 20 volumes of PBS saline and centrifuged

(4000 rpm for 10 min). The absorption (A) of the supernatant was read at 540 nm. The absorption (B), corresponding to a complete hemolysis was acquiered after centrifugation of a RBC suspension previously treated with 20 volumes of ice-cold distilled water. The percentage of hemolysis was then calculated as (A/B)×100.

In order to analyze Hb oxidation, visible absorption spectra (500–700 nm) were measured using hemolysates incubated with different concentrations of wine extracts for 3 h, either in the presence of AAPH (60 mM) or H_2O_2 (7.5 mM), as described by Colado Simão *et al*. (2006). This procedure was performed in samples WPM2, WPS, WPC, PR-E, and APP-E, and it was carried out at “Grupo da Biologia da Inflamação e Reprodução do Instituto de Biologia Molecular e Celular (IBMC) da Universidade do Porto”.

2.12 Statistical Analyses

Differences in anthocyanin content present in dialysate solutions in presence and in absence of WPM, MP and AG were tested by analysis of variance (One-way ANOVA), followed by multiple comparisons test (Tukey's Honestly Significant Difference test) to identify differences in the composition of the dialysate solutions.The percentage of anthocyanins in the dialysate solutions was tested for linearity against dialysis time by simple linear regression.The effects of temperature and polysaccharides-rich fraction on the phenolic composition and antioxidant activity of the wines (W5, W5P, W30 and W30P) were evaluated by a two-way ANOVA followed by multiple comparisons test (Tukey's Honestly Significant Difference test) to identify differences. The Dunnett test was performed to determine the differences against the control wine (WT0). Statistical analyses were tested at 0.05 level of probability.

CHAPTER III - Results and Discussion

3.1 Characterization of Wines, Dealcoholized Wines, and Wine Fractions

3.1.1 Overview

In this work two red wines were used as source of possible bioactive compounds: a wine from 2006 vintage (RW1) and wine 2009 (RW2).

In order to study the material present in the wine non-alcoholized fraction, both wines were heat-evaporated during 1 h at atmospheric pressure at temperatures close to 100 ºC, allowing to obtain the respective dealcoholized wines (DW1 and DW2). The RW and DW wines were analyzed concerning their general phenolic composition (total phenols, tannins, and anthocyanins) and antioxidant activity (DPPH and ABTS assays). In addition, in order to characterize the polysaccharides and the phenolic compounds present in DW, they were fractionated by solid phase extraction (SPE) using C-18 sep-pak cartridges, allowing to obtain the hydrophilic and hydrophobic fractions. The latter were further separated in four main phenolic fractions (phenolic acids, catechins, oligomeric procyanidins, and anthocyanins), that were used to evaluate their contribution to the total antioxidant activity of DW. The detailed phenolic characterization of DW was also obtained using HPLC/UV-Vis.

Moreover, both DW were extensively dialysed and the retentates were concentrated, frozen, and freeze-dried, to obtain the wines polymeric material (WPM1 and WPM2) as a powder. The WPM were characterized in terms of polysaccharides, phenolics, and amino acid content.

As the polymeric material still contained a red color, diagnostic of the presence of anthocyanins and anthocyanins derived pigments, the WPM1 was extracted with methanol to remove them. The insoluble material (PMi), which still presented a pink color, was further purified by C-18 sep-pak cartridges in order to try to separate the phenolic compounds that still remained associated with the polymeric material. The methanol soluble fraction was also fractionated by gradually precipitation with chloroform. The most abundant fraction was purified by C-18 sep-pak cartridges and analysed concerning phenolic compounds and sugars that remained associated with them.

For the specific analysis of DW2, it was centrifuged and the material in the supernatant was fractionated by ethanol precipitations. The polysaccharides and the anthocyanins of each fraction were determined.

In order to study the interactions of different wine polysaccharides (WPS) with the phenolic compounds, four polysaccharides-rich fractions (MP, AG0, AG1, and AG2) were obtained from WPS. In addition, three phenolics-rich fractions (PA-E, PR-E, and APP-E) were prepared from wine phenolic compounds fraction (WPC). Their content in phenolic compounds and polysaccharides was examined.

The overview of the origin of the fractions used in this chapter is shown in Figure 3.1. In addition, DW was used for study retention capacity of anthocyanins by wine polymeric material (section 3.2.2); fractions MP and AG1 were used for the study of retention capacity of anthocyanins by mannoproteins-rich and arabinogalactan-rich fractions (section 3.2.3); fractions WMP and PMi were used to determine the activation energy of release of phenolic compounds (section 3.2.4); fraction WPC was used to study the effect of storage condition on the antioxidant activity (section 3.3.2); fractions DW, F1, F2 and F3 were used for evaluate the stability of phenolic compounds under *in vitro* simulated digestive tract (section 3.4.2); fractions F1, F2 and F3 were used for evaluate the effect of phenolic compounds on cell viability (section 3.4.3) and fractions WPS, WPC, PA-E, PR-E and APP-E were used for biological studies (sections 3.4.4 and 3.4.5).

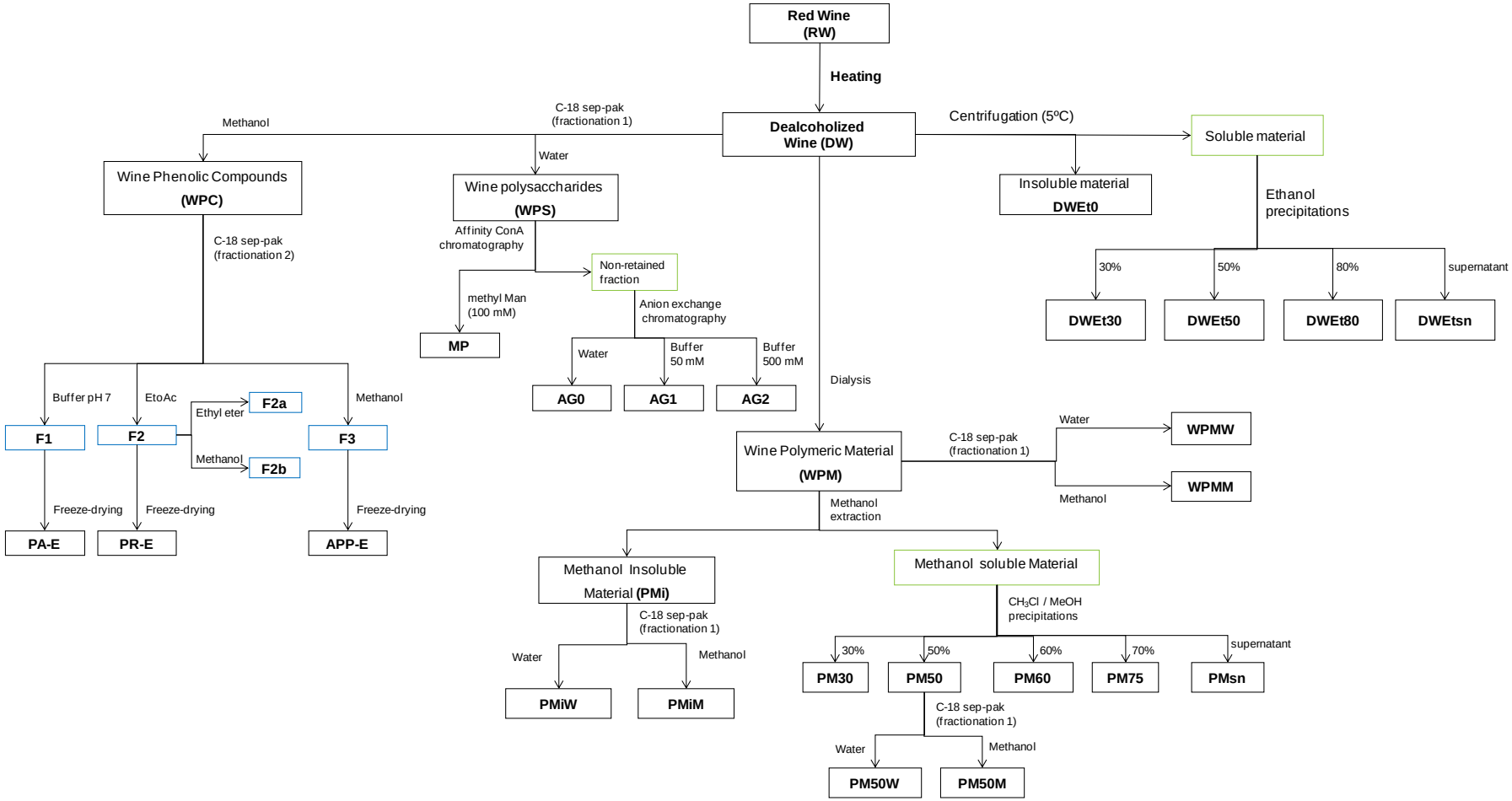


Figure 3.1 : Scheme of the origin of the fractions used in this thesis.

3.1.2 General chemical characterization of wines and dealcoholized wines

3.1.2.1 General phenolic composition and antioxidant activity

The general phenolic composition of the two red wines used in this work and the respective dealcoholized wines is presented in Table 3.1. For comparison of the red wine composition with that of the dealcoholized wine, a volume of distilled water equal to the volume removed during the dealcoholization was added to the dealcoholized wine.

Table 3.1: General phenolic composition of the red wines and dealcoholized wines derived from them.

Samples	Total Phenols (mg/L GAE)	Tannins (g/L)	Total anthocyanins (mg/L Mv3Glc)
RW 1	1318 ± 19	1.21 ± 0.12	241.4 ± 7.3
DW 1	1323 ± 37	1.17 ± 0.18	234.4 ± 4.1
RW 2	3071 ± 81	1.62 ± 0.21	415.4 ± 23.2
DW 2	3040 ± 39	1.56 ± 0.11	421.4 ± 14.7

Mean ± standard deviation. GAE- gallic acid equivalents; Mv3Glc-malvidin 3-glucoside.

The phenolic profile of wines depends on grape phenolic composition, on winemaking techniques and also on reactions that occur during aging and storage (Vinas *et al*., 2000). A large range of values for phenolic compounds concentration in wine has been described in literature for red wines, ranging from 1019 to 4059 mg/L (Aguirre *et al.*, 2010; Frankel *et al.*, 1995; Goncalves & Jordao, 2009; Ivanova *et al.*, 2011; Katalinic *et al.*, 2004; Sanchez-Moreno *et al.*, 1999).

The RW1 contained 1318 mg/L of phenolic compounds, determined by the Folin-Ciocalteu method, expressed as gallic acid equivalents (GAE), while the RW2 contained 3071 mg/L of total phenolic compounds. Among others, two main factors may explain this difference in the concentration of phenolic compounds: the grape variety and the extent of maceration. RW1 was produced from grapes of

Vitis vinifera L. varieties (Touriga Nacional, Tinta Roriz, and Jaen; ~2:1:1) with 8 days of maceration period, whereas RW2 was produced only from grapes of *Vitis vinifera* L. Touriga Nacional, with 12 days maceration period. In accordance, Jordão *et al.*, (2011) reported higher amounts of total phenolics for monovarietal Touriga Nacional wine when compared with monovarietal Tinta Roriz wine (3216 vs 2771 mg GAE/L). The values estimated for DW were 1323 and 3040 mg/L, respectively, for DW1 and DW2, similar for those obtained for wines.

Concerning the tannin content, RW1 (1.21 g/L) and RW2 (1.62 g/L) presented an amount of tannins statistically similar to DW1 (1.17 g/L) and DW2 (1.56 g/L). These values are in accordance with some reported in literature (Bogianchini *et al.*, 2011; Chira *et al.*, 2012) (1.17-2.23 g/L) for red wines from other varieties, but slighty lower than those reported by Jordão *et al.*, (2011) (1.9 and 2.3 g/L) for Touriga Nacional and Tinta Roriz varieties, respectivelly. The RW2 contained 415.4 mg/L of total anthocyanins, a higher value than the 241.4 mg/L obtained for RW1. The DW presented similar amounts of total anthocyanins (421.4 and 234 mg/L) to those of the RW. These findings are in accordance with those obtained by Bogianchini *et al*. (2011) who reported similar values of tannins and anthocyanins for wines and dealcoholized wines (with 2% v/v of ethanol) obtained by reverse osmosis at constant pressure (30 bar) at room temperature. The RW2 contained higher amounts of the total phenols, tannins, and anthocyanins than RW1. This may be explained by the maceration phase that favours the extraction of components, namely anthocyanins and flavanols, from grape solids leading to higher amounts of phenolic compounds in wines (Cheynier *et al.,* 1997).

The comparable amounts of total phenolic compounds, tannins, and anthocyanins obtained for the wines understudy and their corresponding dealcoholized wines shows that the distillation process used to obtain the dealcoholized wines seems not to promote in high extent loss of phenolic compounds.

The antioxidant activity of the wines was performed using DPPH and ABTS assays. According to Wang *et al.* (2004), $ABTS^{\cdot+\bullet}$ and $DPPH^{\bullet}$ radicals have a different stereochemical structure and a different method of genesis, and thus leed to different response to the inactivation of radicals originating different absolute

values of antioxidant activity. In general, there are a large variation of antioxidant activity values mentioned in the literature as a result of the different antioxidant methods used, times of reaction, or the units of expression of the results. Also, varietal and vintages factors, together with winemaking and ageing process, are some of the most influential factors on the antioxidant capacities of wines (Landrault *et al*., 2001), as these results in variability of phenolic composition of wines (Rivero-Pérez *et al.* 2007). Nevertheless, a positive correlation among levels of total phenolic compounds and antioxidant activities has been reported (Katalinic *et al.*, 2004; Lachman *et al.*, 2007; Robards *et al.*, 1999).

The results obtained for red wines and the correspondent dealcoholized wines, determined by ABTS and DPPH assays, expressed as trolox equivalents (TE), are shown in Figure 3.2.

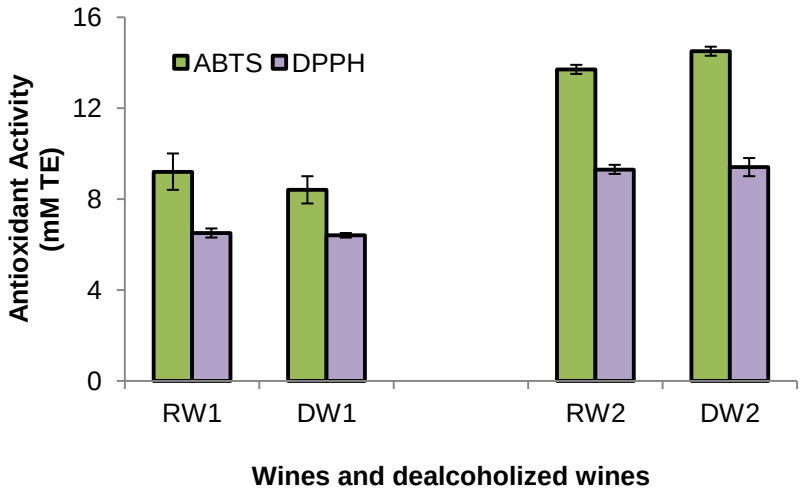


Figure 3.2: Antioxidant activity of wines and dealcoholized wines assayed by ABTS and DPPH methods. RW1- red wine 1; DW1- dealcoholized wine 1; RW2- red wine 2; DW2- dealcoholized wine 2.

The values obtained by ABTS assay were 9.2 (RW1) and 13.7 (RW2) mM of TE, and 6.5 (RW1) and 9.3 (RW2) mM of TE, quantified by DPPH assay. These values are in accordance to those reported for the antioxidant activity of red wines, ranging from 4.0 to 17.4 mM and from 2.2 to 11.1 mM TE for DPPH and ABTS assays, respectively (Fernandez-Pachón *et al*., 2004; Villano *et al.,* 2006). The

values obtained for antioxidant activity of DW were statistically similar to RW, ranging from 91 to 106% the antioxidant activity of wines, independently of the method assayed. During the dealcoholization process the free SO_2, which presents antioxidant activity, is released. However, it was not reflected in a decrease in antioxidant activity of the dealcoholized wines. A possible explanation for this fact having not occurred is the low content in free SO_2 (12 and 10 mg/L) of these wines. Corrales *et al.* (2008) observed a loss of antioxidant capacity in model solutions of Cy3Glc with sodium pyruvate when heating at 70 ºC during 1.5 h. These conditions induced a decrease of the antioxidant activity of the anthocyanin fraction and a consequent lower antioxidant activity of the dealcoholized wines. The induction of these reactions in wines only occurred for periods longer than 1 h. In our study, the wines were submitted to higher heat treatment (close to 100 ºC), but only during 1 h, which may explain the maintenance of their antioxidant properties.

Despite the methodological differences, the results obtained with ABTS or DPPH methods allowed to conclude that the heating process used for dealcoolization had no significant alteration on the antioxidant activity of the compounds present. The time and temperature used to obtain the dealcoholized wines are comparable with those used in distilleries, which can not degrade the bioactive compounds of wine.

To evaluate the contribution of each class of phenolic compounds to the antioxidant activity of DW, the phenolic compounds were fractionated by solid phase extraction allowing to obtain four fractions (F1, F2a, F2b, and F3). The F1 was eluted with pH 7.0 phosphate buffer, F2 was eluted with ethyl acetate, and F3 was eluted with acid methanol. The pH of F1 fraction was adjusted to 2, and re-eluted through the C18 cartridge. The salts were removed with distilled water and the fraction containing the phenolic acids was recovered by elution with acidic methanol. The F2 was re-loaded into the column and fractions F2a and F2b were obtained with diethyl ether and methanol, respectively. According to Sun *et al.* (2006), fraction 1 collects phenolic acids, fraction 2a monomeric flavanols and flavonols, fraction 2b oligomeric procyanidins, and fraction 3 anthocyanins, polymeric proanthocyanidins, and other pigmented complexes. The good

efficiency of separation was confirmed by the chromatographic profile and relative absorbance of fractions at 280, 320, and 520 nm. The presence of phenolic acids was confirmed by the absorbance at 280 and 320 nm (for hydroxybenzoic and hydroxycinnamic acids respectively), the presence of flavan-3-ols was confirmed by the absorbance at 280 nm, and the anthocyanins at 520 nm.

The antioxidant activity (expressed as mmol of TE by mass of phenolic compound expressed as GAE) of the phenolic compounds of each fraction is shown in Figure 3.3.

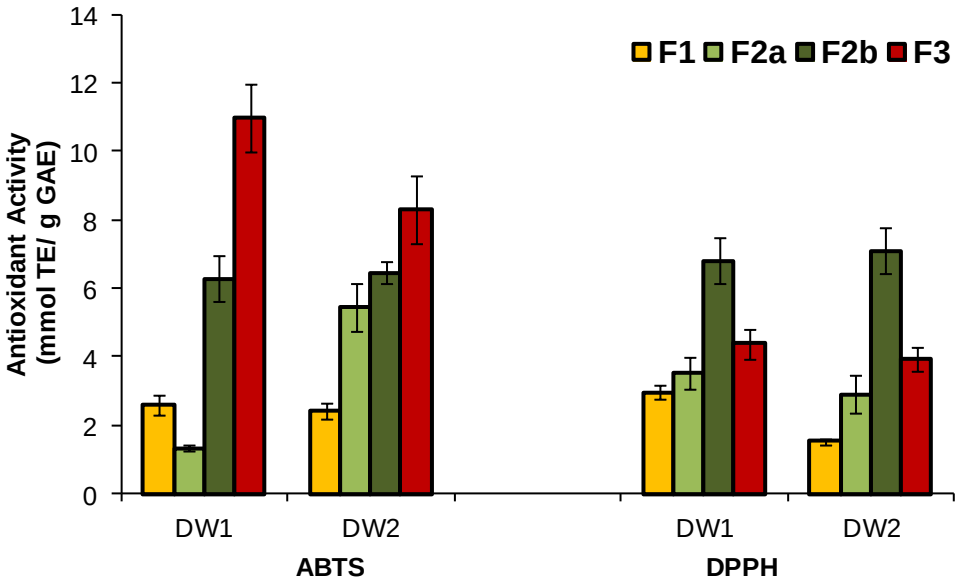


Figure 3.3 : Antioxidant activity (mmol TE/g GAE of fraction) of the phenolic compounds present in the diferent dealcoholized wine fractions obtained by C18 fractionation. F1- eluted with pH 7.0 phosphate buffer; F2- eluted with ethyl acetate; F3- eluted with acidic methanol. Fractions F2a (eluted with diethyl ether) and F2b (with methanol) resulted from a second elution of F2 through the same column.

The results showed that the relative antioxidant activity was dependent of the method assayed. For the ABTS assay, the fraction F3 of both DW1 and DW2, composed by anthocyanins and polymeric procyanidins, showed to be the most efficient. In contrast, for the DPPH assay, the fraction F2b, mainly containing oligomeric procyanidins, exhibited the highest antioxidant activity. In general, the antioxidant activity of the different fractions was comparable in both DW, except

for F2a and F3 when determined by the ABTS assay. The antioxidant activity depends not only of the total of phenolic compounds, but of the relative antioxidant activity of individual phenolic compounds present in each fraction. For example, epicatechin *O*-gallate has twice higher antioxidant activity than catechin and epicatechin (Rice-Evans *et al.,* (1996), compounds that are expected to be recovered in fraction F2a. Also, as the degree of polymerization of the anthocyanins in relevant for their antioxidant activity (Radovanovic & Radovanovic, 2010), once RW2 was a younger wine than RW1, where it is expectable a lower degree of polymerization of anthocyanins, a higher contribution of monomeric anthocyanins for the antioxidant activity of F3 in DW2 than DW1 can be expected.

The contribution of the different fractions of DW obtained by C18 sep-pak cartridge for the total antioxidant activity (expressed as mM of TE) of DW1 and DW2 by the ABTS and DPPH assays is shown in Figure 3.4.

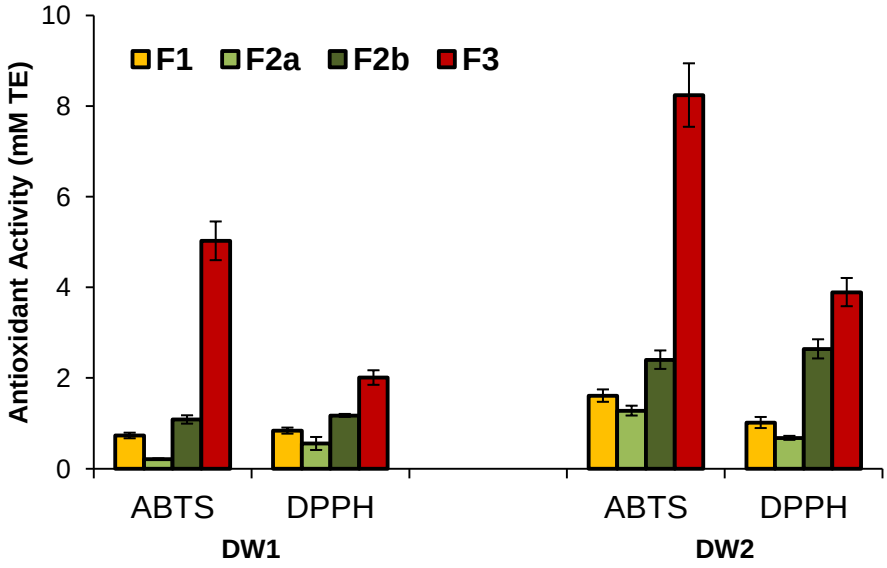


Figure 3.4: Antioxidant activity (mM TE) of the diferent dealcoholized wine fractions obtained by C18 fractionation. F1- eluted with pH 7.0 phosphate buffer; F2- eluted with ethyl acetate; F3- eluted with acidic methanol. Fractions F2a (eluted with diethyl ether) and F2b (with methanol) resulted from a second elution of F2 through the same column.

The two methods offered the same reactivity order for the different analysed fractions: F3 (containing anthocyanins) was the highest, followed by F2b (containing oligomeric procianidins and polymeric procyanidins), F1 (phenolic

acids) and F2a (catechins). As F3 was present in large amount in this DW, even with a relatively lower antioxidant activity than F2b for DPPH assay, it had a higher contribution for the total antioxidant activity of DW. The magnitude of differences was dependent of the method assayed. F3 represented 44-47% of the antioxidant activity of the sum of fractions by the DPPH assay and 61-71% by the ABTS assay. These results suggested that the higher reactivity of these wines for ABTS when compared with DPPH method was mainly due to the anthocyanins and polymeric procyanidins (F3 fraction). The contribution of F2b represented 15 and 18% of antioxidant activity determined by ABTS assay and 26 and 32% by DPPH. The lowest contribution to wine antioxidant activity was conferred by F2a fraction. This is in accordance with some studies (Xanthopoulou *et al.* 2010; Wang *et al.* 1997; Simonetti *et al.* 1997; Ghiselli *et al.* 1998; Beecher, 2003) which showed that the anthocyanin-rich fractions were the most important fractions for red wine antioxidant activity. However other authors (Arnus *et al*. 2002; Burns *et al.* 2000; Kallithraka *et al.* 2005) reported no correlation between antioxidant activity and anthocyanin content of red wines. Thus the data on literature about the contribution of the different classes of phenolic compounds to the total antioxidant activity are still contradictory.

It can be noted that the sum of the four fractions was lower than the antioxidant activity of wines, for both methods assayed. Regarding RW1, the sum of the fractions accounted for 7.0 and 4.6 mM, for ABTS and DPPH, respectively. These values represented 76 and 70% of total wine antioxidant activity. In case of RW2, the sum of fractions accounted for 13.5 and 8.2 mM, which represented 99 and 88% of total antioxidant activity of wine, for ABTS and DPPH, respectively. The possible retention of phenolic compounds in the cartridge can explain the difference observed in antioxidant activity. These rates of recuperation were much higher than those reported by Fernandez-Pachón *et al.* (2004), 49% for ABTS and 46% for DPPH, after separation by C18 sep-pak cartridge, using aqueous acetonitrile followed by ethyl acetate as eluents.

The fractions containing procyanidins (F2b) and anthocyanins and polymeric procyanidins (F3) were those that exhibit the highest antioxidant activity. However, as the concentration of F3 in red wines was higher than that of oligomeric

procyanidins, the major contribution to the total antioxidant activity can be attributed to the anthocyanins and polymeric procyanidins.

3.1.2.2 Characterization of polimeric material of wines

The dealcoholized wines were extensively dialysed, and the resulting retentates were concentrated, frozen, and freeze-dried, to give the wines polymeric material (WPM) as a powder.

The amount of polymeric material recovered accounted for 1.1 g/L and 1.3 g/L for RW1 and RW2, respectively (Table 3.2). These values were lower than the 1.5-2.3 g/L reported for the dialysis of a red wine using the same cut off but performed only during 48 h at 25 ºC, 7 L/h running water (Saura-Calixto & Díaz-Rubio, 2007). Although different wine samples have been used, this difference may also be due to the extensive dialysis that was performed in the present study.

Sugar analysis showed that polysaccharides accounted for 31% (w/w) of WPM1 and 36% of WPM2 (Table 3.2). These values were similar to the 27-38% reported for Baga red wine (Coimbra *et al.*, 2005).

Table 3.2: Sugars composition of wine polysaccharides (mg/g and mol%) present in wine polymeric material of red wine 1 and 2 (WPM1 and WPM2).

	Sugars composition			
	WPM 1		WPM 2	
Sugar residue	mg/g	mol%	mg/g	mol%
Rhamnose	9.8 ± 0.7	3 ± 0	12.0 ± 0.9	4 ± 0
Arabinose	57.9 ± 2.0	22 ± 1	30.5 ± 0.8	10 ± 1
Mannose	77.4 ± 3.1	25 ± 2	96.5 ± 5.1	25 ± 2
Galactose	79.1 ± 2.6	26 ± 1	75.1 ± 7.1	21 ± 1
Glucose	35.3 ± 3.3	11 ± 1	38.6 ± 3.1	11 ± 1
Uronic acids	44.8 ± 2.3	13 ± 1	105.0 ± 3.2	28 ± 2
Total sugars	304 ± 30	--	358 ± 30	--

Mean of three replicates ± standard deviation

The wine polysaccharide fractions have been classified, based on their sugar residue composition, as arabinogalactan-proteins (AGP), type I and type II rhamnogalacturonans (RG-I and RG-II) or mannoproteins (MP). Mannose (Man, 78.4 mg/g), galactose (Gal, 76.8 mg/g), glucose (Glc, 35.0 mg/g), arabinose (Ara, 57.5 mg/g), and rhamnose (Rha, 9.7 mg/g) were the main neutral sugars constituent of the WPM1 and uronic acids (UA) accounted for 44.4 mg/g of total sugars in these polysaccharides (Table 3.2). WPM2 was mainly composed of UA (105.0 mg/g), Man (96.5 mg/g), and Gal (75.1 mg/g). Glc (38.6 mg/g), Ara (30.5 mg/g), and Rha (12.0 mg/g) were also quantified.

The quantification of the polysaccharides may be achieved from the concentration of individual monosaccharides that are characteristic of well-defined wine polysaccharides. All Man content can be attributed to mannoproteins. Arabinogalactans can be quantified by the contribution of Ara, and Gal. Thus, the WPM1 and WPM2 used in this study were composed of 137 and 105 mg/g of pectic polysaccharides and of 77 and 96 mg/g of mannoproteins (excluding the proteic fraction), respectively. Taking into account the amount of polymeric material recovered, these polysaccharides contribute with 150 and 136 mg/L of arabinogalactans and with 85 and 125 mg/L of mannoproteins, respectively in DW1 and DW2. Significant differences of the total polysaccharides concentration between the DW1 and DW2 could be observed, possibly due to the longer maceration time used for preparation of RW2, resulting in wines with higher amount of polysaccharides, as described by Doco *et al*. (2007). The total amount of polysaccharides were present at approximate concentrations of 335 mg/L in RW1 and of 465 mg/L in RW2 analysed in this study. In accordance, Doco *et al.* (1999) described a value of ~485 mg/L for a red wine, where the quantification of polysaccharides was performed after extensive dialysis (cut-off 6-8 kDa). Otherwise, Ayestarán *et al*. (2004) and Guadalupe *et al.* (2012) reported higher values (752-1184 mg/L) for red wines, in which the polysaccharides fraction was obtained by concentration five times the wines before ethanol precipitation. Beyond the influence of grape variety, winemaking technology, and aging conditions on the amount of wine polysaccharides, the isolation procedure used is

also a relevant aspect to take into account (Ayestarán *et al.*, 2004; Doco *et al.*, 2003; Guadalupe *et al.*, 2012; Segarra *et al.*, 1995).

Although WPM2 presented higher amount of polysaccharides than WPM1, the sugar residues were present in similar proportions in both wine samples, except for Ara and UA. Ara residues were present in higher relative amounts in WPM1 when compared with WPM2 (22 vs 10 mol%), while UA residues were present in lower amounts (13 vs 28 mol%). The molar ratio Ara/Gal was 0.84 for RW1 and 0.45 for RW2. This ratio is usually close to 1 for red wine polysaccharides (Brillouet *et al.*, 1990; Doco *et al.*, 2007; Pellerin *et al.*, 1995). This lowest ratio suggested a degradation of wine AGP (Doco *et al.*, 2007), especially in RW2. The relative richness of the wine polysaccharides in homogalacturonans versus rhamnogalacturonans can be deduced from the rhamnose/galacturonic acid ratio (A. Arnous & Meyer, 2009). For RG-I this ratio is 1 and for HG this ratio is 0. The values determined for these wines, 0.25 and 0.13, in RW1 and RW2, respectivelly, indicate that homogalacturonan is predominant in both wines, mainly in RW2. Man accounted for 25 mol% in both wines, which was lower than those reported by Coimbra *et al.* (2005) for Baga red wines (34-39 mol%). When compared with the results described by Brillouet *et al.* (1990), for a red wine extensively dialysed for 6 days, the WPM1 contained more Gal (26 mol%) and Glc (11 mol%), otherwise, possessed less UA. WPM2 had a relative composition similar of that described by the same authors. These results show that the polysaccharides obtained from the two wines contained the same proportion of mannoproteins and pectic polysaccharides although those of RW2 were less branched and contained higher amounts of HG.

In addition to polysaccharides, the WPM1 and WPM2 contained 319 and 427 mg GAE/g of phenolic compounds, determined by Folin-Ciolcateu method, which was consistent with their red color. The amount of phenolic compounds associated with polymeric material represented 28% of the total present in RW1 and 18% of the total of phenolic compounds present in the RW2, a lower value when compared with literature (35-60%) for red wines using the same methodology (Saura-Calixto & Díaz-Rubio, 2007). According these authors, the polysaccharides are the main target for polyphenols association. However, in addition to

polysaccharides, proteins are another constituent of wine polymeric material that can bind polyphenols (Ferreira *et al.*, 2001).

a) Methanol extraction of WPM1

The extraction is the main step in the recovery, isolation, characterization, and quantification of individual and total polyphenolics from various plant-based materials. Phenolic compounds are often most soluble in solvents less polar than water. The most common solvents are methanol, ethanol, acetone, ethyl acetate, and their aqueous solutions. Usually, the acidification of solvent is desirable for the extraction of anthocyanins because the red flavylium cation is more stable.

With the aim of removing the phenolic compounds associated with the high molecular weight material, the wine polymeric material was extracted with acidic methanol. In addition to polysaccharides, the methanol insoluble fraction (PMi) was shown to contain phenolic compounds, while the material soluble in methanol still contained sugars associated with phenolic compounds (Table 3.3). The methanol treatment removed 67% of the phenolic compounds and 27% of the total sugars of WPM1.

Table 3.3: Yield (mg/L) and composition of fractions precipitated in graded concentrations of chloroform/methanol.

Fraction	Solid material (mg/L)	Total Phenols (mg/g)	Total sugar (mg/g)	Sugars Composition (mol %)					
				Rha	Ara	Man	Gal	Glc	UA
PMi	539±86	214±7	452±48	2±0	19±1	30±0	28±3	6±1	14±3
PM30	66	349±12	425±23	2±0	33±2	20±1	18±1	8±0	17±0
PM50	129	510±21	313±8	5±0	38±2	10±1	10±0	19±1	17±1
PM60	64	668±30	196±10	6±0	37±1	6±0	7±0	24±1	19±1
PM75	86	766±65	115±19	5±0	26±1	2±0	3±0	33±17	29±1
PMsn	126	370±10	84±5	10±1	20±1	3±0	5±0	50±3	9±1

Mean ± standard deviation. WPM1- polymeric material of wine 1. PMi- polymeric material insoluble in methanol. PM30, PM50, PM60, PM75 – fractions soluble in methanol precipitated in solutions of chloroform:methanol with 30, 50, 60 and 75 % of chloroform, respectively. PMsn- supernatant fraction of precipitation.

The estimated amount of sugars in the methanolic soluble fraction was 215 mg/g. It is probable that the sugar residues of this fraction were oligosacharides or monosaccharides components of the polyphenols. Also, Doco *et al.* (2003) isolated a fraction with a molecular weight lower than 5 kDa described as containing a complex mixture of polyphenolic compounds, oligosaccharides, and small peptides from wine polymeric material extensively dialyzed using a membrane cut off of 6-8 kDa. In addition, the presence of polysaccharids was already described to remain soluble in 80% ethanolic solution, which induces the possibility of carbohydrates to be present in methanolic solutions associated to phenolic compounds. In particular, arabinans (Vidal *et al.*, 2001) and rhamnogalacturonans and homogalacturonans (Doco *et al.*, 1999) present in the wine were suggested to remain in the supernatant after ethanol precipitation. Also, Cardoso *et al.* (2002) isolated an arabinan from olive pomace by fractionation of a pectic polysaccharide-rich fraction that remained soluble in 80% ethanol.

The PMi was obtained by centrifugation, dissolved with water and freeze dried. This fraction was composed of 452 mg/g of polysaccharides and phenolic compounds reached 214 mg/g (Table 3.3). Sugar analysis showed that Man that arises from mannoproteins was the major sugar residue, representing 30 mol%. Gal, Ara, UA and Rha, sugar residues of pectic polysaccharides, represented 64% of total sugars in PMi. Man (89%), Gal (80%), UA (73%) and Ara (64%) remained preferentially in PMi fraction, while Glc and Rha were mainly removed by methanol, remaining 39% in PMi.

The methanolic material was submitted to successive additions of chloroform. The material was precipitated by gradually addition of chloroform performing solutions with 30%, 50%, 60%, and 75% (v/v, chloroform/methanol) according to the procedure proposed by Saucier *et al.* (2001). From the successive additions of chloroform resulting the fractions PM30, PM50, PM60, PM75, and PMsn. The material soluble in methanol was preferencially recovered in PM50 and PMsn fractions, 129 mg/L and 126 mg/L, respectively.

The amount of sugars in fractions decreased as the concentration of chloroform increased, ranging from 425 mg/g in PM30 to 115 mg/g in PM75. In contrast, the amount of phenolic compounds, expressed as GAE, increased from

349 mg/g in PM30 to 766 mg/g in PM75. The PMsn contained 370 mg/g of phenolic compounds, representing 13% of the PM content.

Sugar analysis showed that the relative amount of Man and Gal exhibited a tendency to decrease in fractions precipitated with higher concentration of chloroform. Man represented 20 mol% of PM30 and 3% of PMsn. The relative amount of Gal was 18 mol% in PM30 and 5 mol% in PMsn. Otherwise, the relative amount of Glc had an opposite behaviour, reaching a maximum of 50 mol% on PMsn. The presence of Glc in these WPM1 fractions could be attributed to phenolic compounds, namely anthocyanins. The amount of UA ranged from 17 to 29 mol% in the precipitated fractions, accounting for 9 mol% in PMsn. The lower amount of UA in PMsn was in accordance with the charge characteristic of these sugar residues. The ratio of (Ara+Gal)/(Rha+UA) can be used to estimate the relative proportion of neutral arabinogalactan side-chains to the rhamnogalacturonan backbone that can occur in alcohol-rich solutions (Doco *et al.,* 1999). This ratio was 3.0 in PMi fraction, allowing to infer a highly branched structure. The decrease of this ratio from 2.7 to 0.8, respectively from PM30 to PM75 induced a decrease of the neutral side chains of rhamnogalacturonan structure. The higher relative amount of Rha (10 mol%) and lower of UA (9 mol%) present in PMsn fraction when compared with the PM75 fraction (5 mol% of Rha and 29 mol% of UA) may be due to the enrichement in phenolic compounds as Rha as been described as one component (Kosir & Kidric, 2002). The ratio Ara/Gal was 0.7 in PMi, similar to the value for WPM1, and lower than those observed for the metanolic fractions. This ratio increased from 1.8 in PM30 to 8.3 in PM75, which is much higher than that usually described for wine polysaccharides (~1) and is close to that described for wine oligosaccharides (2.8 and 3.1) (Ducasse *et al.*, 2010). These results allowed to infer the presence of Ara-rich oligosaccharides and/or Ara-rich phenolic compounds.

b) Separation of sugars from phenolic compounds by SPE

In order to try to remove the remaining phenolic compounds associated to the polymeric matrix, the WPM1 and PMi fractions were further purified by solid phase extraction using a C-18 sep-pak cartridge. In addition, the most abundant fraction soluble in methanol (PM50), still containing 31% of sugar, was also loaded into the C18 sep-pak cartridge. This procedure allowed obtaining hydrophylic polysaccharide-rich fractions eluted with water (WPMW, PMiW and PM50W) with a slightly brown-rose color and hydrophobic phenolic-rich fractions (WPMM, PMiM and PM50M) eluted with acidic methanol with a purple color. The yield, the total phenols and sugar composition of each fraction is described in Table 3.4.

Table 3.4: Yield (mg/L) and composition of fractions obtained from C-18 extraction of WPM1, PMi and PM50 fractions.

Fraction	Solid material (mg/L)	Total Phenols (mg/g)	Total sugars (mg/g)	Sugars Composition (mol %)					
				Rha	Ara	Man	Gal	Glc	UA
WPMW	642 ±15	92±7	372±23	6±1	25±2	24±3	28±1	7±1	10±2
WPMM	259±11	574±34	200±16	3±0	30±2	9±0	20±2	24±1	13±1
PMiW	198±13	39±3	560±22	2±0	20±1	28±1	28±2	3±1	18±3
PMiM	144±15	416±21	259±17	2±0	29±1	20±1	16±1	15±1	17±5
PM50W	38	107±7	401±13	8±1	18±1	20±1	17±1	17±1	18±1
PM50M	59	637±25	110±8	4±0	15±2	10±1	9±0	42±2	20±1

Mean ± standard deviation. WPMW- wine 1 polymeric material fraction eluted with water; WPMM- wine 1 polymeric material fraction eluted with acidic methanol; PMiW- insoluble polymeric material fraction eluted with water; PMiM- insoluble polymeric material fraction eluted with acidic methanol; PM50W- fraction precipited with 50% (v/v, chloroform/methanol) eluted with water; PM50M- fraction precipited wiyh 50% (v/v, chloroform/methanol) eluted with acidic methanol.

The percentage of recovery of solid material, phenolic compounds and total sugars after C-18 SPE purification of fractions WPM, PMi and PM50 is shown in Figure 3.5.

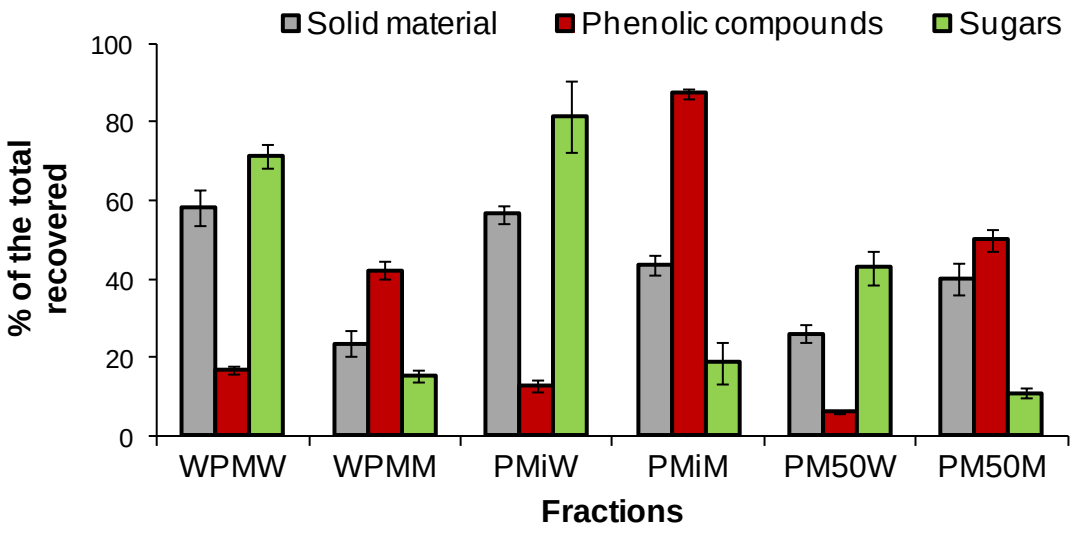


Figure 3.5: Percentage of recovery of solid material, phenolic compounds and total sugars after C-18 SPE purification of fractions WPM1, PMi and PM50. WPMW- wine 1 polymeric material fraction eluted with water; WPMM- wine 1 polymeric material fraction eluted with acidic methanol; PMiW- insoluble polymeric material fraction eluted with water; PMiM- insoluble polymeric material fraction eluted with acidic methanol; PM50W- fraction precipited wiyh 50% (v/v, chloroform/methanol) eluted with water; PM50M- fraction precipited with 50% (v/v, chloroform/methanol) eluted with acidic methanol.

The solid material of WPMW represented 58% of WPM1, while WPMM represented 24%. This apparent loss of 18% of polymeric material not recovered after C-18 purification step may be due to the higroscopicity of the material that easily hydrates upon storage in anhydrous conditions. WPMW recovered 71% of polysaccharides and 17% of phenolic compounds present in WPM1. The WPMM fraction recovered 15% of total sugars and 42% of phenolic compounds initially present in WPM1.

Regarding the methanol insoluble polymeric material, it can be seen that PMiW contained 58% of the material recovered (Figure 3.5). Seven percent of the phenolic compounds initially present in PMi were recovered in this fraction, allowing to infer that they are strongly associated to polysaccharides. The PMiW was also richer in polysaccharides (56%) than PMiM (26%). The total sugars recovered in PMiW represented 46% of those initially present in PMi fraction. In contrast with WPM1 and PMi fractions, the solid material of PM50 was preferencially recovered in the fraction eluted with acidic methanol (PM50M). The

PM50M fraction recovered 11% of total sugars and 50% of phenolic compounds. This fraction was composed by 64% of phenolic compounds and 11% of polysaccharides.

Based on the amounts of mass obtained for each fraction and its relative sugar composition it can be observed that all sugar residues were preferentially recovered in the hydrophilic fractions, except for Glc. Part of Glc residues of hydrophobic fractions may arise from phenolic compounds, namely from anthocyanins. Analysing the relative sugar composition (mol%), it was observed that the hydrophilic fractions contained higher amounts of Man and Gal when compared with the hydrophobic fractions. In contrast, the relative amount of UA was similar in hydrophilic and hydrophobic fractions, while the latter fractions were composed of higher amounts of Glc, representing 15, 24 and 41 mol% for WPMM, PMiM, and PM50M, respectively. The retention of both, carbohydrates and phenolic compounds, allows concluding that they were tightly bound.

c) Amino acid composition of WPM1, WPM2 and PMi fractions

The total amount of amino acids of WPM1, WPM2 and PMi fractions were carried out by gas chromatography performed after acid hydrolysis and derivatized to the heptafluorobutyric derivatives. The acid exposure results in the deamination of the amide groups of asparagine and glutamine to yield aspartic acid and glutamic acid, respectivelly. Thus Aspargic acid and asparagine were quantified together and assigned by Asx, while glutamic acid and glutamine were named Glx. In addition, more eight amino acids [alanine (Ala), glycine (Gly), valine (Val), treonine (Thr), serine (Ser), leucine (Leu), isoleucine (Ile), and proline (Pro)] were identified in these fractions. Based on their hydropathy index, the amino acids can be classified as hydrophilic (Gly, Thr, Ser, Asx and Glx) or as hydrophobic (Ala, Val, Leu, Iso and Pro). The WPM1 contained 98.6 mg/g of amino acids, which represented 108 mg/L (Table 3.5).

Table 3.5: Amino acids composition (mg/g) of WPM1, WPM2 and PMi fractions.

	Samples		
Amino acid	WPM1	WPM2	PMi
Ala	8.9 ± 0.3	7.5 ± 1.7	6.1
Gly	14.9 ± 2.0	23.7 ± 3.1	11.0
Val	14.1 ± 1.6	14.2 ± 1.4	9.3
Thr	9.7 ± 0.9	7.1 ± 1.3	18.3
Ser	10.3 ± 0.6	8.7 ± 2.9	19.8
Leu	11.7 ± 2.4	16.6 ± 2.8	9.5
Iso	9.2 ± 1.6	10.6 ± 0.9	6.9
Pro	4.4 ± 1.0	15.0 ± 1.8	5.1
Asx	6.2 ± 1.5	8.3 ± 0.7	13.8
Glx	8.9 ± 1.7	15.4 ± 3.1	18.1
Total (mg/g)	98.6 ± 6.6	126.9 ± 5.3	117.9

Ala- alanine, Gly- glycine, Val- valine, Thr- treonine, Ser- serine, Leu- leucine, Ile- isoleucine, Pro- proline, Asx – aspartic acid + asparagine; Glx- glutamic acid + glutamine. WPM1- polymeric material of wine 1; WPM2- polymeric material of wine 1. PMi- polymeric material insoluble in methanol.

The WPM1 was composed by 15% of Gly, 14% of Val, 12% of Leu, 11% of Ser, and 9% of Ala, Ile, and Glx. Asx (6%) and Pro (4%) were present in minor amounts. The hydrophilic amino acids accounted for 64 % and the hydrophobic for 36%. When compared with WPM1, WPM2 contained a higher amount of amino acids, 126.9 mg/g, which represented 165 mg/L. WPM2 was mainly composed by Gly (23.7 mg/g), Leu (16.6 mg/g), Glx (15.4 mg/g), Pro (15.0 mg/g), and Val (14.2 mg/g). Together they represented 66% of total amino acids quantified. When compared with WPM1, WPM2 contained higher amounts of Pro (+ 9.3 mg/g), Gly (+ 8.1 mg/g), Glx (+ 6.3 mg/g) and Leu (+ 4.8 mg/g), whereas the others amino acids were present in comparable amounts.

The PMi fraction contained 11.8 mg/g of the amino acids representing 59% of WPM1. Ser (17%), Thr (16%), Glx (15%), and Asx (12%) were the amino acids present in higher amounts in PMi. Ser, Thr, Glx and Asx were preferentially (> 90%) recovered in PMi. The hydrophobic amino acids were preferentially removed

by methanol, in agreement with the higher hydrophobicity of this solvent compared with water.

d) Antioxidant activity of fractions obtained by precipitation in solutions of chloroform:methanol

The antioxidant power of WPM1 and of fractions obtained by precipitation in solutions of chloroform:methanol was evaluated as antioxidant efficacy (AE) by the DPPH assay (Sanchez-Moreno *et al.*, 1999). The antioxidant efficacy was determined using the expression $AE = 1/(EC_{50} \times T_{EC50})$. The EC_{50} is defined as the amount of antioxidant needed to decrease 50% the initial concentration of the $DPPH^{\bullet}$ radical, and (Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995)(Brand-Williams *et al.*, 1995). The T_{EC50} is the time needed to the decrease occurs. For each extract, five different concentrations were tested, and the kinetic behavior was used to determine the time needed to reach the steady state. Concentrations of the phenolic compounds in the reaction medium were plotted against the percentages of $DPPH^{\bullet}$ remaining in order to obtain the EC_{50} value. The T_{EC50} obtained for the extracts understudy ranged from 30 to 120 min. According to Sánchez-Moreno *et al.* (1998), the kinetic behavior can be classified as rapid for periods lower than 5 min, intermediate for 5-30 min and slow for periods longer than 30 min. Thus, according this classification, all extracts exhibited a slow kinetic behavior. The higher concentration of extract, which means higher ratio extract / DPPH, the lower the T_{EC50}. Also, a large range of T_{EC50} values was obtained by Villano *et al.* (2006) for 20 phenolic compounds varying from 11 min to caftaric acid until 180 min for catechin.

Figure 3.6 shows the AE values determined to the different fractions, expressed as mass (g) of DPPH by mass (g) of extract by time (min) of reaction.

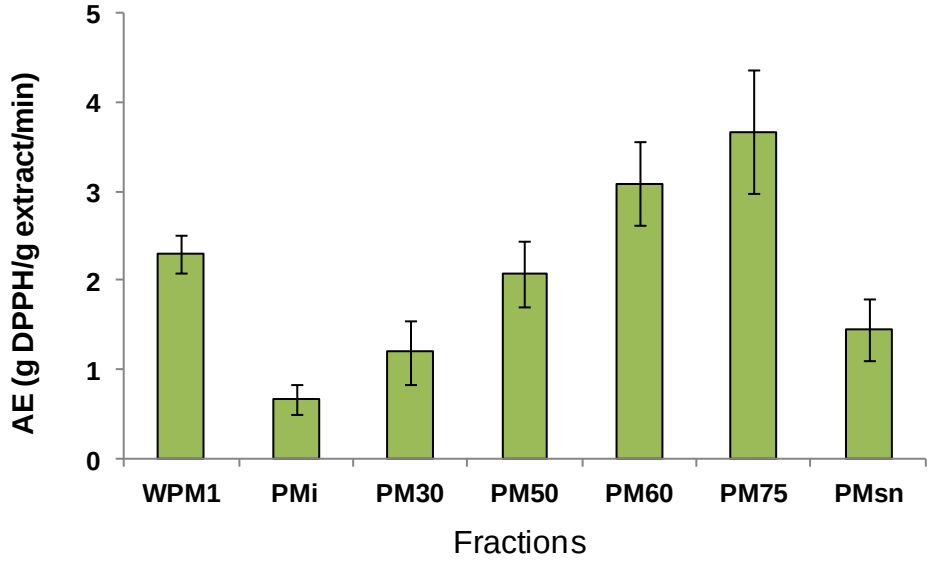


Figure 3.6: Antioxidant efficacy of fractions precipitated in graded concentrations of chloroform:methanol. WPM1- polymeric material of wine 1. PMi- polymeric material insoluble in methanol. PM30, PM50, PM60, PM75 – fractions soluble in methanol precipitated in solutions of chloroform:methanol with 30, 50, 60 and 75 % of chloroform, respectively. PMsn- supernatant fraction of precipitation.

The fraction PM75 exhibited the highest AE, reaching 3.7 g DPPH/g extract/min. The AE values showed a good correlation (r^2=0.8047) with the total phenols present in each fraction, showing that these compounds were the main responsible for the antioxidant power of the fractions. However, the AE value of WPM1 was 2.3 g DPPH/g extract/min higher than those obtained for PM30 (1.2 g DPPH/g extract/min) and PM50 (2.1 g DPPH/g extract/min) fractions that contained higher amounts of phenolic compounds (Table 3.3). This fact may be due to the contribution of different phenolic compounds to antioxidant activity, and also to the contribution of polysaccharides. In accordance, Aguirre *et al.* (2009) isolated an arabinogalactan as a the neutral fraction of wine polysaccharides, which possessed antioxidant capacity. These findings allow to infer the possible contribution of polysaccharides to antioxidant activity of wine polymeric material.

3.1.3 Procyanidin composition composition of dealcoholized red wine 1

The procyanidin composition of dealcoholized red wine 1 was performed by analysis of fraction eluted with ethyl acetate (Figure 3.7). The procedure allowed to quantify catechin monomers ((+)-catechin, (-)-epicatechin and (-)-epicatechin *O*-gallate.), procyanidins dimers (procyanidin B1, procyanidin B2, procyanidin B3, procyanidin B4, procyanidin B5, procyanidin B7 and procyanidin B2 *O*-gallate) and a trimer (C1). A large range of individidual flavanols has been described but, in general, catechin and procyanidin B1 are the most abundant monomers and oligomers, respectively, identified in wines.

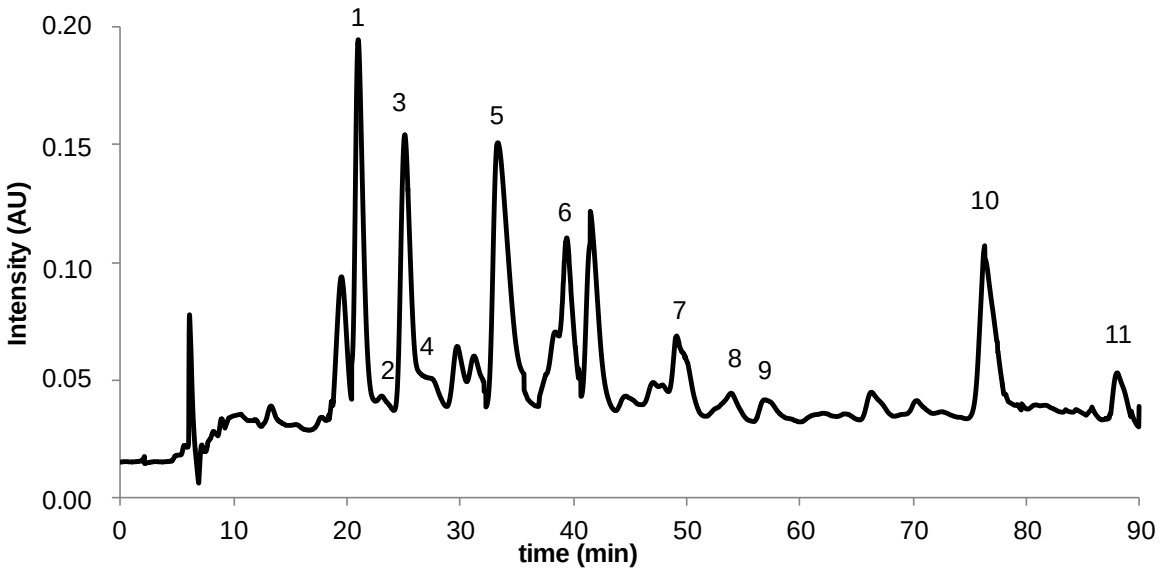


Figure 3.7: HPLC chromatogram of procyanidin fraction recorded at 280 nm. Identification: 1. procyanidin B1, 2. procyanidin B3, 3. (+)-catechin, 4. procyanidin B4, 5. procyanidin B2+B6, 6. (-)-epicatechin, 7. Trimer C1, 8. procyanidin B2 *O*-gallate, 9. procyanidin B7, 10. (-)-epicatechin *O*-gallate, 11. procyanidin B7.

The procyanidin concentration (Table 3.6) was expressed in mg/L of each compound, obtained by standard calibration curves. It can be seen from Table 3.6 that the total amount of procyanidin was 83.7 mg/L which was in accordance with the values described by Monagas *et al*., (2003) (76.9–133.2 mg/L) and Mateus *et al.* (2001) (68.0 and 93.4 mg/L). Otherwise, Garcia-Falcón *et al.* (2007) quantified lower values of monomers plus oligomers in red wines (31–53 mg/L). The amount

of monomers was 11.1 mg/L, and accounted for 13% of procyanidins quantified. Cosme *et al.* (2009) described similar values (2.2–30.4 mg/L) of monomeric flavanols (quantified by vanillin assay) for Portuguese red wines. Catechin represented almost half of monomeric flavanols, accounted by 5.1 mg/L, a lower value when compared with those reported by Monagás *et al*. (2003) (17.2–29.5 mg/L) and by Garcia-Falcón *et al.* (2007) (19-21 mg/L).

Table 3.6: Amount of procianidins (expressed as mg/L of each compound) of red wine 1.

Peak number	Flavan-3-ols	Concentration (mg/L)
	Monomers	
3	(+)-catechin	5.1 ± 0.1
6	(-)-epicatechin	3.6 ± 0.3
10	(-)-epicatechin *o*-gallate	2.4 ± 0.4
	Oligomers	
1	procyanidin B1	32.7 ± 2.9
2	procyanidin B3	0.7 ± 0.2
5	procyanidin B2 + B6	27.0 ± 0.5
4	procyanidin B4	2.3 ± 0.1
11	procyanidin B5	3.9 ± 0.7
9	procyanidin B7	0.8 ± 0.1
8	procyanidin B2 *O*-gallate	1.5 ± 0.2
7	Trimer C1	3.8 ± 0.6
	Total	83.7 ± 2.1

Mean ± standard deviation

Regarding dimers, they account for the most part of procyanidins quantified, 82%, reaching 68.2 mg/L. Lower values of oligomeric procyanidins, ranging from 10.9 to 228.3 mg/L have been described for red wines (Cosme *et al*., 2009;

García-Falcón *et al*., 2007). Procyanidin B1 accounted for 32.7 mg/L, was the dimer present in higher concentration in DW1. Accordingly, procyanidin B1 has been described as the main dimer present in red wines (Mateus *et al*., 2001; García-Falcón *et al.,* 2007; Sun *et al.,* 2011). Procyanidin B3 accounted for 0.6 mg/L. In accordance, values of 20.2–35.0 mg/L for procyanidin B1 have been reported (García-Falcón *et al.*, 2003; Monagás *et al*., 2003) and of 0.9–6.9 mg/L for procyanidin B3 (Mateus *et al.,* 2001; Monagás *et al*., 2003). The value of procyanidin B2 plus procyanidin B6 was 27.0, whereas procyanidin B2 contributes in larger amount. Accordingly, procyanidin B2 was described as the second most abundant in wines in concentrations ranging from 11.9 to 31.7 mg/L (Monagás et al., 2003). Procyanidin B5 (3.9 mg/L), procyanidin B4 (2.3 mg/L), procyanidin B7 (0.8 mg/L), and procyanidin B2 *O*-gallate, (1.5 mg/L, probably co-eluted with other compound) also were present in this wine. The trimer C1 was present in 3.8 mg/L, similar to the 1.6 to 5.6 mg/L found by Mateus *et al.* (2001). The obtained value was lower when compared with those reported by Garcia-Falcón *et al*. (2003) (9–20 mg/L), but slightly higher than 0.6 to 2.4 mg/L described by Monagás *et al.* (2003).

3.1.4 Detailed phenolic composition of red wine 2

3.1.4.1 Phenolic acids and monomeric flavan-3-ols

The identification and quantification of phenolic acids and catechins of DW2 was determined by analysis of the mixture of the fractions F1 and F2a obtained from DW2 after C18 sep-pak cartridge purification. The hydroxybenzoic acids, hydroxycinnamic acids, and catechins were quantified as gallic (GAE), caffeic acid (CAE), and catechin equivalents (CE), respectively, all in mg/L. The fraction F1 was obtained by elution with phosphate buffer pH 7. The pH of fraction was adjusted to pH 2, and reloaded into the C18 cartridge. The salts were removed with distilled water and the fraction containing the phenolic acids was recovered by elution with methanol. The monomeric and oligomeric flavan-3-ols were eluted with ethyl acetate. This fraction was taken to dryness, redissolved in phosphate buffer, pH 7.0, and finally reloaded into the C18 sep-pak cartridge. The monomeric flavanols (catechins) were recovered by elution with diethyl ether. This fraction was evaporated to dryness and redissolved in methanol. Both fractions, containing phenolic acids and catechins were combined and then analyzed by HPLC. The chromatographic profile of phenolic acids and catechins fractions at 280 nm and 320 nm is described in Figure 3.8.

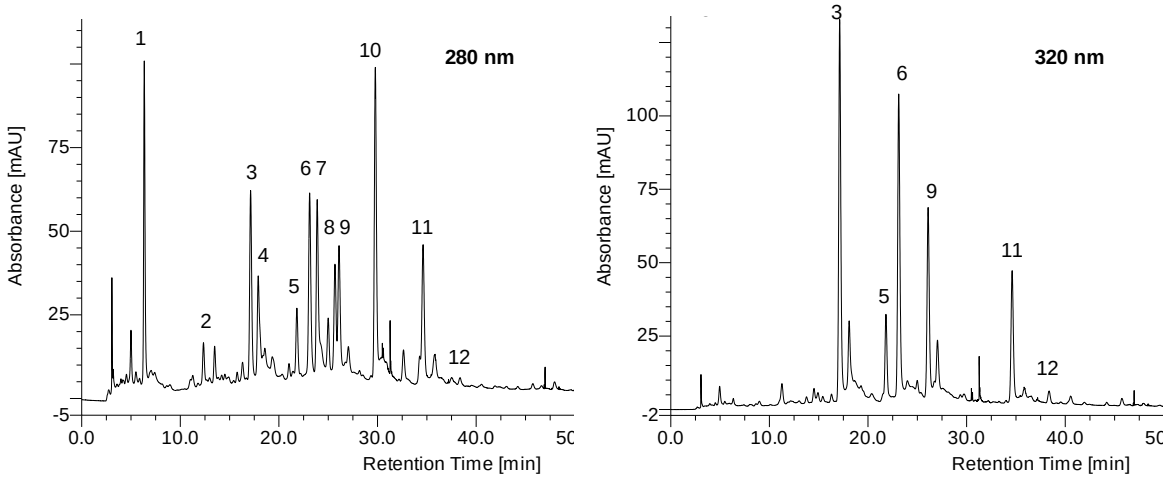


Figure 3.8: HPLC chromatograms of phenolic acids and catechins fractions recorded at 280 and 320 nm. Identification: 1. gallic acid, 2. protocatechuic acid, 3. *trans*-caftaric acid, 4. *p*-hydroxybenzoic, 5. *trans*-coutaric, 6. chlorogenic acid, 7. (+)-catechin, 8. syringic acid, 9. caffeic acid,10. (-)-epicatechin, 11. *p* –coumaric acid, 12. ferulic acid.

A total of four hydroxybenzoic acids (gallic, protocatechuic, *p*-hydroxybenzoic and syringic acids) and six hydroxycinnamic acids (*trans*-caftaric, *trans*-coutaric, chlorogenic, caffeic, *p*–coumaric and ferulic acids) were found at detectable levels in the wine sample (Table 3.7). In addition, catechin and epicatechin were also quantified.

Table 3.7: Concentration (mg/L) of phenolic acids and cathechins in red wine 2.

Peak number	Phenolic Compounds	Concentration (mg/L)
Hydroxybenzoic acids		28.6
1	Gallic	14.8
2	Protocatechuic	1.9
4	*p*-Hydroxybenzoic	9.0
8	syringic	2.9
Hydroxycinnamic acids		46.9
3	*trans*-Caftaric	13.8
5	*trans*-Coutaric	3.3
6	Chlorogenic	11.3
9	Caffeic	9.1
11	*p*-Coumaric	9.0
12	*trans* -Ferulic	0.3
Catechins		37.8
7	(+)-Catechin	15.2
10	(-)-Epicatechin	22.6

The DW2 was composed of 28.6 mg GAE/L of benzoic acid, in accordance with the values obtained by García-Falcón *et al.* (2007) (28-42 mg/L) for benzoic acids of red wines. However, Arnous *et al.* (2001) reported levels of benzoic acids (average content of 338 mg/L GAE) that were much higher than those found in DW2. Gallic acid accounted for the largest concentration among the benzoic acids, 14.8 mg/L, representing 52% of all quantified. Its presence in wine is mainly due to the hydrolysis of flavonoid gallate esters (Frankel *et al*., 1995). The concentration

of *p*-hydroxybenzoic acid was 9.0 mg/L, which is higher than those quantified by García-Falcón *et al.* (2007) (1-2 mg/L) and Minussi *et al.* (2003) (0.7–1.3 mg/L). Syringic and protocatechuic acids were the two benzoic acids found at the lowest levels in RW2, 2.9 and 1.9 mg/L, respectively. Minussi *et al.* (2003) found similar values (2.2-3.9 mg/L) of syringic acid, and higher values (2.6–7.2 mg/L) of protocatechuic acid. A wide range of values for gallic acid concentration in wine has been described, ranging from 528 to 1267 mg/L (Arnous *et al.*, 2001; García-Falcón *et al.*, 2007; Minussi *et al.*, 2003), quantified using different methodologies. For instance, Arnous *et al.* (2001), employed a direct-injection method to quantify (126–528 mg/L in case of GA), while Garcia-Falcon *et al.* (2007) used a liquid-liquid extraction to obtain phenolic acids fraction. Minussi *et al.* (2003) measured the amounts of phenolic acids by capillary zone electrophoresis.

Regarding hydroxycinnamic acids, the DW2 was composed of 46.9 mg CAE/L of hydroxycinnamic acids, a value that was lower than the values reported by Alén-Ruiz *et al.* (2009) (82–219 mg/L) and by Arnous *et al*. (2001) (average content of 500 mg/L) for red wines. The *trans*-caftaric acid was the most abundant compound, with a value of 13.8 mg/L, a lower value when compared with 27-106 mg/L reported by García-Falcón *et al.* (2007) and with 134–562 reported by Arnous *et al.* (2001), also expressed as CAE. Also, substantial amounts of clorogenic acid (11.3 mg/L), caffeic acid (9.1 mg/L) and *p*-coumaricc acid (9.0 mg/L) were also found in this wine. Arnous *et al.* (2001) and Minussi *et al.* (2003) reported lower values of caffeic acid (1.0–2.2 and 2.7–3.6 mg/L) and *p*-coumaric acid (1.4 – 3.1 and 1.0 – 2.9 mg/L) for 8 red wines. However, Arnous *et al.* (2001) found 143.9 and 105.9 mg/L of caffeic acid and *p*-coumaricc acid, respectively, for one wine.

The amount of catechin, expressed as catechin equivalents, in RW2 were 15.2 slightly lower than those (17–28 mg/L) reported in literature (Garcia-Falcón *et al.,* 2007; Monagas *et al.,* 2003). Epicatechin accounted for 22.6 mg/L, comparable to the 2–20 mg/L described by Garcia-Falcón *et al.* (2007). Otherwise, the average values reported by Arnous *et al.* (2001) for catechin (83.3 mg/L) and epicatechin (62.6 mg/L) were much higher than those quantified in RW2.

3.1.4.2 Monomeric anthocyanins composition of red wine 2 and the corresponding dealcoholized wine

The determination of anthocyanins was performed by direct-injection of wine and dealcoholized wine. The chromatographic profile is shown in Figure 3.9. It was possible to quantify a total of four anthocyanin 3-glucosides, four anthocyanin 3-acetylglucosides and two anthocyanin 3-coumaroylglucosides.

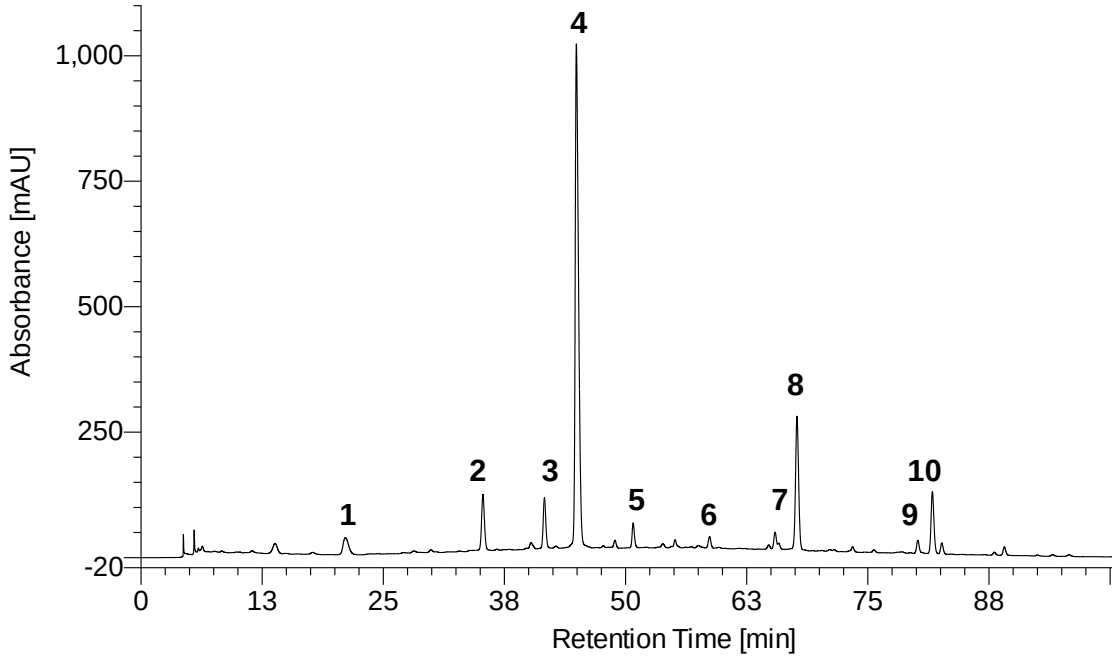


Figure 3.9: HPLC chromatogram of red wine recorded at 520 nm. Identification: 1. Dp3Glc, 2. Pt3Glc, 3. Pn3Glc, 4. Mv3Glc, 5. Dp3AcGlc, 6. Pt3AcGlc, 7. Pn3AcGlc, 8. Mv3AcGlc, 9. Pn3CmGlc, 10. Mv3CmGlc.

The amount of individual monomeric anthocyanins of red wine and dealcoholized wine, quantified by HPLC and expressed as equivalents of malvidin 3-glucoside, is shown in Figure 3.10. The RW2 and the DW2 contained 379.1 mg/L and 375.3 mg/L of anthocyanins, respectively. The similar amount of each anthocyanin quantified in each sample (Figure 3.10), allowed to conclude that the amount and composition of anthocyanins were not affected by the wine dealcoholization conditions used. This result is in accordance to the results obtained by Tsai and Huang (2004), who reported the conversion for Roselle

variety anthocyanin model system, of only 1% of monomeric anthocyanins into polymeric anthocyanins after 2 h of heating at 90 ºC.

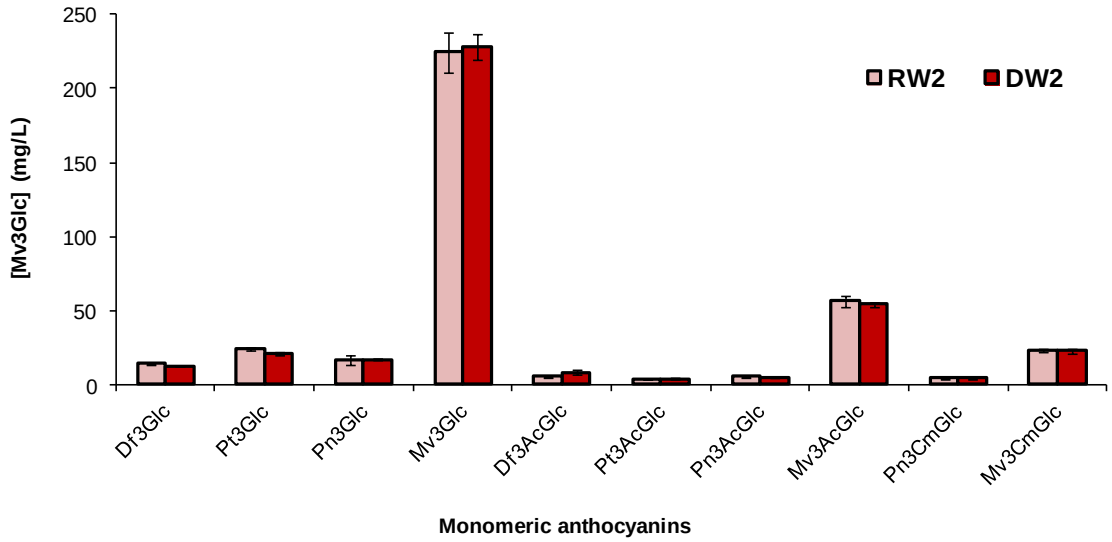


Figure 3.10: Concentration (expressed as mg/L of Mv3Glc) of individual monomeric anthocyanins present in the RW2 and in DW2.

The RW2 contained 224.6 mg/L of malvidin-3-glucoside (Mv3Glc), representing around 60 % of the anthocyanins quantified. This value is in accordance with those reported by Teissedre and Landrault (2000) (90-382 mg/L) and is higher than those reported by Lorenzo *et al.,* (2005) (71 mg/L), also for red wines. In addition, the RW2 contained 56.4 mg/L of malvidin 3-(6-acetyl)glucoside (Mv3AcGlc), 23.9 mg/L Petunidin 3-glucoside (Pt3Glc) and 23.5 mg/L of malvidin 3-(6-coumaroyl)glucoside (Mv3CmGlc). Peonidin 3-glucoside (Pn3Glc) and delphinidin 3-glucoside (Dp3Glc) range from 14.4 mg/L to 16.9 mg/L, slightly higher than the values (6.2-10.2 mg/L) found in Monastrell red wines (Tsai & Huang, 2004). Delphinidin 3-(6-acetyl)glucoside (Df3AcGlc), petunidin 3-(6-acetyl)glucoside (Pt3acGlc), peonidin 3-(6-acetyl)glucoside (Pn3AcGlc), and peonidin 3-(6-coumaroyl)glucoside (Pn3CmGlc) were present in the range of 3.8-8.3 mg/L, slightly higher than the values found by Monagas *et al.* (2005) for red wines (~0.2-5.0 mg/L). As verified with the general phenolic composition, the distillation process preserved the monomeric anthocyanins of red wines in dealcoholized wines.

3.1.4.3 Fractionation of red wine 2 by graded ethanol precipitation

Polysaccharides are one of the main target for the association of phenolic compounds to polymeric material. For the quantification of polysaccharides in a very complex matrix, such as wine samples, a preliminary extraction is necessary. Dialysis (Brillouet *et al.*, 1990; Coimbra *et al.*, 2002; Doco *et al.*, 2003) and precipitation with ethanol (Ayestarán *et al.*, 2004; Guadalupe *et al.*, 2012; Segarra *et al.*, 1995) are two methods commonly used. Ayestarán *et al.,* (2004) obtained larger amounts of polysaccharides when the precipitations with ethanol were performed at 4 ºC in comparison with room temperature. Moreover, the precipitation of polysaccharides can be achieved by direct precipitation of polysaccharides in non-concentrated or in concentrated wines before ethanol addition.

In order to isolate and characterize the polysaccharides present in DW2, the DW2 was submitted to successive additions of ethanol. The precipitation in graded ethanol solutions has been described to be selective for precipitation of wine polysaccharides, as the mannoproteins tend to precipitate at 60% of ethanol and the AG tend to precipitate at 80% (Coimbra *et al.*, 2002). Prior to the ethanol precipitation, the DW2 was cooled at 5ºC, and the insoluble material was recovered by centrifugation (fraction $DW_{Et}0$). The soluble material was fractionated according to its solubility in ethanol, allowing to obtain the fractions precipitated with 30, 50 and 80% (v/v, ethanol/water) assigned respectively as Et30, Et50, Et80. The fraction that remained soluble was named Etsn and was also analyzed. The yield of solid material (mg/L), total sugars (mg/L), and total monomeric anthocyanins (expressed as mg/L of Mv3Glc) is presented in Table 3.8.

Table 3.8: Yield (mg/L) and composition of fractions obtained by precipitation in ethanol solutions.

Concentration (mg/L)	Fractions					
	$DW_{Et}0$	$DW_{Et}30$	$DW_{Et}50$	$DW_{Et}80$	$DW_{Et}sn$	Total
Solid material	300 ± 32	157± 8	557 ± 23	383 ± 24	1635 ± 87	3025 ± 190
Carbohydrates	59 ± 10	16 ± 5	218 ± 11	48 ± 1	124 ± 5	466 ± 26
Monomeric anthocyanins *	20 ± 1	8 ± 0	9 ± 1	9 ± 0	225 ± 1	270 ± 8

* expressed as malvidin 3-Glc equivalents.

The total material of lyophilized fractions accounted for 3.0 g/L, and was preferentially (54%) recovered in the $DW_{Et}sn$ fraction. The fractions precipitated with 50 and 80% of ethanol represented 18 and 13%, respectively of the obtained material. The amount of polysaccharides, expressed as mg/L of wine was estimated based on the solid material obtained and on the richness of carbohydrates (mg/g) of each fraction. The total amount of carbohydrates was 466 mg/L, of which 47% were recovered in fraction $DW_{Et}50$. The $DW_{Et}sn$ and $DW_{Et}80$ contained 23% and 10% of the total obtained. The insoluble fraction ($DW_{Et}0$) contained 300 mg/L, representing 13% of carbohydrates. Based on the amount of monomeric anthocyanins of this fraction (Table 3.10) it is possible that these sugars could arise from co-precipitation with phenolic compounds. The soluble fraction, which contained 87% of carbohydrates, was used for further experiments. The amount (expressed as mg sugars by g of dry material) and the relative percentages (mol%) of sugar residues in fractions obtained by graded precipitation in ethanol are presented Table 3.9.

The insoluble fraction in cold water ($DW_{Et}0$) contained 19.8% of polysaccharides, mainly composed by Glc (62 mol%), in accordance with the high amount of anthocyanins. Ara, Man, Gal and UA ranged from 8 to 10 mol%. Similarity, Ayestarán *et al.* (2004) described Glc as the main sugar residue (~45%) followed by Gal (~20%) of the insoluble fraction of two red wines. $DW_{Et}30$ contained 41 mol% of Glc and the relative amounts of Ara, Man, Gal and UA ranged from 13 to 16 mol%. $DW_{Et}50$ accounted for the highest percentage of

sugars (39%), and was composed by Man (38 mol%), Gal (19 mol%), UA (15 mol%), Ara (13 mol%), Glc (11 mol%), and Rha (4 mol%).

Table 3.9: Amount (mg/g) and sugar composition (mol%) of fractions obtained by precipitation in ethanol solutions.

		Fractions				
		$DW_{Et}0$	$DW_{Et}30$	$DW_{Et}50$	$DW_{Et}80$	$DW_{Et}sn$
	Total sugar (mg/g)	198 ± 20	103±8	391±20	127±4	76±2
Sugar composition (mol%)	Rha	3 ± 0	2 ± 0	4 ± 1	3 ± 0	3 ± 0
	Ara	8 ± 0	13 ± 3	13 ± 1	8 ± 1	8 ± 1
	Man	8 ± 2	16 ± 1	38 ± 3	8 ± 1	6 ± 0
	Gal	10 ± 1	14 ± 3	19 ± 3	11± 2	7 ± 1
	Glc	62 ± 3	41± 5	11 ± 2	46 ± 5	68 ± 3
	UA	9 ± 3	13 ± 2	15 ± 2	24 ± 3	8 ± 2

Mean three replicates ± standard deviation.

Man, arising from mannoproteins, was preferencially (83%) recovered in $DW_{Et}50$, in accordance to the results obtained by Coimbra *et al.* (2002; 2005) for precipitation in ethanol of wine polysaccharides. The $DW_{Et}SN$ contained 68 mol% of Glc, and represented 90 mg/L, half of total Glc quantified in DW2. The ratio (Ara + Gal) / UA was lower in $DW_{Et}50$ (0.8) when compared with the other fractions (1.8 – 2.2), which was indicative of the presence of pectic polysaccharides in this fraction. The higher relative amounts of Glc was observed in $DW_{Et}0$ and $DW_{Et}SN$ in accordance to the high concentration of anthocyanins in those fractions.

The sugar composition of the DW polymeric fractions confirms the presence of mannoproteins, arabinogalactanas, and rhamnogalacturonans, in addition with glucose. The total amount of Glc (183 mg/L) quantified in the RW2 was similar to the 136, 280 and 315 mg/L of Glc reported by Guadalupe *et al.* (2012) in red wines. The high quantities of glucose were attributed to the presence of grape and yeast polysaccharides but also to phenolic compounds. However, these values can be

considered high when compared with those usually reported for polysaccharides of red wines (Boulet *et al.*, 2007; Doco *et al.*, 2007; Guadalupe *et al.*, 2007; Vernhet *et al.*, 1999; Vidal *et al*., 2003). The highest contribution of supernant fraction to the total of Glc (~50%) associated with the richness of this fraction in anthocyanins allowed to conclude that the contribution of anthocyanins to the total Glc present in wine is high.

The monomeric anthocyanins were mainly (83%) recovered in the ethanol soluble fraction ($DW_{Et}sn$). The insoluble residue ($DW_{Et}0$) contained 7% and the other fractions contained only 3% of monomeric anthocyanins. The relative amount of each anthocyanin present in the five fractions is shown in Table 3.10. The sum of anthocyanins obtained was 270.4 mg/L, representing 72% of those initially present in the DW2.

Table 3.10: Monomeric anthocyanins composition (relative amount) of fractions obtained by precipitation in ethanol solutions.

	Fractions				
Anthocyanin	$DW_{Et}0$	$DW_{Et}30$	$DW_{Et}50$	$DW_{Et}80$	$DW_{Et}sn$
Df3Glc	3.8±0.3 ^{a}	1.8±0.2 ^{bc}	2.3±0.6 ^{b}	1.9±0.6 ^{ab}	1.0±0.2 ^{c}
Pt3Glc	5.9±0.1 ^{a}	5.2±0.4^{ab}	4.7±0.5 ^{bc}	5.6±0.4 ^{ab}	4.0±0.2 ^{c}
Pn3Glc	4.6±0.5 ^{a}	4.7±0.4 ^{a}	4.8±0.3 ^{a}	4.5±0.3 ^{a}	4.9±0.2 ^{a}
Mv3Glc	59.3±0.9 ^{a}	60.9±2.5 ^{a}	61.4±2.8 ^{a}	59.4±1.9 ^{a}	51.0±1.2 ^{b}
Df3AcGlc	1.3±0.1 ^{a}	1.7±0.2^{a}	1.6±0.1^{a}	2.2±0.3 ^{b}	1.7±0.1 ^{a}
Pt3AcGlc	1.1±0.0 ^{a}	0.9±0.1 ^{a}	1.1±0.1 ^{a}	1.1±0.1 ^{a}	0.8±0.1 ^{a}
Pn3AcGlc	1.7±0.1 ^{a}	1.9±0.2 ^{a}	1.7±0.2 ^{a}	1.8±0.1 ^{a}	1.9±0.1 ^{a}
Mv3Acglc	14.1±1.3 ^{a}	14.6±0.8^{a}	15.2±0.6 ^{a}	14.4±0.5 ^{a}	20.7±1.0 ^{b}
Pn3CmGlc	1.3±0.3 ^{a}	1.3±0.1 ^{a}	1.2±0.0 ^{a}	1.3±0.2 ^{a}	2.4±0.2 ^{b}
Mv3CmGlc	6.8±0.3 ^{a}	7.0±0.1 ^{a}	6.0± 1.0^{a}	6.9±0.3 ^{a}	11.6±0.3 ^{b}

Mean of three replicates. For each row, the means value followed by the same letter are not significantly different.

The relative composition of monomeric anthocyanins in $DW_{Et}0$, $DW_{Et}30$, $DW_{Et}50$, and $DW_{Et}80$ fractions was similar. Mv3Glc was the main anthocyanin

present (59.3-61.4%), followed by Mv3Acglc (14.1-15.2%) and Mv3CmGlc (6.0-7.0%). Df3Glc, Pt3Glc and Pn3Glc ranged between 1.8 and 5.9%. Df3AcGlc, Pt3AcGlc, Pn3AcGlc and Pn3CmGlc were present in lower relative amounts, varying between 0.9 and 2.2%. The DWEtsn was mainly composed by 51.0% of MvGlc, 20.7% of Mv3Acglc, and 11.6% of Mv3CmGlc.

For all samples, anthocyanin 3-glucosides (61-74%) were present in higher amounts, followed by anthocyanin 3-(6-acetyl)glucosides (18-25%) and anthocyanins 3-coumaroylglucoside (7-14%). However, significant differences were obtained between the composition of the precipitates and the supernatant as the DWEtsn contained a statistically lower relative amount of anthocyanin 3-glcucosides and higher relative amounts of anthocyanin 3-acylglucosides than the precipitates (Figure 3.11). This distribution may be due to the higher hydrophobicity of anthocyanins 3-acylglucosides that are more soluble in ethanol, when compared with anthocyanin 3-glucosides.

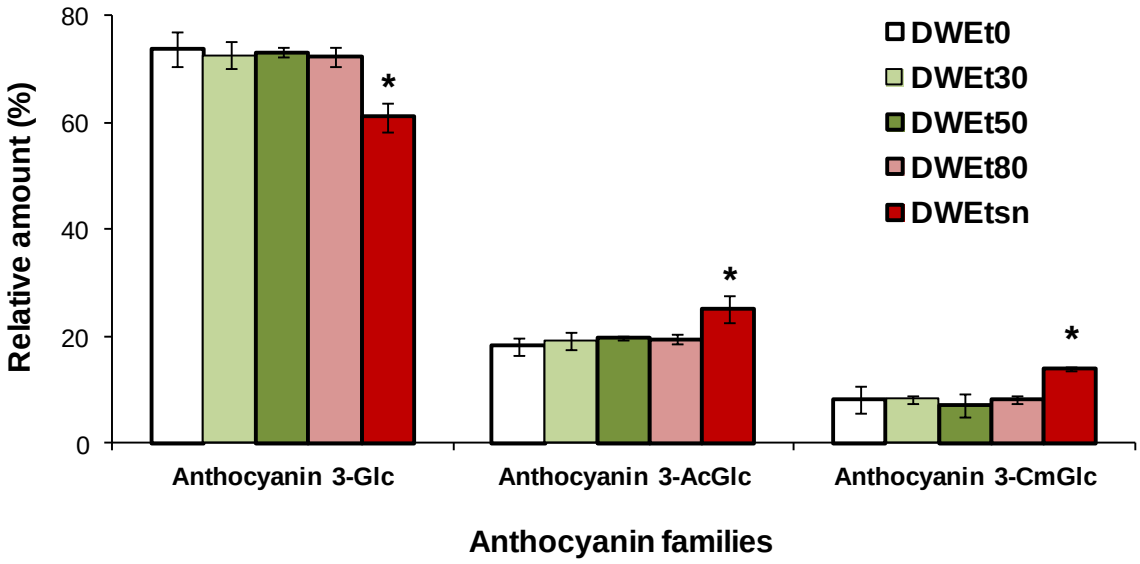


Figure 3.11: Relative amount of glucosilated (anthocyanins 3-Glc), acetylglucosilated (anthocyanins 3-AcGlc) and coumaroylglucosilated anthocyanins (anthocyanins 3-CmGlc). * statistically different according to Tukey test ($p < 0.05$).

3.1.4.4 Fractionation of DW2 by solid phase chromatographies

a) Separation of polysaccharide-rich material

The combination of anion-exchange with size-exclusion and affinity chromatographies to obtain different classes of wine polysaccharides has been suggested previously (Pellerin *et al.,* 1995).

The DW2 was loaded into the C18 sep-pak cartridge, at wine pH, in order to separate the hydrophylic from the hydrophobic fraction. The unbound material was eluted with water, recovered, concentrated, dialyzed and freeze dried allowing to obtain the hydrophylic polymeric material, mainly composed by polisaccharides (WPS). After concentration, this fraction still presented a slighty pink color, indicative of the presence of anthocyanins and derived pigments associated to the material. The separation of polysaccharides was carried out by affinity concanavalin A, and anion-exchange chromathographies resulting in one mannoprotein-rich fraction (MP) retained in the concanavalina A, and three arabinogalactan rich fractions, namely a neutral fraction (AG0) eluted with water from anion-exchange column, and two acidic fractions obtained in a stepwise elution (AG1 and AG2), with 50 and 500 mM of phosphate buffer. All fractions were dialyzed to remove the salts, freeze-dried, and recovered as solid material.

The WPS fraction contained the high molecular weight material, which accounted for 464 mg/L of solid material. This material contained 734 mg/g of sugars. Man (32 mol%), Gal (23 mol%), Glc (17 mol%), Ara (13 mol%) and UA (11 mol%) were present in higher amounts. In addition to polysaccharides, WPS contained 166 mg/g of phenolic compounds, expressed as GAE, and 138 mg/g of proteins using the bicinchoninic acid (BCA) method and bovine serum albumin (BSA) as standard. The WPS material was only composed by 17% of phenolic compounds, comparing with the 43% of phenolics present in WPM2, this suggests that a major part of the phenolic compounds of WPM2 was retained in the C18 sep-pak.

Table 3.11: Yield and composition of fractions isolated from DW2 by SPE.

Fraction	Yield	Total Phenols	Protein	Total sugar	Carbohydrate sugars (mol %)					
	(mg/L)	(mg/g)	(mg/g)	(mg/g)	Rha	Ara	Man	Gal	Glc	UA
WPS	464±27	166±8	138±9	734±45	4±1	13±2	32±2	23±0	17±1	11±1
MP	153	--	94±12	622±17	0±0	1±0	93±1	2±0	1±0	2±0
AG0	99	--	49±2	570±39	4±0	35±1	4±2	31±2	14±1	10±2
AG1	107	--	70±5	602±28	6±1	23±1	4±1	50±4	7±2	9±2
AG2	23	--	65±2	450±40	4±0	32±3	3±0	35±3	3±0	20±3
WPC	3332±111	605±24	145±3	74±7	2±0	8±1	5±1	22±2	78±4	6±1
PA-E	443±21	152±15	6±0	--	--	--	--	--	--	--
PR-E	870±49	556±8	105±10	41±4	1±0	7±2	25±3	7±2	37±2	20±3
APP-E	2187±123	631±17	215±9	62±5	tr	11±1	tr	tr	84±4	4±1

Mean of three replicates ± standard deviation. WPS- wine polysaccharides fraction, MP mannoproteins fraction, AG0- neutral arabinagalactan fraction, AG1- acidic arabinogalactan fraction eluted with 50 mM of phosphate buffer, AG2- acidic arabinogalactan fraction eluted with 500 mM of phosphate buffer, WFC- wine phenolic compounds extract, PA-E phenolic acids extract, PR-E procyanidins-rich extract , APP-E anthocyanins-rich fraction. tr-traces.

The material retained by the concanavalin A medium (MP fraction) accounted for 153 mg/L of wine, representing 33% of the WPS material. It was rich in sugars (62%), mainly Man (93 mol%), which represented more than 80% of Man present in WPS. The amount of mannoproteins, estimated by the amount of Man and protein, was 102 mg/L of wine, in which protein represented 14%. This value was similar to the ~110 mg/L described by Doco *et al.* (2003) but lower than those reported by Ayestarán *et al.* (2004) (141-171 mg/L) for red wines. The percentage of protein was higher than the 1.4-9.3% reported in literature (Boulet *et al.*, 2007; Vernhet *et al.*, 1999; Vidal *et al.*, 2003) for different fractions of mannoproteins isolated from red wines.

Concerning arabinogalactans, a neutral (unretained) fraction (AG0) eluted with water and two acidic fractions (AG1 and AG2) were obtained. Together, they represented 49% of WPS material. The most abundant were AG1 (107 mg/L of wine) and AG0 (99 mg/L), contrasting with AG2 presenting only 23 mg/L. The fractions AG0, AG1, and AG2 were composed of 570 mg/g, 602 mg/g and 450 mg/g of polysaccharides respectively. The arabinose and galactose residues were almost totally recovered in these fractions. The estimated values for the arabinogalactans were lower (108 mg/L) than that obtained by Doco *et al.* (2003) (~200 mg/L) and Ayestarán *et al.* (2004) (308-445 mg/L). The protein content

ranged from 4.9 to 7.0% of dry matter, which was slightly higher than 2.4 to 3.7 previously described (Pellerin *et al.*, 1995; Pellerin *et al.*, 1993; Vernhet *et al.*, 1999). The AG0 was mainly composed of Ara (35 mol%), Gal (31 mol%), Glc (14 mol%) and UA (10 mol%). Lower relative amounts of UA for the neutral AG fraction were described, 2.7 mol% (Pellerin *et al*., 1995), and 4 mol% (Coelho *et al.*, 2011). Glucuronic acid has been described to be the prominent uronic acid of all AGP whereas galacturonic acid was only present in AG fractions with Rha content was higher than 7 mol% (Pellerin *et al.,* 1995; Vidal *et al.,* 2003), derived from pectic polysaccharides. Thus, as the molar percentage of Rha was 4 and 6 %, it can be inferred the possible presence of GalA. The retention in the anion-exchange column is essentially based on the UA groups of arabinogalactanas of the pectic polysaccharides. Thus the high value of UA in the AG0 fraction could signify that UA were unavailable to bind to the column, possibly due to steric hindrance. The strength of the ionic interaction of each polysaccharide towards the anionic resin groups was described to be more related to the density of charges and distribution of the uronosyl groups than to the global net charge (Vidal *et al.,* 1996). Also, the retention on the anion-exchange resin may be related to the sizes and composition of neutral sugars of AGP. The results obtained by Vidal *et al.* (2003) suggested that a lower ratio of Aral/Gal could also explain a lower retention of AGPs. However, an opposite behavior was verified to occur with AGP0 and AGP1, which presented molar ratios of 1.1 and 0.5, respectively.

The AG1 was mainly composed by Gal (50 mol%), Ara (23 mol %) and UA (9 mol%), a sugar composition characteristic of arabinogalactanas and rhamnogalacturonans (Coimbra *et al*., 2002), as also observed by Coelho *et al.* (2011). Otherwise, Pellerin *et al.* (1993), obtained an arabinogalactan fraction (also eluted with 50 mM phosphate buffer) with a composition richer in arabinose, containing 50 mol% of Gal, 46 mol% of Ara and 5 mol% of UA. The AG2 had a higher UA content (21 mol%) than AG1, in accordance with its stronger binding capacity. Also, Pellerin *et al.* (1995), obtained five different fractions of arabinogalactan proteins based on the strength of their binding to the column, due their increasing UA content. Table 3.11 shows that Gal and Ara represented 69 mol% of the sugars present in AG2 fraction. Among the AG fractions, AG1

contained the highest percentage of proteins (7%) and of polysaccharides (60%). The sugar composition of fractions described above showed that this fractionation allowed to separate the mannoproteins components from arabinogalactans arising from DW. When the WPS fraction was loaded in concanavalin A medium, it was possible to observe a red stain on the top of the column, correspondent to the phenolic compounds. This material remained on the column and was not recovered.

Only 48% of the UA present WPS was recovered in MP, AG0, AG1 and AG2. This showed that an important part of the pectic polysaccharides was state in the column. Also, a part was co-eluted with the AG rich fractions.

Glycosidic linkage analysis of MP fraction showed that Man residues represented 94 mol%, which was similar to the previous results of sugar analysis by GC-FID. Terminally linked Man*p* (29 mol %), 2,6-Man*p* (27 mol %), 2-Man*p* (27 mol %), and 3-Man*p* (11 mol %) were the most abundant linkages, which are linkages usually described for mannoproteins from yeast origin (Vidal *et al*., 2003). These results were in good accordance with those obtained by Coelho *et al*. (2011).

b) Separation of phenolic-rich material

The wine phenolic compounds (WPC) were eluted from C18 column with acidic methanol. The methanol was removed by rotary-evaporation, with successive water additions to assure methanol remotion. The pH was adjusted to 7 and loaded into the C18 sep-pak cartridge, resulting in three different fractions, eluted with phosphate buffer pH 7, ethyl acetate, and acidic methanol. The organic solvents used to obtain the fractions were removed and then freeze-dried to be recovered as solid material. The resulting extracts were named phenolic acids extract (PA-E), procyanidin-rich extract (PR-E) and anthocyanin-rich extract (APP-E), respectively. All fractions were characterized concerning their abundance in the wines of origin, sugar composition, and content in protein and phenolic compounds (Table 3.11).

Regarding the phenolic fractions, WFC accounted for 3.3 g of dry material per liter of wine, which was higher than the 2.9 g/L described by Auger *et al*. (2010). The WFC was composed of 605 mg GAE/g of phenolic compounds, 145 mg/g of proteins and 74 mg/g of polysaccharides. Glc accounted for 78 mol% of total sugars, probably most of then resultant from phenolic compounds. The largest fraction recovered was APP-E (2187 mg/L, 62%), followed by PR-E (879 mg/L, 25%) and PA-E (443 mg/L, 13%). The sum of phenolic compounds recovered accounted for 1931 mg/L, 96% of WFC. The phenolic compounds present in APP-E extract represented 71% of WFC, contrasting with 3% of PA-E.

The extracts WFC, PR-E and APP-E contained 14.5%, 10.5% and 21.5% of protein. However, it is possible that the values achieved may be overestimated, because when phenolic compounds are present in high amounts, they may interfere with the color used to determine the protein. The PR-E was composed of 4.1% of polysaccharides. Glc (37 mol%), Man (25 mol%) and UA (20 mol%) were the sugars present in highest relative amounts. In addition, it contained 7 mol% of Ara and Gal. The fraction of phenolic compounds retained by the C18 stationary phase and eluted with acid methanol (APP-E) was shown to contain only 62 mg/g of sugars. This fraction was composed of 84 mol% of Glc, 11 mol% of Ara and 4 mol% of UA, and only traces of Rha, Man and Gal were found. It is possible that the retention of these small amounts of sugars in the C18 column may be due to the covalent linkage of the carbohydrates to the hydrophobic material, namely, phenolic compounds and/or protein. The sugar composition of these hydrophobic fractions showed different relative amounts of sugars.

c) Determination of EC_{50} of wine extracts

The antioxidant activity of the wine extracts were evaluated by the concentration of each extract necessary to decrease the initial $ABTS^{+\bullet}$ radical concentration by 50% (EC_{50}). For each extract, five concentrations were used and their percentages of inhibition were determined. The EC_{50} parameter was calculated from a linear-dose response obtained by plotting the percentage of

inhibition against the concentration of each extract. Table 3.12 shows the range of concentrations, the percentages of inhibition and the EC_{50} values (expressed on the weight basis of the freeze-dried extracts by mL of solution) for each extract understudy.

Table 3.12: Concentrations (mg/mL), percentages of inhibiton (%) and EC_{50} values for extracts used in biological assays.

Extract	Concentration (mg/mL)	Inhibition (%)	EC_{50} (mg/mL)
WPM2	0.064 - 0.384	12 - 82	0.24
WPS	0.072 - 0.434	12 - 77	0.27
WFC	0.081 - 0.407	21 - 80	0.22
PA-E	0.565 – 3390	15 - 57	3.07
PR-E	0.053- 0.263	23 - 83	0.15
APP-E	0.048 - 0.191	21 - 72	0.12

WPM2- wine polymeric material, WPS- wine polysaccharides fraction, WPC- wine phenolic compounds fraction, PA-E phenolic acids extract, PR-E procyanidins-rich extract, APP-E anthocyanin-rich extract.

A lower EC_{50} value represents a higher antioxidant activity. The APP-E exhibited the lowest EC_{50} (0.12 mg/mL), followed by the PR-E (0.15 mg/mL) in accordance with their high amount of phenolic compounds (63 and 56%, respectively). The EC_{50} of WFC and WPM2 was 0.22 and 0.24 mg/mL. Taking in consideration the amount of phenolic compounds of WPS, a lower value of EC_{50} (0.27 mg/mL) was obtained, inducing the possibility of the presence of phenolic compounds with relative higher antioxidant activity. The PA-E was the extract with higher amount of phenolic compounds necessary to to decrease in 50% the initial amount of $ABTS^{+\bullet}$. In general the highest antioxidant activity were obtained for the samples with highest phenolic content.

3.1.5 - Final remarks

The results obtained in the characterization of wines, dealcoholized wines, and wine fractions, allowed to conclude that:

- The two wines and the correspondent dealcoholized wines contained comparable amounts of total phenolic compounds, tannins and anthocyanins showing that the distillation process used to obtain the dealcoholized wines did not promote relevant loss of phenolic compounds. Moreover, similar amounts of monomeric anthocyanins were observed in RW2 and DW2.

- The antioxidant activity was not affected by the distillation process. The anthocyanins-rich fraction achieved the highest contribution to wine antioxidant activity.

- From above, it is possible to infer that the dealcoholization process carried out, is a fast and simple methodology that preserved the main characteristics of wines. Also, the compounds that result from the distillation process of distillaries maintain the properties, allowing to define methodologies for their extraction and valorization.

- The WPM1 and WPM2 were composed by polysaccharides and proteins associated with phenolic compounds and accounted for 1.1 and 1.3 mg/L of solid material. The WPM were composed of 31 and 36% of polysaccharides, 32 and 43% of phenolic compounds, and 9.9 and 12.7% of amino acids. The sugar analysis showed the presence of mannoproteins, arabinogalactanas, and rhamnogalacturonans.

- When WPM1 was extracted with methanol, 67% of the phenolic compounds and 27% of polysaccharides were removed. The methanol soluble fraction revealed the presence of carbohydrate residues, mainly Glc, but also other residues arising from carbohydrates. The retention of both, carbohydrates and phenolic compounds after further purification step on C18 sep-pak allowed to conclude that some part of them were tightly bound.

- The analysis by HPLC showed that procyanidin B1, procyanidin B2 and cathechin were the procyanidins present in higher amounts in DW1.

- DW2 was composed of 28.6 mg GAE/L of benzoic acids and 46.9 mg CAE/L of hydroxycinnamic acids. Gallic acid and *p*-hydroxybenzoic acid were the hydroxybenzoic acids present in highest amount, while regarding hydroxycinnamic acids, *trans*-caftaric, chlorogenic, caffeic, and *p*-coumaric acids were in highest concentration. The amount of catechin and epicatechin, expressed as catechin equivalents, was 37.8 mg/L. Moreover, this wine contained 379.1 mg Mv3Glc/L of monomeric anthocyanins, of which Mv3Glc represented 60%.

- The material resulting from the selective precipitation of DW2 with ethanol solutions was preferentially recovered in the DW_{Et}sn (54%) and DW_{Et}50 (18%) fractions. Fourty seven percent of the total amount of carbohydrates (466 mg/L) was recovered in fraction DW_{Et}50 and 83% of the monomeric anthocyanins (270.4 mg/L) were obtained in the DWEtsn. The results showed a high concentration of Glc in wine (183 mg/L), attributed mainly to anthocyanins.

- From the elution of DW2 into C18 sep-pak cartridge resulted the polysaccharide-rich fraction (WPS) and the phenolic-rich fraction (WPC). The WPS accounted for 464 mg/L and was composed by 73% of polysaccharides and 17% of phenolic compounds. The WPC accounted for 3.3 g/L and was composed by 60% of phenolic compounds and 7% of carbohydrates.

- Four fractions with polysaccharides ranging between 45 and 62% were prepared from WPS. Mannoproteins were obtained in the first fraction, while arabinogalactans and rhamnogalacturonans were recovered in the latter fractions.

- The separation of WPC resulted in three fractions, where the anthocyanin-rich fraction was the most abundant (62%). This fraction was composed by 63% of phenolic compounds and 6% of carbohydrates, of which Glc accounted for 84%.

- The results showed that non-alcoholic fraction of wine could be an important source of phenolic compounds. Also, the results showed that they are associated with wine polymeric material with different intensities. Thus it is important to evaluate the interactions between wine polymeric material and phenolic compounds. For that, the fractions here obtained and characterized will be used.

3.2 Study of Interactions Between Polymeric Material and Phenolic Compounds

3.2.1 Overview

The results described in the previous chapter showed that fractions of dealcoholized wine phenolic compounds are strongly associated with the polymeric material. In order to evaluate possible selectivity of these interactions, a set of experiments were performed. The diffusion through a dialysis membrane of nine anthocyanins, comprising glucosylated, acetylglucosylated, and coumaroylglucosylated structures, in presence and in absence of the wine polymeric material was followed and the retention capacity for the different anthocyanins was determined. Also, the diffusing anthocyanins through a dialysis membrane were studied in presence of mannoprotein- and arabinogalactan-rich fractions.

To determine the velocity, the kinetic constant and the activation energy of phenolic release from the wine polymeric material, the dialysis of dealcoholized wine 1 (DW1), polymeric material of wine 1 (WPM1) and methanol insoluble polymeric material (PMi), using four different concentrations of DW1, WPM1, and five temperatures (5, 25, 30, 35 and 40 ºC) were performed until a steady state was reached. The amount of phenolic compounds diffused through the membrane dialysis was quantified.

3.2.2 Study of the retention capacity of anthocyanins by wine polymeric material

3.2.2.1 Study of the diffusion rates of phenolic compounds in presence of wine polymeric material

In order to study the retention capacity of monomeric anthocyanins by the wine polymeric material, two dialyses were performed. As a blank experiment, a dialysis was performed without the presence of the WPM2. The effect of the anthocyanin structure on the diffusion rates was evaluated performing a dialysis in presence of wine polymeric material. The pH of the started material was 3.60 and at the end of dialysis the pH of retentate was 3.90. At this pH range the anthocyanins are in the flavylium cation form.

3.2.2.2 Total phenolic and monomeric anthocyanins composition of dialysate solutions

Table 3.13 shows the amount of total phenolic compounds released in presence of the WPM2 into the different dialysate solutions. From those values, it was possible to determine the percentage of recovery in the different dialysate solutions.

The amount of phenolic compounds present in the first dialysate solution ($W01_{WPM}$) represented 45% of phenolic compounds initially present in the dialysis bag. The $W02_{WPM}$, $W03_{WPM}$ and $W04_{WPM}$ contained 19, 11 and 10% of initial phenolic compounds, respectively, while the following dialysis waters contained less than 5% each.

The concentration of each one of the 9 anthocyanins released in presence of the WPM and present in each dialysate solution and in the retentate, expressed as mg/L of wine in Mv3Glc equivalents, is also shown in Table 3.13. In general, the total amount of antocyanins recovered in each dialysate solution showed a

continuous decrease along time. It is possible to see that the major part (73%) of anthocyanins was obtained in the first 5 water exchanges. The $W01_{WPM}$ contained 98.7 mg/L of anthocyanins, which represented 27% of total anthocyanins present inside the dialysis bag. The amount of anthocyanins in $W02_{WPM}$ and $W03_{WPM}$ represented 15 and 12% of the initial anthocyanins; 10% of the amount of anthocyanins was recovered in the $W04_{WPM}$ and $W05_{WPM}$.

The $W01_{WPM}$ was mainly composed by Mv3Glc (73%), Mv3AcGlc (8%), Pt3Glc (7%), Pn3Glc (5%) and Df3Glc (4%). The $W02_{WPM}$ contained 68% of Mv3Glc, 14% of Mv3AcGlc, 6% of Pt3Glc, 5% of Pn3Glc, and 3% of Df3Glc. The main anthocyanins present in the first two dialysate solutions were the main anthocyanins present in wine. However, as the changes in dialysis waters proceed, the relative amount of Mv3Glc, tend to decrease, reaching a minimum value of 58% in $W07_{WPM}$ whereas the acetylated form of Mv3Glc (Mv3AcGlc) tend to increase, reaching a relative content of 19% in $W08_{WPM}$. If the driving force for the separation process was mainly the concentration gradient, the amount of anthocyanins released to the dialysate solutions should be proportional to the concentration inside the dialysis bag. As this was not observed, the data concerning the diffusion behavior of the individual anthocyanins along the successive dialysis steps was analyzed in detail and was related with their structural features.

Table 3.13: Amount of monomeric anthocyanin (expressed as mg/L of equivalents of Mv3Glc) and the total phenolic compounds (expressed as mg/L of equivalents of gallic acid) present in the dialysate solutions in presence of wine polymeric material (W01WPM to W11WPM)

Fraction	Df3Glc	Pt3Glc	Pn3Glc	Mv3Glc	Df3AcGlc	Pt3AcGlc	Pn3AcGlc	Mv3AcGlc	Mv3CmGlc	Total monomerix anthocyanins	Total Phenolic compounds
Dialysis in presence of wine polymeric material											
W01$_{WPM}$	4.40±0.37^{a}	6.97±0.46^{a}	5.27±0.19^{a}	72.00±2.76^{a}	1.02±0.02^{a}	0.46±0.04^{ab}	0.42±0.02^{ab}	7.61±0.37^{a}	0.48±0.03^{a}	98.71±13.98^{a}	1361.9±26.3^{a}
W02$_{WPM}$	1.80±0.23^{b}	3.47±0.18^{b}	2.71±0.13^{b}	38.71±1.27^{b}	0.79±0.02^{ab}	0.52±0.05^{a}	0.45±0.04^{a}	7.83±0.32^{a}	0.56±0.08^{a}	57.09±4.50^{b}	575.5±29.2^{b}
W03$_{WPM}$	1.27±0.01^{c}	2.43±0.05^{c}	1.86±0.05^{c}	28.19±1.17^{c}	0.90±0.01^{a}	0.48±0.04^{ab}	0.53±0.01^{b}	7.26±0.19^{a}	3.70±0.53^{b}	46.02±2.26^{c}	324.7±5.1^{c}
W04$_{WPM}$	0.87±0.02^{cd}	1.37±0.13^{de}	1.75±0.03^{cd}	22.03±1.19^{d}	1.26±0.09^{c}	0.34±0.06^{ab}	0.53±0.05^{b}	4.65±0.12^{b}	3.02±0.27^{b}	35.64±0.94^{c}	291.0±16.8^{c}
W05$_{WPM}$	0.96±0.12^{cd}	1.44±0.25^{de}	2.00±0.21^{c}	22.97±3.43^{cd}	1.46±0.09^{c}	0.33±0.13^{ab}	0.54±0.07^{b}	6.06±0.72^{ab}	0.64±0.03^{a}	36.38±6.37^{c}	124.2±7.7^{d}
W06$_{WPM}$	0.47±0.03^{de}	1.04±0.21^{ef}	1.36±0.10^{d}	13.50±0.82^{e}	1.35±0.18^{c}	0.12±0.06^{bc}	0.28±0.08^{c}	3.28±0.06^{c}	0.64±0.03^{a}	21.44±4.92^{d}	114.5±5.1^{d}
W07$_{WPM}$	0.06±0.00^{f}	0.54±0.05^{ef}	0.55±0.05^{ef}	5.40±0.36^{fg}	0.83±0.09^{ab}	0.03±0.00^{c}	0.05±0.01^{d}	1.44±0.08^{d}	0.39±0.05^{ac}	9.32±1.18^{e}	60.9±2.4^{de}
W08$_{WPM}$	0.28±0.01^{f}	0.66±0.00^{def}	0.75±0.03^{e}	7.66±0.17^{f}	0.58±0.10^{bd}	0.07±0.02^{c}	0.05±0.02^{c}	2.35±0.53^{cd}	0.32±0.03^{c}	12.61±0.75^{de}	71.5±3.0^{d}
W09$_{WPM}$	0.35±0.05^{ef}	0.64±0.01^{ef}	0.48±0.04^{ef}	6.45±0.37^{fg}	0.57±0.12^{bd}	0.04±0.01^{c}	0.12±0.04^{c}	1.35±0.11^{de}	0.12±0.02^{d}	10.09±1.33^{de}	44.8±4.4^{e}
W10$_{WPM}$	0.01±0.00^{g}	0.12±0.02^{g}	0.20±0.00^{fg}	1.82±0.06^{gh}	0.35±0.01^{de}	0.03±0.01^{c}	0.01±0.00^{d}	0.36±0.01^{ef}	0.08±0.01^{d}	2.93±3.81^{f}	34.8±0.3^{e}
W11$_{WPM}$	0.04±0.01^{g}	0.09±0.02^{g}	0.12±0.02^{g}	1.19±0.13^{h}	0.24±0.02^{e}	0.03±0.00^{c}	0.04±0.01^{d}	0.27±0.03^{f}	0.06±0.01^{d}	2.07±0.41^{f}	4.8±1.2^{f}
Retentate	0.15±0.00	0.31±0.02	0.10±0.00	1.21±0.05	0.31±0.05	0.10±0.01	0.45±0.01	0.62±0.02	1.14±0.06	4.38±0.37	13.6±2.3
Dialysis in the absence of wine polymeric material											
W1$_{FPC}$	7.23±0.01^{a}	12.61±0.13^{a}	11.09±0.02^{a}	141.41±7.20^{a}	4.25±0.12^{a}	1.72±0.21^{a}	2.33±0.08^{a}	28.22±2.10^{a}	6.78±1.24^{a}	215.64±1.38^{a}	2364.0±91.0^{a}
W2$_{FPC}$	1.41±0.17^{b}	2.46±0.25^{b}	2.14±0.25^{b}	31.61±1.05^{b}	1.08±0.11^{b}	0.39±0.03^{b}	0.56±0.03^{b}	6.49±0.50^{b}	1.47±0.03^{b}	47.62±2.66^{b}	342.1±14.7^{b}
W3$_{FPC}$	0.49±0.06^{c}	0.89±0.13^{c}	0.78±0.11^{c}	11.50±0.71^{c}	0.70±0.10^{b}	0.14±0.02^{bc}	0.24±0.02^{bc}	2.63±0.10^{c}	0.72±0.09^{c}	18.10±1.31^{c}	67.0±3.2^{c}
W4$_{FPC}$	0.27±0.01^{c}	0.54±0.02^{c}	0.50±0.02^{c}	6.61±0.16^{d}	0.40±0.02^{b}	0.08±0.01^{c}	0.13±0.01^{c}	1.45±0.06^{d}	0.23±0.01^{d}	10.20±0.38^{d}	50.5±5.2^{c}
W5$_{FPC}$	0.22±0.01^{c}	0.43±0.03^{c}	0.39±0.02^{c}	5.36±0.37^{d}	0.33±0.03^{b}	0.06±0.01^{c}	0.11±0.01^{c}	1.18±0.07^{d}	0.25±0.01^{d}	8.34±0.65^{d}	53.8±3.1^{c}

Values of each anthocyanin (columns) for each dialysate solution (in presence or in absence of polymeric material) showing the same letter are not significantly different according to the Tukey test ($p \leq 0.05$

3.2.2.3 Effect of anthocyanin structure on diffusion rate

Figure 3.12 shows the chromatogram of the anthocyanins obtained for $W01_{WPM}$ and $W10_{WPM}$. It is possible to observe that the relative abundance of the anthocyanin 3-glucosides (anthocyanin 3-Glc) tend to decrease more than the anthocyanin 3-(acyl)glucosides. The ratio between Mv3Glc and anthocyanins 3-(acyl)glucosides (Table 3.13), exhibited a tendency to decrease as the number of dialyses performed increases. For example, according to Table 3.13, the ratio Mv3Glc/Df3AcGlc was 79 in $W01_{WPM}$ and 6 in $W10_{WPM}$; the ratio Mv3Glc/Mv3AcGlc was 9 in $W01_{WPM}$ and 5 in $W10_{WPM}$; and the ratio Mv3Glc/ Mv3CmGlc was 151 in $W01_{WPM}$ and 24 in $W10_{WPM}$.

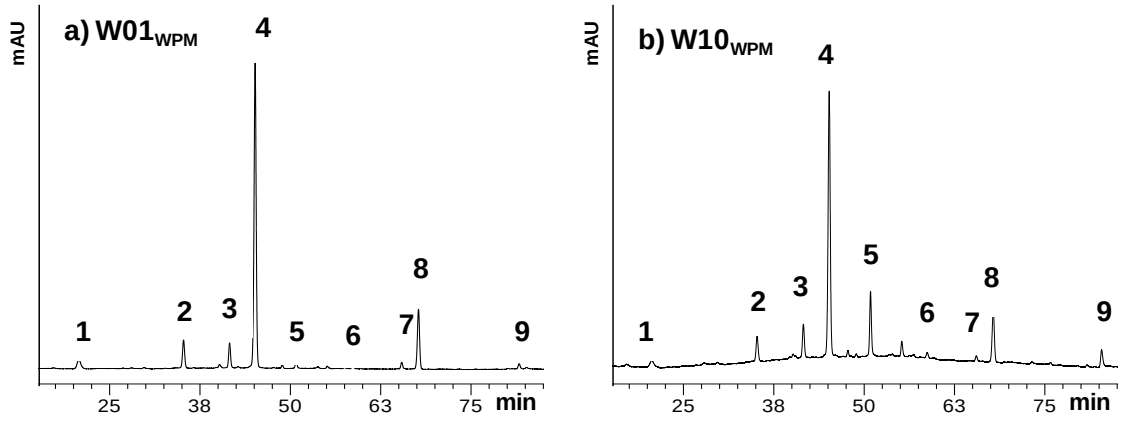


Figure 3.12: HPLC chromatograms of $W01_{PWM}$ and $W10_{PWM}$ fractions recorded at 520 nm. 1. Dp3Glc, 2. Pt3Glc, 3. Pn3Glc, 4. Mv3Glc, 5. Dp3AcGlc, 6. Pt3AcGlc, 7. Pn3AcGlc, 8. Mv3AcGlc, 9. Mv3CmGlc.

Figure 3.13 shows the recovery of each anthocyanin in the different dialysate solutions. The behaviour for anthocyanin 3-Glc that is different from that of the anthocyanin 3-(acyl)glucosides. For instance, 31-36% of the anthocyanin 3-Glc were recovered in $W01_{WPM}$ Figure 3.13a, while for anthocyanin 3-(6-acetyl)glucosides (anthocyanins 3-AcGlc) the values ranged from 9 to 14% (Figure 3.13b).

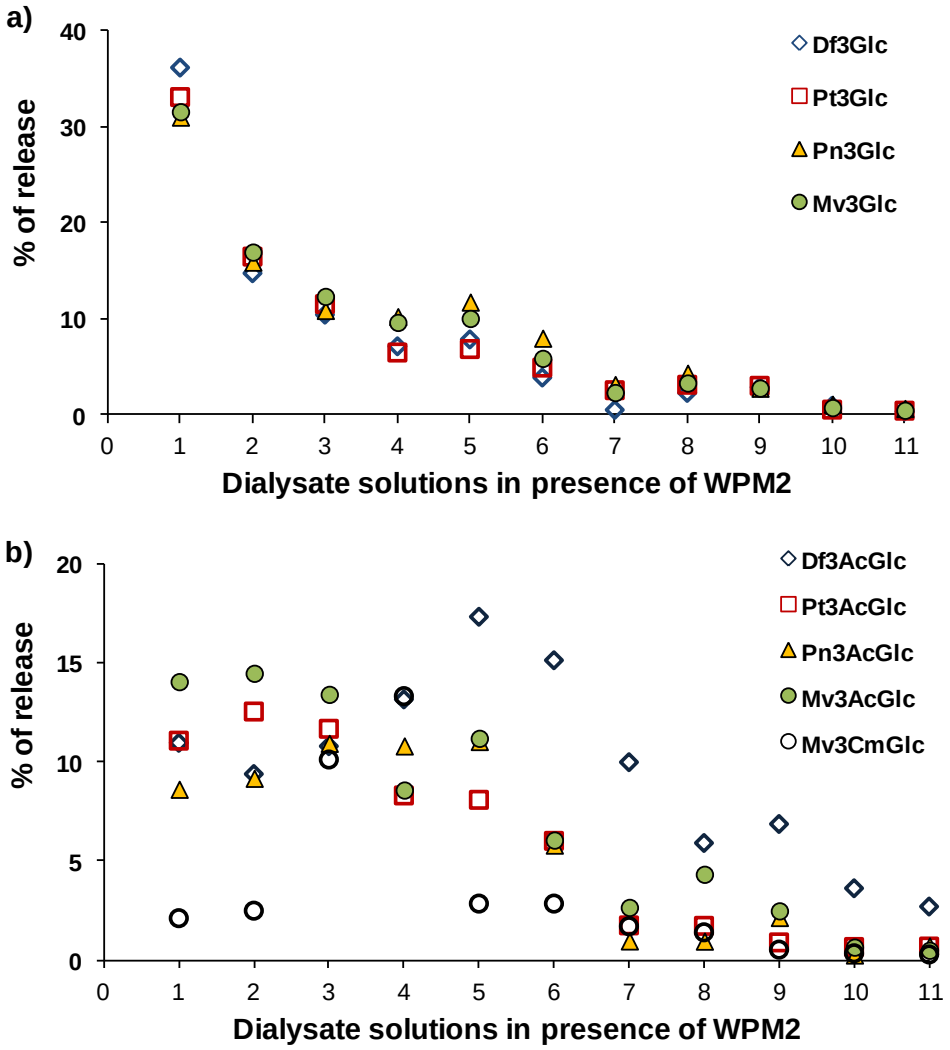


Figure 3.13: Percentage of release for each anthocyanins into the different dialysate solutions in the presence of wine polymeric material ($W01_{WPM}$ to $W11_{WPM}$). a) anthocyanin 3-glucosides b) anthocyanin 3-(acyl)glucosides.

The recovery on the $W02_{WPM}$ of anthocyanins 3-Glc decrease to 15-17% while for the anthocyanins 3-AcGlc the values were similar to those of $W01_{WPM}$. In the $W03_{WPM}$, the percentage of recovery was similar for both types of anthocyanins (10-12%), while for $W04_{WPM}$ the percentage of anthocyanins 3-Glc (6-10%) was lower than the acetylated anthocyanins (8 to 15%). At the 11th dialysis, the retentate contained 4.4 mg/L of anthocyanins. Mv3Glc (28%) was the main anthocyanin present inside the dialysis bag, however in lower relative

amount than that observed in all the dialysis waters analysed (Table 3.13). Three acylated anthocyanins, Mv3CmGlc (26%), Mv3AcGlc (24%) and Pn3AcGlc (10%) were also present in relative high amounts when compared with the diffused material. These results suggest that the acylation of glucose residue of anthocyanins could difficult their release through the dialysis membrane.

Table 3.14 shows the correlation coefficient of the regression curve that best represented the diffusion for each anthocyanin from the WPM2. All anthocyanins 3-Glc (Mv3Glc, Df3Glc, Pt3Glc, and Pn3Glc) followed a logarithmic relationship, exhibiting a very similar behaviour and correlation coefficients that ranged from 0.8616 for Df3Glc to 0.9507 for Mv3Glc. On the contrary, for their acetylated derivatives (Mv3AcGlc, Df3AcGlc, Pt3AcGlc, and Pn3AcGlc), linear decays have been observed (Table 3.14).

Table 3.14: Equation and correlation coefficient of percentage of release of anthocyanins in the dialysate solution and retention coefficient of each anthocyanin by WPM2.

	WPM dialysis		**FPC dialysis**		**Retention coefficient (RC)**
	Equation (Y=)	**Correlation coefficient (r^2)**	**Equation (Y=)**	**Correlation coefficient (r^2)**	
Df3Glc	-13.0ln(x) + 28.6	0.8616	-44.7ln(x) + 62.8	0.8319	**0.52**
Pt3Glc	-12.3ln(x) + 27.6	0.9165	-44.2ln(x) + 62.3	0.8293	**0.56**
Pn3Glc	-11.1ln(x) + 26.9	0.9298	-44.0ln(x) + 62.1	0.8269	**0.58**
Mv3Glc	-21.1ln(x) + 27.9	0.9507	-42.7ln(x) + 60. 9	0.8470	**0.56**
Df3AcGlc	-0.88x + 14.9	0.4282	-35.3ln(x) + 53.8	0.8539	**0.83**
Pt3AcGlc	-1.37x + 13.9	0.9091	-43.0ln(x) + 61.2	0.8535	**0.85**
Pn3AcGlc	-1.192x + 12.7	0.7314	-40.9ln(x) + 59.2	0.8553	**0.88**
Mv3AcGlc	-1.56x + 16.5	0.9166	41.7ln(x) + 59.9	0.8508	**0.80**
Mv3CmGlc	-0.43x + 6.4	0.1507	41.7ln(x) + 59.9	0.8508	**0.97**

3.2.2.4 Dialysis in absence of wine polymeric material

To disclose if the different behaviour of anthocyanins 3-Glc and anthocyanins 3-AcGlc could be attributed to the retention of phenolic compounds by the WPM2 or to diffusion factors that could selectively retain the passage of the different anthocyanins throughout the dialysis membrane, a dialysis was performed without the presence of the WPM2. In this experiment, the phenolic

compounds released from the wine were mixed, concentrated and the solution obtained was named as free phenolic compounds (FPC) solution. The FPC dialysed in the absence of the WPM2, using the same conditions as previously described, allowed to obtain five dialysate solutions ($W1_{FPC}$ to $W5_{FPC}$), comprising 90% of the amount of anthocyanins initially present in the dialysis bag. The $W1_{FPC}$ solution accounted for 215.6 mg/L (Table 3.13) of anthocyanins which represented 65% of the total anthocyanins, while $W2_{FPC}$ accounted for 14%. Five percent of the initial total anthocyanins were recovered in the third dialysate while 3 % was recovered in the fourth and fifth solutions. This sharp release of anthocyanins to the first dialysate solution contrasted with the observed in the presence of the WPM, allowed to infer a retention of the anthocyanins.

The dialysate fractions $W1_{FPC}$-$W5_{FPC}$ (Table 3.13) were mainly composed by Mv3Glc (64-66%), Mv3AcGlc (13-15%), Pt3Glc (5-6%), Pn3Glc (4-5%), and Df3Glc (3%), showing a homogeneous composition among them. This contrasts with the differences observed in the presence of the WPM2 previously discussed. Figure 3.14 shows the percentage of initial Mv3Glc and Mv3AcGlc diffused through the dialysis membrane in the different dialysis performed in the absence of the WPM2 for the 5 dialysate solutions.

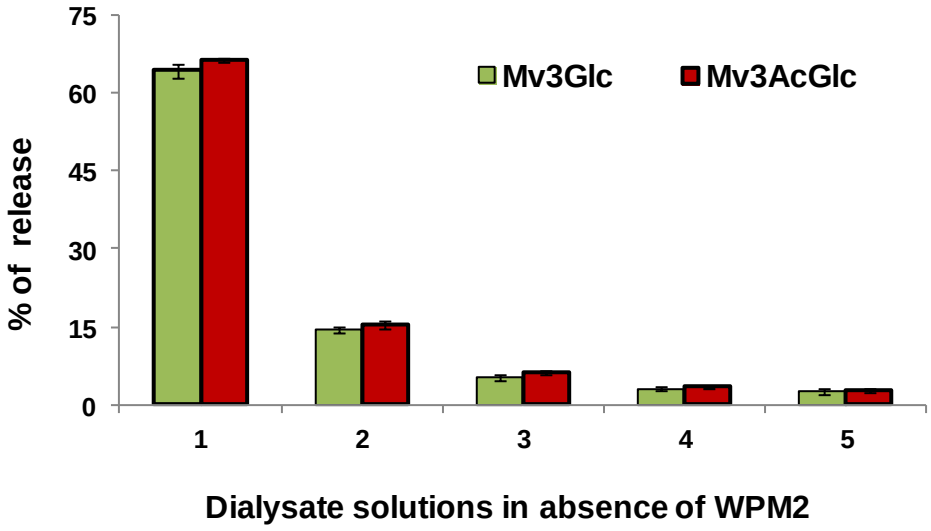


Figure 3.14: Percentage of release of malvidin 3-glucoside and malvidin 3-(6-acetyl)glucosides in the absence of polymeric material, for the 5 dialysate solutions (W1FPC to W5FPC).

A similar behaviour of Mv3Glc and Mv3AcGlc was observed in $W1_{FPC}$, whereas 64 and 66%, respectively, were recovered. These higher percentages showed that these compounds were selectively retained by the WPM. This was also stated in the percentages of the initial material recovered in the following dialysate solutions. For $W05_{WPM}$, 10% of Mv3Glc and 11% of Mv3AcGlc were recovered whereas for $W5_{FPC}$ only 2% Mv3Glc and Mv3AcGlc were obtained. The retention of Mv3Glc and Mv3AcGlc by the WPM prevents their release in relative higher amounts in the early stages, as observed in $W1_{FPC}$, and promotes their release in relative higher amounts in the later stages, as observed in $W05_{WPM}$. Comparable results have been reported for the retention of esters by the wine polymeric material (Rocha *et al.*, 2007).

For all anthocyanins under study, the percentage of each compound in the successive dialysate solutions obtained in the absence of the WPM2 showed a logarithmic decay (Table 3.14). This behaviour can be explained by the drastic decay observed in the first dialysis, leaving in the dialysis bag a small amount of phenolic compounds that did not significantly vary their amount in the following dialyses performed. This contrasted with the behaviour observed for these compounds when dialysed from the WPM2, especially the acetylated anthocyanins that previously presented a linear decay. Also, for the anthocyanins 3-Glc, the logarithmic decay was lower than that observed in the absence of the WPM2.

3.2.2.5 Determination of the retention capacity of monomeric anthocyanins by the wine polymeric material

In order to quantify the retention capacity of WPM2 towards the anthocyanins, for each anthocyanin a retention coefficient (RC) was calculated based on the percentage of its release to the dialysis water during the first 6 h period in the presence of WPM2 and in its absence.

The mean value of RC of anthocyanins 3-Glc (Table 3.14) was 0.50 ranging from 0.47 to 0.52. For acetylated anthocyanins the mean value obtained was 0.82 ranging from 0.77 to 0.87. These results showed that the WPM2 has a capacity to partially retain all anthocyanins. Also, a higher retention capacity was obtained when the anthocyanins were acetylated. The acetylation of the glucose residue enhances the hydrophobicity of the anthocyanins suggesting that the association of polymeric material with anthocyanins could be mainly due to hydrophobic interactions. In addition, CH–π interactions can occur between the aromatic rings on anthocyanins and carbohydrates, namely the β-galactopyranoside residues components of the wine arabinogalactans, in a similar behaviour as reported to occur when simple carbohydrates are mixed in aqueous solutions with phenol and aromatic amino acids (Vandenbussche *et al.*, 2008).

3.2.3 Study of retention capacity of anthocyanins by mannoprotein-rich and arabinogalactan-rich fractions

Having shown that different anthocyanins interact differently with the wine polymeric material, in this study, the interactions that can occur between the main polysaccharides families and anthocyanins were also studied. For this purpose, the diffusion performance of anthocyanins through a dialysis membrane was evaluated in presence of a mannoprotein-rich fraction as well in presence of an arabinogalactan-rich fraction, eluted with 50 mM. The chemical composition of these two fractions has been described in section 3.1.4.3. MP fraction contained 62% of polysaccharides, of which 93 mol% mannose. The AG fraction contained 60% of polysaccharides, of which 73 mol% were Ara + Gal. A solution containing the phenolic compounds without polymeric material was prepared by dialysis, collecting the material that diffused through the membrane. This material was used for a dialysis performed in the absence of the wine polysaccharides and defined as the blank (B) experiment for the release of phenolic compounds. In addition, was also used for a dialysis in the presence of MP (MP dialysis) and AG (AG dialysis). In case of MP and AG dialysis, polysaccharides were added at a concentration of 150 mg/L to the phenolic solution, and stand on agitation during one hour before being insert in the dialysis bag to start the dialysis. Each dialysis trial was performed during 15 h, with water renewals every 3 h, allowing to obtain five dialysate solutions for each experimental condition.

3.2.3.1 Total anthocyanins and monomeric anthocyanin composition of dialysate solutions

The 5 dialysate solutions obtained for each experiment were named $W1_B$ - $W5_B$ for blank trial, $W1_{MP}$ - $W5_{MP}$ for MP trial, and $W1_{AG}$ - $W5_{AG}$ for AG trial, in accordance with the serial number of the water changes performed. Each one of the five dialysate solutions obtained was concentrated for analysis of total and individual anthocyanins.

The concentration of total anthocyanins and of each one of the 9 monomeric anthocyanins (expressed as mg/L of solution in Mv3Glc equivalents) in the initial phenolic solution and in dialysate solutions obtained from the different dialysis is shown in Table 3.13. From those values, it was possible to determine the percentage of release through the membrane for the different dialysate solutions. The total amount of anthocyanins estimated colorimetricaly, initially present in the phenolic solution was 280.9 mg Mv3Glc/L, of which 65%, estimated by HLPC, to be in monomeric state. Mv3Glc accounted for 129.5 mg Mv3Glc/L, representing 71% of total monomeric anthocyanins quantified. Mv3AcGlc accounted for 16%, Mv3CmGlc 5%, and Pt3Glc 4%. Pn3Glc, Df3Glc and their acetylated derivatives accounted for 1-2% of monomeric anthocyanins. The amount of total anthocyanins present in the first blank dialysate solution ($W1_B$) represented 51% of the initially present in the dialysis bag whereas the $W1_{MP}$ and $W1_{AG}$ contained only 34 and 28% of initial anthocyanins, respectively. These values were significantly different. In the second dialysate solutions the amount of anthocyanins released from blank was 52.5 mg Mv3Glc/L (Table 3.15), showing a decrease in the amount released in relation to the first dialysate solution of 33%. However, $W2_{MP}$ and $W2_{MP}$ contained 40.4 and 48.9 mg Mv3Glc/L of anthocyanins, which represented a sifnificant decrease of 19 and 11% respectively in relation to $MP1_{AG}$ and $W1_{AG}$ which is lower than observed for $W2_B$. For the first four dialysate solutions, the total amount of anthocyanins present in the blank dialysate solutions were higher than in presence of MP and AG. The sum of anthocyanins recovered in the five dialysate solutions of the blank experiment represented 83% of the anthocyanins initially present inside the dialysis bag, whereas for MP and AG dialyses that value was ~58% (Figure 3.15). The results obtained indicated a slowly release of the total anthocyanins into dialysate solutions in presence of both polysaccharides.

Figure 3.15 shows the percentage of recovery of total monomeric anthocyanins in the different dialysate solutions for the blank, MP and AG experiments. It was possible to observe that in the first dialysate solution the amount of anthocyanins recovered was higher in the blank than in MP and AG solutions. Also, this behaviour is maintained until the 4^{th} water exchange, at 12 h. These results are in accordance with the observed for total anthocyanins.

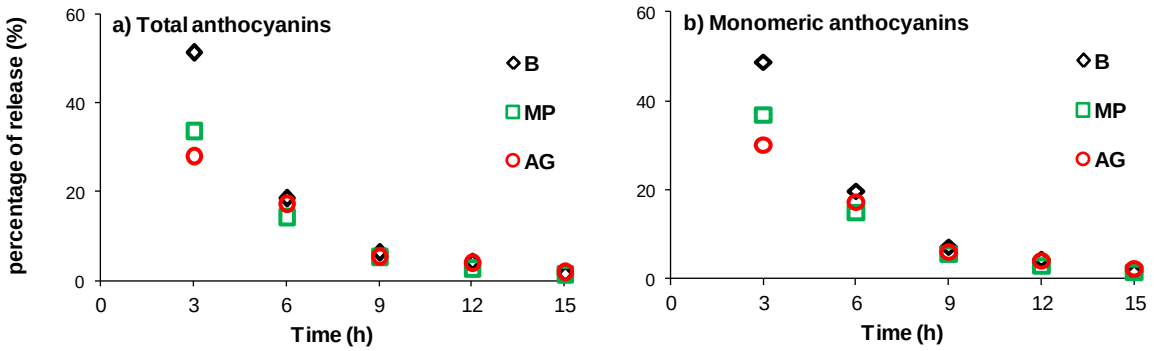


Figure 3.15: Percentage of recovery of total anthocyanins (a) and monomeric anthocyanins (b) in the different dialysate solutions for the blank (B), mannoproteins (MP) and arabinogalactan (AG) experiments.

The percentage of total monomeric anthocyanins in the successive dialysate solutions showed a logarithmic decay. For instance, 49% of the monomeric anthocyanins were recovered in $W1_B$, while for $W1_{MP}$ was 37% and for $W1_{AG}$ was 31%, showing that AG interact in higher extent with anthocyanins than MP. The release of anthocyanins on the $W2_B$ decreased to 20 %, while for $W2_{MP}$ and $W2_{AG}$ the decrese was 15 % and 17%, respectively. For the other dialysate solutions, a similar percentage of recovery was observed, 6-7, 3-5 and 2% for W3, W4, and W5, respectively. The total amount of monomeric anthocyanins present in the five dialysate solutions of MP and AG accounted for 114.8 and 111.2 mg Mv3Glc/L which represented ~75% of blank dialysate solutions. The retention of anthocyanins by MP are in accordance with the observations made by Guadalupe and Ayestaran (2008) showing that the addition of commercial mannoprotein to wines exhibited a slight delay (*ca* ~30%) in the extraction of pomace anthocyanins at the end of alcoholic fermentation, when compared with control wines (no addition of mannoproteins) which could induce a similar tendency of partial retention of anthocyanins by mannoproteins. The results obtained.

3.2.3.2 Effect of anthocyanin structure on diffusion rate

It can be seen, from Table 3.15, that the main anthocyanin present in the dialysate solutions was Mv3Glc, ranging from 61-71% in blank experiment, and from 63-73% and 68-76%, respectively for MP and AG dialysate solutions, followed by Mv3AcGlc which accounted of 15-24% for W_B, 15-21% for W_{MP} and 10-17% for W_{AG}. Mv3CmGlc and Pt3Glc ranged from 3-8 % and from 2-4%, respectively, in the dialysate solutions. The ratio between Mv3Glc and Mv3CmGlc exhibited a different tendency along the number of dialyses performed. For example, according to Table 3.15, the ratio Mv3Glc / Mv3CmGlc was 16 in $W1_B$, which was similar to $W5_B$. In contrast, the ratio Mv3Glc / Mv3CmGlc decreased from 21 in $W1_{MP}$ to 15 in $W5_{MP}$ and from 27 in W1 to 10 in W5 of AG dialysate solutions. These results showed that the interaction of MP and AG with anthocyanins may depend of their structure.

In order to relate the interactions of the anthocyanin families with MP and AG, the amount of anthocyanin 3-Glc (Figure 3.16a), anthocyanin 3-AcGlc (Figure 3.16b), and anthocyanin 3-CmGlc (Figure 3.16c) retained inside the dialyses bag was determined. It is possible to observe a retention of all anthocyanins families by MP and AG. MP and AG exhibited a similar retention of anthocyanins 3-Glc and anthocyanin 3-CmGlc, but the latter in higher extend when compared with the blank experiment. At end of 15 h of dialyses, the amount of anthocyanins retained was ~1.5 and ~2.7 fold for anthocyanins 3-Glc and anthocyanin 3-CmGlc, respectively. For anthocyanin 3-AcGlc, MP and AG exhibited clearly a different behavior, where higher retention was observed for AG.

Table 3.15: Amount of monomeric anthocyanin and the total anthocyanin (expressed as mg/L of equivalents of Mv3Glc) present in the initial phenolic solution (IPS) and in the dialysate solutions in absence of polysaccharides (B), and in presence of mannoproteins (MP) and in presence of arabinigalactans (AG). (Mean of three replicates ± standard deviation)

Fraction	Df3Glc	Pt3Glc	Pn3Glc	Mv3Glc	Df3AcGlc	Pn3AcGlc	Mv3AcGlc	Pn3CmGlc	Mv3CmGlc	Total monomeric anthocyanins	Total anthocyanins
IPS	1.63±0.13	7.42±0.23	1.62±0.12	129.46±7.9	1.88±0.05	3.39±0.13	28.61±1.13	1.62±0.16	9.24±0.45	183.2±4.2	280.9±13.3
Dialysis of blank experiment											
$W1_B$	1.09±0.02	2.78±0.11	0.75±0.01	61.00±3.28	0.93±0.01	1.56±0.07	16.78±0.24	0.98±0.03	3.83±0.04	89.7±1.9	144.6±4.0
$W2_B$	0.48±0.04	0.90±0.01	0.24±0.03	23.31±1.15	0.32±0.02	0.41±0.03	6.89±0.10	0.50±0.01	1.72±0.03	36.4±2.1	52.5±1.4
$W3_B$	0.17±0.00	0.45±0.02	0.12±0.01	8.19±1.20	0.09±0.01	0.14±0.00	3.17±0.50	0.15±0.01	0.91±0.05	13.4±0.4	18.2±1.1
$W4_B$	0.08±0.01	0.24±0.00	0.06±0.00	5.47±0.08	0.06±0.00	0.06±0.02	1.82±0.09	0.11±0.00	0.44±0.02	8.4±0.3	12.1±0.8
$W5_B$	0.05±0.02	0.14±0.01	0.04±0.02	2.33±0.12	0.02±0.01	0.02±0.01	0.47±0.03	0.03±0.00	0.15±0.02	3.3±0.1	4.5±0.1[d]
Dialysis in the presence of mannoproteins fraction											
$W1_{MP}$	0.74±0.09	2.39±0.16	0.61±0.03	48.00±2.89	0.56±0.05	1.03±0.04	11.85±0.41	0.52±0.01	2.24±0.06	67.9±1.5	94.9±3.9
$W2_{MP}$	0.37±0.02	0.95±0.01	0.24±0.05	20.26±1.00	0.16±0.01	0.37±0.01	4.13±0.21	0.21±0.01	0.99±0.10	27.7±0.5	40.4±3.0
$W3_{MP}$	0.15±0.02	0.39±0.02	0.10±0.02	6.59±0.09	0.08±0.00	0.23±0.01	2.18±0.13	0.03±0.00	0.79±0.05	10.5±0.6	15.3±0.4
$W4_{MP}$	0.05±0.00	0.18±0.01	0.04±0.01	3.94±0.38	0.03±0.00	0.08±0.01	0.92±0.03	0.05±0.01	0.35±0.07	5.6±0.2	8.0±0.3
$W5_{MP}$	0.19	0.08	0.04	2.06	0.02	0.00	0.47	0.03	0.14	3.0	4.3±0.2
Dialysis in the presence of arabinogalactan fraction											
$W1_{AG}$	0.72±0.05	2.31±0.01	0.61±0.0	42.49±0.23	0.34±0.02	0.58±0.01	6.54±0.12	0.35±0.00	1.59±0.02	55.5±2.3	79.0±2.2
$W2_{AG}$	0.52±0.04	1.36±0.10	0.33±0.0	24.43±1.06	0.19±0.03	0.39±0.05	3.34±0.24	0.34±0.04	1.09±0.28	32.0±0.2	48.9±1.7
$W3_{AG}$	0.14±0.02	0.41±0.01	0.10±0.0	7.36±0.17	0.08±0.01	0.17±0.03	2.38±0.18	0.23±0.01	0.56±0.12	11.4±1.0	15.5±1.2
$W4_{AG}$	0.11±0.06	0.30±0.01	0.06±0.0	5.80±0.44	0.05±0.01	0.08±0.00	0.82±0.6	0.07±0.00	0.49±0.06	7.8±0.7	11.6±0.9
$W5_{AG}$	0.03	0.18	0.05	3.25	0.03	0.06	0.49	0.10	0.32	4.5	5.9±0.5

Values of each anthocyanin (columns) for each dialysate solution showing the same letter are not significantly different according to the Tukey test ($p \leq 0.05$)

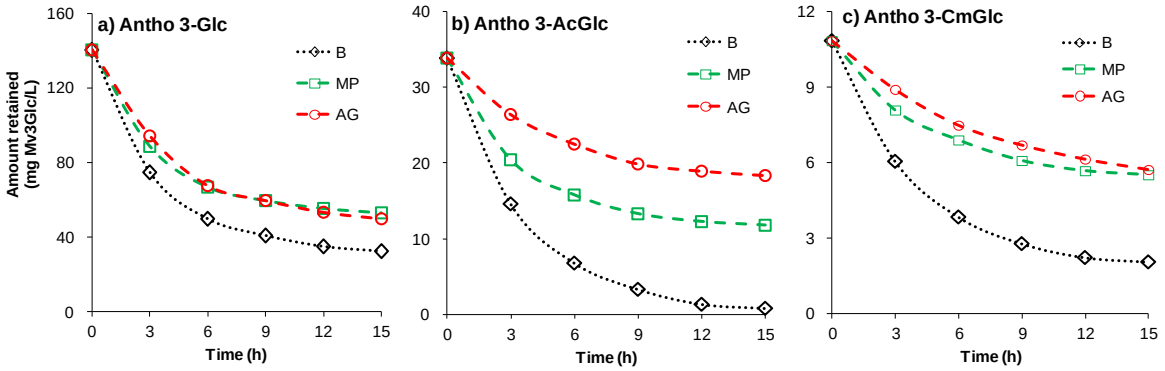


Figure 3.16: Amount (mg/L) of anthocyanins retained inside dialyses bag for blank (B), mannoproteins (MP) and arabinogalactan (AG) experiment of each anthocyanin families: a) anthocyanin 3-glucosides (Antho 3-Glc); b) anthocyanin 3-(acetyl)glucosides (Antho 3-AcGlc); c) anthocyanin 3-(coumaroyl)glucosides (Antho 3-CmGlc).

The percentage of the sum of released into dialysate of anthocyanins 3-Glc (Figure 3.17a) and their acetyl (Figure 3.17b) and coumaroyl (Figure 3.17c) derivatives showed a different behavior according to their structures. Regarding anthocyanins 3-Glc, the percentage of release in $W1_B$ (after 3h) was 61%, 57% in $W1_{MP}$ and 50% $W1_{AG}$. These values were statistically different from $W1_B$. In contrast, in second dialysate solutions (after 6h), a significantly higher relative amount of anthocyanins was observed in AG (31%) when compared with B (22%) and MP (24%) dialysis. No significant differences were observed for 3rd (after 9h), 4th (after 12h) and 5th (after 15h) dialysate solutions.

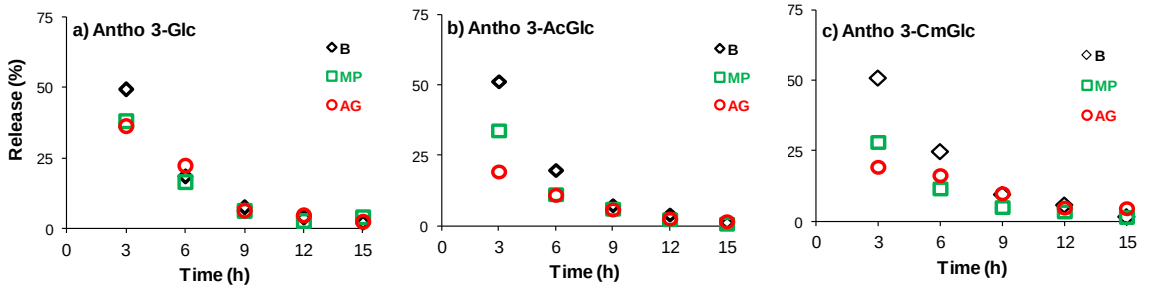


Figure 3.17: Percentage of release into the different dialysate solutions for blank, MP and AG experiment of each anthocyanin families: a) anthocyanin 3-glucosides (Antho 3-Glc); b) anthocyanin 3-(acetyl)glucosides (Antho 3-AcGlc); c) anthocyanin 3-(coumaroyl)glucosides (Antho 3-CmGlc).

In case of anthocyanins 3-AcGlc, a similar behavior for B and MP experiments was observed, with relative releases of 58-65% and 60-67%, respectively, however the absolute amount was lower for MP. For the AG dialysate solution those values ranged from 46 to 49%, showing a higher retention of anthocyanins by AG comparing with MP. The values of percentage of release of anthocyanins 3-AcGlc found for $W2_B$, $W2_{MP,}$ and $W2_{AG}$ were 23-25%, 19-22% and 25-30%, respectively. It can be seen from Figure 3.17c, that the release of anthocyanins 3-CmGlc in presence of AG is clearly different than the observed for B and MP dialysis. The relative amount of anthocyanins released for $W1_{AG}$ was 36%, less ~20% than for $W1_B$ and $W1_{MP}$. In contrast, the relative amount of anthocyanins in $W3_{AG}$ was 17%, more ~7% than for $W2_B$ and $W2_{MP}$.

These results showed that the AG promote the retention of anthocyanins in a higher extent than MP. These interactions were higher for anthocyanin 3-CmGlc, exhibiting a slowly release through the dialyses membrane, comparing with anthocyanin 3-Glc and anthocyanin 3-AcGlc. For instance, the ratio anthocyanin 3-Glc/anthocyanin 3-CmGlc was 1.4 in $W1_{AG}$, while for $W5_{AG}$ was 0.4. These findings confirmed the previous results of the higher retention of coumaroyllated anthocyanins by polymeric material and shows that the polysaccharides mainly responsible for this effect are the AG.

3.2.3.3 Determination of the retention capacity of monomeric anthocyanins by the MP and AG polysaccharides

In order to quantify the retention capacity of MP and AG towards the anthocyanins, for each anthocyanin a retention coefficient (RC) was calculated based on the percentage of its release to the dialysis water during the first 3 h period in the presence of polysaccharide fractions and in its absence. A RC=0 means that the polysaccharides did not have any capacity of retention while RC=1 represents 100% of retention. For each anthocyanin a retention coefficient was calculated (Table 3.16).

Table 3.16: Retention coefficient of each anthocyanin by MP and AG.

RC	MP	AG
TA	0.34±0.03	0.45±0.02
TMA	0.24±0.01	0.38±0.02
Df3Glc	0.32±0.04 [a]	0.34±0.03 [a]
Pt3Glc	0.14±0.01 [b]	0.17±0.02 [a]
Pn3Glc	0.19±0.02 [b]	0.19±0.02 [a]
Mv3Glc	0.21±0.01 [b]	0.30±0.02 [a]
Df3AcGlc	0.39±0.04 [ac]	0.63±0.12 [b]
Pn3AcGlc	0.34±0.03 [a]	0.63±0.05 [b]
Mv3AcGlc	0.29±0.02 [a]	0.61±0.05 [b]
Pn3CmGlc	0.47±0.02 [d]	0.64±0.01 [b]
Mv3CmGlc	0.41±0.02 [c]	0.58±0.02 [b]

TA- total anthocyanins; TMA- total monomeric anthocyanins. Values of RC (columns) for each monomeric anthocyanin showing the same letter are not significantly different according to the Tukey test ($p \leq 0.05$).

The RC of total anthocyanins was 0.34 and 0.45 for MP and AG, respectively. Also, retention of the total monomeric anthocyanins was observed by MP and AG, the latter in higher extent. The mean RC of anthocyanins 3-Glc was 0.21 and 0.25 for MP and AG respectively. For anthocyanin 3-AcGlc and anthocyanin 3-CmGlc the values obtained were 0.34 and 0.44 for MP and 0.62 and 0.61 for AG. These results showed that MP and AG had both the capacity to retain anthocyanins, and that this capacity is dependent on their structure. For AG, statistically higher values RC were obtained for the acylated derivatives anthocyanins, when compared with the glucoside anthocyanins. The results obtained by Ortega-Regules *et al*. (Ortega-Regules *et al.*, 2006) suggested that the lowest extraction of anthocyanins from cell-wall grapes was correlated, among others, with high values of galactose of the cell-wall material, which is in accordance with the higher retention of anthocyanins by the arabinogactan-rich fraction. The results obtained allowed to infer that both, MP and AG contributed for the retention capacity of wine polymeric material. Also, in case of acylated anthocyanins, a statistically higher retention capacity was observed for AG when compared with MP. In case of AG, this retention can reach 60% of anthocyanins when they are acylated.

3.2.4 Determination of kinetic constant and activation energy of release of phenolic compounds from wine polymeric material

The results obtained previously showed that different phenolic compounds were retained with different strengths by polymeric material,. The intensity of these interactions was studied by measuring the kinetic constant and activation energy. For this purpose, 3 or 4 concentrations of dealcoholized wine (DW1), wine polymeric material (WPM1), and methanol insoluble polymeric material (PMi) were dialysed at five different temperatures (5, 25, 30, 35, and 40 ºC) until a plateau state was reached. The amount of phenolic compounds diffused through the membrane dialysis was quantified allowing to determine the velocity of release of phenolic compounds, determined colorimetricaly, for each experiment. The kinetic constant was determined plotting the velocities of release against the initial amount of phenolic compounds inside the dialysis bag. The energy necessary for that release, e.g. the activation energie (E_a) was calculated through linear regression of the logarithm of the kinetic constants versus the inverse of absolute temperature.

3.2.4.1 Determination of the velocity of release of phenolic compounds

For determination of the velocity of release of phenolic compounds from the red wine polymeric material through a dialysis membrane, the DW1, WPM1 and PMi were dialysed (12-14 kDa cut-off) and the amount of phenolic compounds released into the dialysate solution was measured. The chemical composition of these two fractions is described in section 3.1.2.2. The cumulative amount of the phenolic compounds difused increased linearly along time until reaching a stage where the amount of release of these compounds was very much lower, seeming to tend to a plateau.

It is possible to see from 3.17 that the time needed to reach the plateau differed according to each sample, amount of phenolic compounds inside dialysis bag and temperature. For DW1 fraction, the plateau state was obtained at 12-24 h of dialysis depending on the temperature of the assay. In case of WPM1, the

steady state ranged from 46 to 56 h of dialysis, and for PMi fraction it was necessary 64-86 h. In general, the time needed to reach this point followed the order PMi > WPM1 > DW1. Also, as temperature increased, lowest times were needed to reach plateau. The amount of phenolic compounds present in the dialysate solution increased as a function of temperature and time. At plateau, the amount of phenolic compounds released into dialysate solutions represented 24.6-32.3% of the amount initially quantified in the dialysis bag for DW1. When this point was reached in WPM1 and PMi dialyses, the relative amount of phenolic compounds released represented 6.2-10.6 % and 2.6-5.1%, respectively, showing a higher release of compounds from DW1 than from WMP1 and PMi, in this order. For a higher concentration of phenolic compounds inside the dialysis bag, higher the amount of phenolic compounds were released.

3.17: Dialyses conditions (temperature, amount of phenolic compounds inside the dialyses bag, amount of phenolic compounds released and velocity of release)

T (K)	Fraction	PC_i (mg GAE)	Time (h)	$PC_{released}$ (μg GAE)	r^2	$v_{released}$ (μg/h)
	DW1	0.42 - 1.69	18 - 24	101 - 306	0.82 - 0.91	5.6 - 25.0
278	WPM1	0.42 - 0.83	48 - 54	29 - 54	0.84 - 0.97	0.5 - 0.9
	PMi	0.92 - 1.22	71 - 83	22 - 30	0.88 - 0.95	0.3 - 0.4
	DW1	0.73 - 1.71	16 - 24	166 - 426	0.82 - 0.91	9.4 - 28.5
298	WPM1	0.42 - 0.83	46 - 54	31 - 63	0.84 - 0.97	0.5 - 1.1
	PMi	0.74 - 0.90	75 - 86	22 - 32	0.81 - 0.97	0.3 - 0.4
	DW1	0.73 - 1.71	15 - 18	193 - 429	0.68 - 0.94	11.4 - 29.2
303	WPM1	0.42 - 0.83	48 - 56	35 - 69	0.86 - 0.89	0.5 - 1.5
	PMi	0.61 - 1.22	80 - 90	23 - 41	0.82 - 0.93	0.2 - 0.5
	DW1	0.68 - 1.69	12 - 16	172 - 445	0.81 - 0.97	12.4 - 29.0
308	WPM1	0.52 - 0.97	46 - 54	38 - 57	0.67 - 0.91	0.6 - 1.2
	PMi	0.61 - 1.22	64 - 72	30 - 57	0.80 - 0.85	0.3 - 0.5
	DW1	0.68 - 1.69	12 - 16	215 - 477	0.87 - 0.91	16.0 - 38.0
313	WPM1	0.52 - 0.97	48 – 49	56 - 73	0.85 - 0.96	1.0 - 1.4
	PMi	0.83 - 1.10	63 - 67	28 - 43	0.93 - 0.98	0.4 - 0.6

PC_i – phenolic compounds present initially inside dialysis bag; Time- time needed to reach the plateau state; $PC_{released}$ – total amount of phenolic compounds released into dialysate solution at plateau state.r^2- correlation coefficient of the curve of phenolic compounds released against time; $v_{released}$- velocity of phenolic compounds through dialysis membrane.

The slope of the linear initial region (Figure 3.18) corresponds to the initial velocity of release of phenolic compounds. Good correlation coefficients were obtained for all curves, ranging from 0.67 to 0.98 (3.17). Each sample was assayed using 3 or 4 concentration levels, allowing the determination of 3 or 4 velocities (3.17). As example, Figure 3.18 shows the amount of phenolic compounds released along time for DW1, WPM1, and PMi fractions at 30 ºC. All dialysis experiments exhibited patterns similar to those presented in Figure 3.18. In case of DW1, the amount of phenolic compounds inside the dialysis bag ranged from 0.73 to 1.71 mg and their velocities of release ranged from 11.4 to 29.2 μg/h. For WPM1, the velocities ranged from 0.5 to 1.5 μg/h, and for PMi from 0.2 to 0.5 μg/h.

It was possible to observe higher velocities of release for higher amounts of material inside the dialysis bag. Also,when the same concentration of material was used, higher velocities were determined for higher temperatures, showing a fastest release with the increase in temperature.

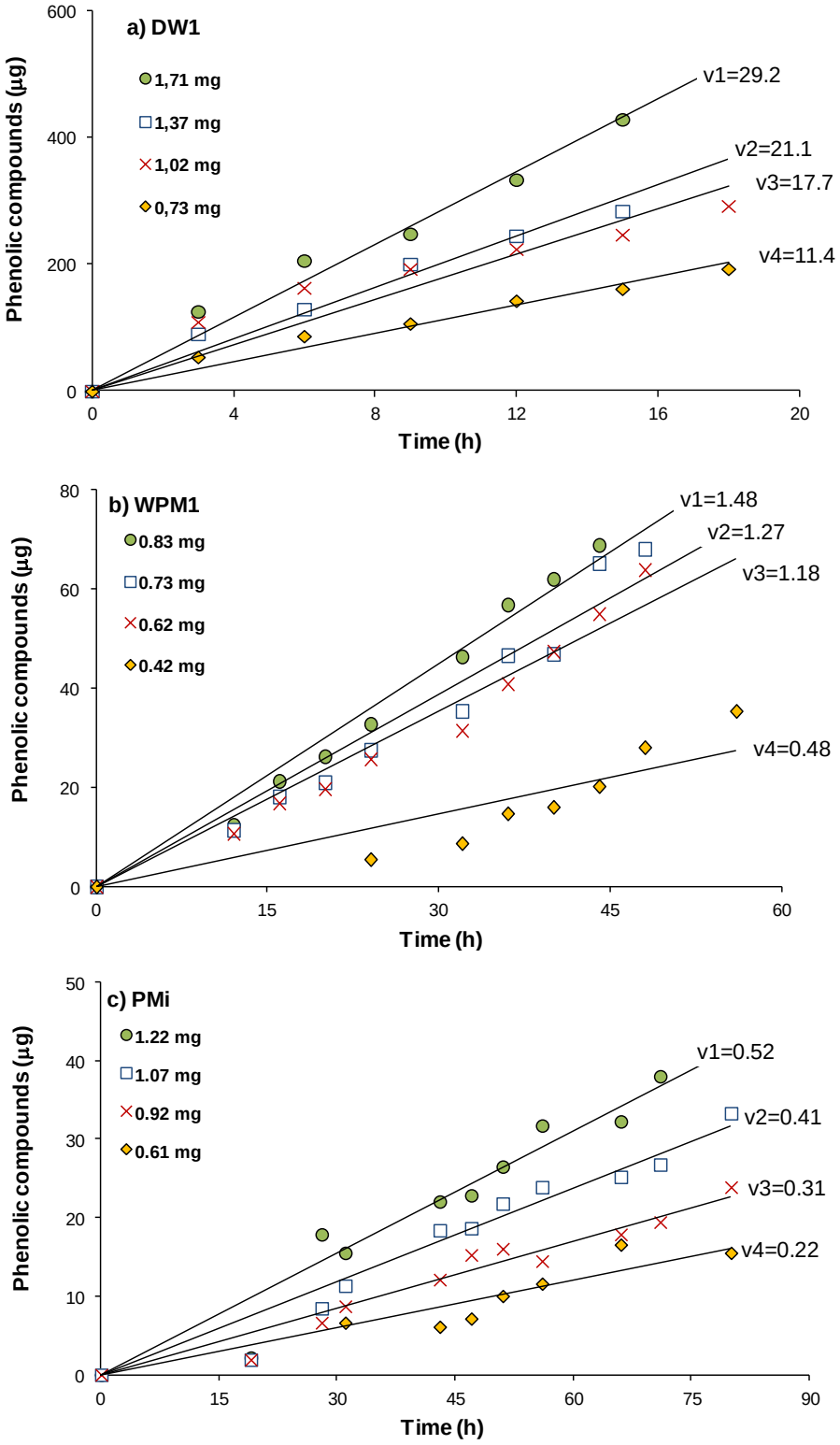


Figure 3.18: Phenolic compounds present in the dialysis solution plotted against their release time, for a) DW1, b) WPM1 and c) PMi fractions, at 303 K. 0.73-1.71 mg, 0.83-0.42 mg, and 0.61-1.22 mg were the initial amounts of phenolic compounds inside dialysis bag, for DW1, WPM1and PMi, respectively.

3.2.4.2 Determination of the kinetic constants and activation energy

The kinetic constant for each temperature was determined plotting the initial velocities of release against the initial amount of phenolic compounds inside the dialysis bag. The Ea values for the phenolic release process over the temperature range of 278 – 313 K were calculated through the slope of the logarithm of kinetic constants versus the inverse of temperature, applying the Arrhenius equation. Correlation coefficients from the curve fits were > 0.85 in all experiments. The kinetic constant value at different temperatures and the activation energy for the different fractions are shown in Table 3.18.

Table 3.18: Kinetic constants (k_c) for different temperatures (T) and activation energy (E_a) for dealcoholized wine 1 (DW1), wine polymeric material 1 (WPM1), and polymeric material insoluble in methanol (PMi).

	DW1		WPM1		PMi	
T (K)	k_c ($\times 10^{-7}$ s^{-1})	E_a (kJ/mol)	k_c ($\times 10^{-7}$ s^{-1})	E_a (kJ/mol)	k_c ($\times 10^{-7}$ s^{-1})	E_a (kJ/mol)
278	40.0		2.28		0.34	
298	42.5		3.40		0.64	
303	46.2	11.5	3.69	35.7	0.89	72.3
308	50.3		3.77		1.28	
313	59.3		5.08		1.37	

The kinetic constant increased as the temperature increased, indicating a higher rate of release of the phenolic compounds. For DW1, the k_c increased from 40.0 x 10^{-7} s^{-1} to 59.3 x 10^{-7} s^{-1} when temperature increased from 278 (5 ºC) to 313 K (40 ºC). The k_c increased from 2.28 x 10^{-7} s^{-1} to 5.08 x 10^{-7} s^{-1} and from 0.34 x 10^{-7} s^{-1} to 1.37 x 10^{-7} s^{-1} for WPM1 and PMi respectively, which means that kc increased as a result of temperature increasing. The resulting E_a for DW1 was 11.5 kJ/mol, expressed as mol of gallic acid, the lowest value determined. The E_a was 35.7 and 72.3 kJ/mol for WPM1 and PMi fraction, respectively. A lower value of activation energy means that the phenolic compounds present in the fraction were easierly released allowing to infer that they were weakly associated to

polymeric material. The results allowed inferring that the phenolic compounds present in PMi fraction were strongerly linked to the polymeric material than in WPM1 fraction.

In order to mimic physiological conditions, the pH of DS was adjusted to 2.0 or 7.0 (initial value of pH 3.48), and dialyses were performed at 37 ºC (310 K). The pH 2.0 mimics the pH of stomach and pH 7.0 mimics the intestinal tract. For each condition, the amount of phenolic compounds released at the end of 3h and the kinetic constant were determined. The results showed a tendency for a faster rate in case of pH 2.0. At the end of 3 h, 24.5% of phenolic compounds were released at pH 2.0, and 20.0% at pH 7.0. The kinetic value obtained for pH 2.0 was 59.8 $\times 10^{-7}$ s^{-1}, slightly higher than the 54.7$\times 10^{-7}$ s^{-1} obtained for pH 7.0 or 50.3 $\times 10^{-7}$ s^{-1} and 59.3 $\times 10^{-7}$ s^{-1} obtained at pH 3.48, at 35 and 40 ºC, respectively. Although a small tendency was observed for the release of the phenolic compounds for DW1 at pH 7.0, the k_c associated to the process was very similar. These results does not allow to conclude about a selective release of phenolic compounds at the two pHs.

3.2.5 Final remarks

The results obtained from the study of interactions between the polymeric material and the phenolic compounds, allowed to conclude that:

- The wine polymeric material had the capacity to interact with the phenolic compounds, partially retaining them.

- All anthocyanins were retained by the WPM, although in different extents. The higher retention capacity (RC from 0.77 to 0.87) was observed for anthocyanins 3-AcGlc when compared with the anthocyanins 3-Glc (RC from 0.47 to 0.52).

- Both mannoprotein and arabinogalactan fractions showed capacity for retaining the different anthocyanins. AG fraction have a higher contribution than mannoproteins for retention capacity of wine polymeric material, especially when the anthocyanins were acylated.

- The highest retention observed for acylated anthocyanins suggests hydrophobic associations of anthocyanins with the wine polymeric material.

- Higher velocities of release were determined as temperature increased, showing a fastest release with temperature, characteristic of endothermic processes.

- The Ea for the realese of phenolic compounds from polymeric material was for DW1 11.5 kJ/mol, the lowest value determined. The E_a was 35.7 and 72.3 kJ/mol for WPM1 and PMi fraction, respectively. The results allowed concluding that the phenolic compounds can present different strengths of association to the wine polymeric material.

3.3 Effect of Storage Conditions on Antioxidant Activity and Phenolic Content

3.3.1 Overview

The results obtained in section 3.1 showed that the antioxidant properties of the compounds initially present in the wine were not lost by the dealcoholization process. In order to study forms to preserve the antioxidant properties of the bioactive compounds obtained from the dealcoholized wine, this section is focused on the evaluation of phenolic composition and antioxidant activity of wine and wine extracts in long-term storage conditions. For that purpose, two experiments were carried out:

1) The objective of the first experiment was to evaluate the effect of light and atmosphere composition on the antioxidant activity of phenolic extract. For that, it was used the fraction of phenolic compounds (PCE) of DW1. The PCE, as a powder, was submitted to different storage conditions of light and atmosphere, at room temperature for approximately 1 year. During that storage period, the antioxidant activity of the extracts was determined.

2) The results obtained in section 3.1 showed that the fraction containing anthocyanins was the main responsible for the wines antioxidant activity. In addition, mannoprotein and arabinogalactan fractions, showed capacity for interacting with wine anthocyanins (see section 3.2). Thus, in the second experiment, performed at two storage temperatures (5 and 30 ºC) during 3 months, it was evaluated the effect of the addition of 0.5 g/L of polysaccharide-rich fraction to the red wine, on the phenolic composition and on antioxidant activity (DPPH and ABTS assays). Total phenols, tannins and anthocyanins were determined spectrophotometrically, while the amount of monomeric anthocyanins was quantified by HPLC.

3.3.2 Effect of storage conditions on antioxidant activity of phenolic extracts

Temperature, light, and atmosphere composition, among others, are factors that contribute for degradation of phenolic compounds (Cavalcanti *et al.*, 2011; Maccarone *et al.*, 1985; Maier *et al.*, 2009) and also for the decrease of their antioxidant activity (Gris *et al.*, 2007). In order to understand how can the antioxidant properties of bioactive compounds be preserved, a phenolic fraction was stored under different conditions of light and atmosphere composition. The phenolic fraction used in this study was obtained from dealcoholized red wine 1. The dealcoholized wine was loaded into the C18 Sep-pak cartridge, washed with distilled water to remove sugars and salts, and eluted with acidic methanol to recover the phenolic-rich fraction. The methanol fraction was evaporated, dissolved in water, and lyophilized to obtain the phenolic compounds fraction (PCE) as a powder.

The phenolic compounds (3 mg of powder) were stored in plastic tubes (5 mL of head-space) with stopper and the following conditions were assayed:

1) Controlled atmosphere (N_2), in dark, stored in a desiccator containing P_2O_5 in solid state.
2) Controlled atmosphere (N_2), exposed to day light, stored in a desiccator containing P_2O_5 in solid state.
3) Air atmosphere in the dark.
4) Air atmosphere exposed to day light.
5) Open tubes exposed to day light.

All conditions were performed at room temperature. The storage conditions are summarized in Table 3.19.

Table 3.19: Storage conditions of phenolic compounds extract.

Condition	Ligth / Dark (L / D)	Atmosphere N_2 / Air (N / A)	Open / Sealed (O / S)
Condition 1 (DNS)	Dark	N_2	Sealed
Condition 2 (LNS)	Light	N_2	Sealed
Condition 3 (DAS)	Dark	Air	Sealed
Condition 4 (LAS)	Light	Air	Sealed
Condition 5 (LAO)	Light	Air	Open

The PCE used contained 491 mg of phenolic compounds per g of sample, estimated as gallic acid equivalents. At day 0, the PCE exhibited an antioxidant activity of 170 mmol of trolox equivalents (TE) by 100 g of dry sample, evaluated by the DPPH method. For each storage condition, 25 individual tubes were prepared. Table 3.20 shows the values of antioxidant activity of PCE, stored in different conditions, evaluated periodically during 342 days of storage period. For each time point, two tubes were used to evaluate the antioxidant activity.

Table 3.20: Antioxidant activity of extract stored at different conditions along 342 days.

	Antioxidant activity (mmol TE/ 100 g of extract) for each storage condition				
Day	DNS	LNS	DAS	LAS	LAO
0	170±5	170±5	170±5	170±5	170±5
12	170±5 ^{a}	169±2 ^{a}	169±5 ^{a}	169±4 ^{a}	167±7 ^{a}
19	166±4 ^{a}	164±9 ^{a}	165±8 ^{a}	157±5 ^{a}	160±9 ^{a}
26	167±8 ^{a}	160±7 ^{a}	166±5 ^{a}	158±4 ^{a}	152±7 ^{a}
40	165±8 ^{a}	166±2 ^{a}	166±9 ^{a}	163±9 ^{ab}	151±8 ^{b}
47	168±9 ^{a}	165±7 ^{a}	168±6 ^{a}	158±7 ^{ab}	154±9 ^{b}
57	169±5 ^{a}	163±8 ^{a}	164±4 ^{a}	151±6 ^{b}	131±4 ^{c}
71	167±4 ^{a}	163±5 ^{a}	163±2 ^{a}	142±8 ^{b}	122±4 ^{c}
92	168±3 ^{a}	162±3 ^{a}	162±3 ^{a}	140±3 ^{b}	118±2 ^{c}
106	168±5 ^{a}	162±4 ^{b}	163±3 ^{ab}	140±2 ^{c}	119±2 ^{d}
142	166±2 ^{a}	161±4 ^{a}	161±2 ^{a}	137±4 ^{b}	108±4 ^{c}
162	165±9 ^{a}	158±2 ^{a}	158±9 ^{a}	142±6 ^{a}	85±13 ^{c}
252	163±7 ^{a}	156±9 ^{a}	150±4 ^{a}	142±9 ^{a}	83±2 ^{b}
342	162±5 ^{a}	155±6 ^{ab}	147±9 ^{ab}	141±8 ^{b}	83±5 ^{c}

Values are mean ± standard deviation of two samples of each condition, analyzed individually in triplicate. Different letters within each row represent significant difference according to the Tukey test ($p \leq 0.05$).

It can be seen, from Table 3.20, that at day 26 of storage, the highest antioxidant activity was achieved by samples DNS and DAS, respectively, 167 and 166 mmol TE/ 100 g. The sample stored outside the desiccator and in a open tube (LAO), possessed the lowest value of antioxidant activity (152 mmol TE/ 100 g). However, no statistical differences were observed for antioxidant activity between any of the different storage conditions. At day 40, it was observed for LAO a statistical lower value of antioxidant activity. For day 57, the values for DNS, LNS and DAS samples ranged from 169, 163 and 164 mmol TE/ 100 g, statistically higher than the 151 mmol TE/ 100 g of LAS, and also statistically different from the 131 mmol TE/ 100 g of LAO. From day 57 until day 142, the antioxidant activity followed the order DNS, LNS, DAS > LAS > LAO, with significant differences.

On day 142, the antioxidant activity of DNS, LNS and DAS samples ranged from 161 to 166 mmol TE/ 100 g, which represented 92 to 97% of the initial antioxidant activity. In addition, the antioxidant activity of the sample kept outside the desiccator and exposed to the light (LAS) was 137 mmol TE/ 100 g, corresponding to 85% of initial value, whereas the value for the sample kept open outside the desiccator was 108 mmol TE/ 100 g, representing only 61% of the initial antioxidant activity. After 342 days, the sample DNS exhibited the highest antioxidant value (162 mmol TE/ 100g), followed by LNS (155 mmol TE/ 100g) and DAS (147 mmol TE/ 100g), Although, no significant differences ($p \leq 0.05$) observed.

From the data in Table 3.20 it was possible to calculate the percentage of remaining antioxidant activity after each storage condition. Figure 3.19 shows the evolution of those values along the storage period. For the sample kept outside the desiccator and exposed to the light (LAS) it was observed, between day 40 and 71 of storage, a loss (13%) of its antioxidant capacity, followed by stabilization until the end of the study.

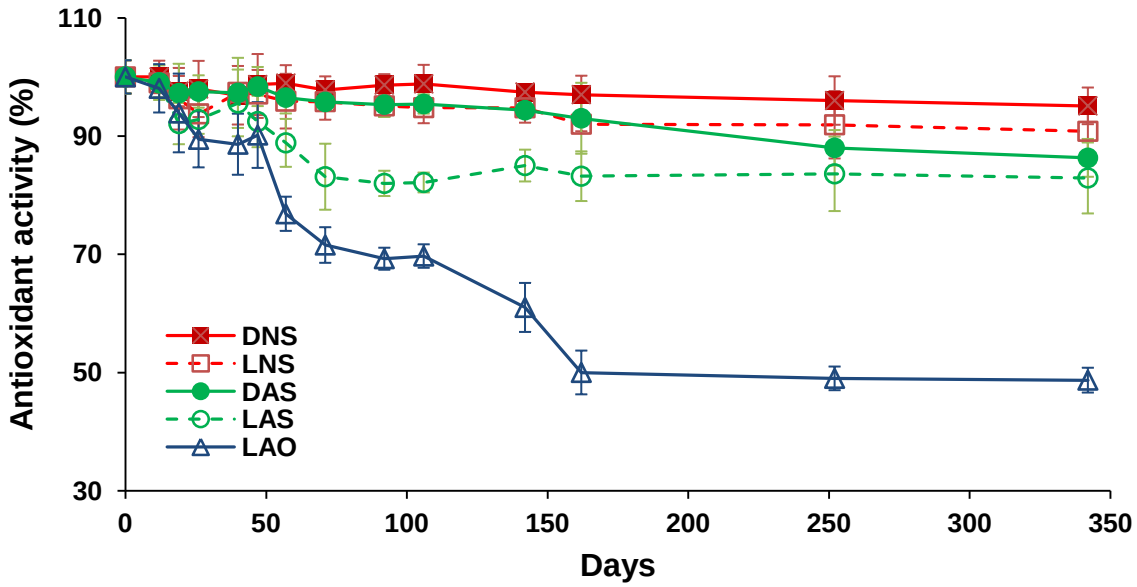


Figure 3.19: Evolution of antioxidant activity (%) of an extract rich in phenolic compounds stored in different conditions, during 342 days. DNS, sample stored in dark, inside desiccator under nitrogen and sealed; LNS, sample stored at light, inside desiccator under nitrogen and sealed; DAS, sample stored in dark, outside desiccator and sealed; LAS, sample stored at light, outside desiccator and sealed; LAO, sample stored at light, outside desiccator and opened.

The sample stored in the non stoppered tube (LAO) exhibited a marked loss (40%) on its antioxidant activity from day 47 to day 162, and then remained constant until the end of the time understudy. Along the storage period, it was possible to observe that the antioxidant activity of DNS was the highest in nearly all points analyzed, with the exception of day 40. Excluding the sample maintained in open air, the percentage of antioxidant preservation ranged from 83 to 95%, at the end of the 342 days, showing a good preservation of antioxidant activity. Accordingly, the maintenance or slightly decrease on antioxidant activity of spray dried powders of phenolic extract of black currant during 12 months at 8 and 25 ºC was also described (Bakowska-Barczak & Kolodziejczyk, 2011).

The results showed that the substitution of air by nitrogen atmosphere leaded to higher preservation of antioxidant activity (DNS and LNS *vs* DAS and LAS). Also, the reduced air exposure inside desiccator could contribute to these differences. It is possible that air transmission through the plastic tubes could be the responsible for the different evolution of the antioxidant activity. Comparing samples kept in dark (DNS and DAS) with those exposed to light (LNS and LAS),

it was possible to verify that the samples kept in the dark possessed higher values of antioxidant activity. Accordingly, Bakowska *et al.* (2003) reported a degradation (*ca.* 40%) for a solution of Cy3Glc kept in dark after 3 months of storage, however that decrease was twofold higher when the sample was exposed to sunlight.

In conclusion, the results obtained showed that, among the conditions studied, the antioxidant activity was best preserved when the phenolic extracts were stored in dark, inside the desiccator and under controlled atmosphere (nitrogen).

3.3.3 Effect of storage temperature and polysaccharide-rich fraction on wine phenolic composition

During the storage period, the total amount of phenolic compounds (on weight basis) in wine is not changed, but its phenolic compounds were shown to undergo continuous changes leading to modification on wine composition (Zafrilla *et al.*, 2003). These reactions, such as polymerization, oxidation, and condensation are responsible for the formation of pigments (Bakker & Timberlake, 1997; Dallas *et al*., 1996). This may cause different reactivity responses using non-specific quantification methods, such as Folin-Ciocalteu reagent. In addition, these reactions affect color and colloidal stability and the antioxidant properties of wine.

The results obtained in section 3.2 showed that mannoprotein and arabinogalactan fractions, had the capacity for interacting with wine anthocyanins. Thus, the impact of a polysaccharide-rich fraction on the phenolic composition and on antioxidant activity of a red wine was assessed after 3 months of storage. Samples of wine were stored under refrigeration at 5 ºC, a temperature close to cellars, and at 30 ºC simulating a faster aging of wine, in presence (0.5 g/L) and absence of a polysaccharides-rich wine material. Five conditions were used:

1) WT0 - initial wine;
2) W5 - wine stored at 5 ºC;
3) W5P - wine stored at 5 ºC, with addition of 0.5 g/L of polysaccharides-rich material;
4) W30 - wine stored at 30 ºC;
5) W30P - wine stored at 30 ºC, with addition of 0.5 g/L of polysaccharides-rich material.

This polysaccharides-rich material added to the wine were composed by 780 mg/g of polysaccharides, of which of 33 mol% of Man, 22 mol% of Ara, 20 mol% of Gal, 10 mol% of UA, 9 mol% of Glc and 5 mol% of Rha, showing that 33% were mannoproteins and 42% of arabinogalactans.

The amount of phenolic compounds of wine used in this study, and it variations according to the storage temperature and influence of the addition of the polysaccharide-rich material is shown in Table 3.21. The results obtained after 3 months of storage (W5, W5P, W30 and W30P) were compared with the initial values (WT0) by Dunnett test. Two-way ANOVA followed by a Tukey test ($p < 0.05$) was used to evaluate the effect of temperature and polysaccharide-rich material addition.

Table 3.21: General phenolic composition of wines at time zero (WT0), and after 3 months of storage, at 5 ºC (W5 and W5P) and at 30 ºC (W30 and W30P).

	Total Phenols (mg/L GAE)	Tannins (g/L)	Total anthocyanins (mg/L Mv3Glc)	AA (mM Trolox)	
				ABTS	DPPH
WT0	1803±142^{a}	1.23±0.08^{a}	411.6±19.8^{a}	9.3±0.3^{a}	8.5±0.2^{a}
W5	1820±53^{a}	1.04±0.03^{ab}	330.3±10.6^{b}	9.1±0.2^{a}	8.3±0.4^{a}
W5P	1892±76^{a}	1.06±0.07^{ab}	344.5±4.6^{b}	8.8±0.9^{a}	8.6±0.2^{a}
W30	1400±52^{b}	0.81±0.02^{c}	184.5±8.0^{c}	6.8±0.4^{b}	6.1±0.3^{b}
W30P	1530±84^{ab}	0.87±0.07^{bc}	212.5±12.2^{d}	7.0±0.3^{b}	6.2±0.4^{b}

mean ± standard deviation of triplicates. Different letters within each column represent significant statistically differences. The results obtained after 3 months were compared with the initial values according to the Dunnet test; Two-way ANOVA followed by a Tukey test ($p < 0.05$) was used to evaluate the effect of temperature and polysaccharide-rich material addition. For each column, the means value followed by the same letter are not significantly different ($p<0.05$).

The initial amount of phenolic compounds was 1803 mg GAE/L of wine. No statistical differences were observed between this value and the values obtained for the wines stored at 5 ºC. Compared with WT0, the wines stored at 30 ºC exhibited lower values of phenolic compounds quantified by the Folin-Ciocalteu reagent. However, only in the case of W30 it was statistically different. Accordingly, Gómez-Plaza *et al.* (2002) described a decrease from 1520 to 1291 mg GAE/L of phenolic compounds for red wines stored at 15-20 ºC during 3 months.

The amount of tannins (1.23 g/L vs 1.04 and 1.06 g/L) showed a slight but not significant variation after storage at 5 ºC, which is in accordance with the results obtained by Atanasova *et al.* (2002) for red wines after 1 and 7 months of

storage and also with data reported by Guadalupe and Ayestaran (2008a) for the maintenance of total tannins during 2 years of aging. The concentration of tannins significantly decreased during storage at 30 ºC. As regards phenolic compounds, no significant effect of polysaccharide-rich fraction was observed.

Regarding anthocyanins, it is known that anthocyanins also depend on the storage conditions decreasing under the effect of temperature (Maccarone *et al.*, 1985). However, their stability depends of the interaction with other molecules, phenolic or non-phenolics, namely polysaccharides. The previous results showed the interaction of polymeric material with anthocyanins, thus it may influence their stability. The initial amount of total anthocyanins, expressed as Mv3Glc equivalents, was 411.6 mg/L. All storage conditions induced significant decreases on the total amount of anthocyanins, especially at 30 ºC. The values found for the wines stored at 5 ºC corresponded to 80 and 84% of the initial amount anthocyanins while for the wines stored at 30 ºC they corresponded to 45 and 52%. The highest values were obtained for the wines stored in presence of polysaccharides-rich material. In case of the wines stored at 30 ºC it was possible to verify a significant difference in the anthocyanin concentration. The addition of the polysaccharide-rich material to the wines originated lower decreases of anthocyanins, suggesting that polysaccharides may interact with anthocyanins contributing for their stabilization. This decrease of anthocyanins content was consistent with the data presented by Wirth *et al.* (2010), who described decreases of anthocyanins after 5 and 10 months of bottling storage at 23 ºC. The decrease in anthocyanin content was probably due to anthocyanin condensation and polymerization reactions that occurred during wine storage (Cheynier *et al.*, 1994; Mazza, 1995).

The amount of monomeric anthocyanins present in the wines at time 0 and after 3 months of period storage is represented in Table 3.22. The wine (WT0) used in this work contained 294.2 mg/L of total monomeric anthocyanins, of wich Mv3Glc accounted the largest percentage (64%). This wine was composed by 234.1 mg/L of anthocyanins 3-Glc, 40.1 mg/L of anthocyanins 3-AcGlc, and 20.0 mg/L of anthocyanins 3-CmGlc.

Table 3.22: Monomeric anthocyanins composition (mg/L) of wines at time zero (WT0), and after 3 months of storage at 5 ºC (W5 and W5P) and at 30 ºC (W30 and W30P).

	WT0	W5	W5P	W30	W30P
Df3Glc	9.5±0.7^{a}	8.8±1.2^{a}	9.2±0.7^{a}	3.5±0.2^{b}	4.5±0.6^{c}
Pt3Glc	17.3±2.3^{a}	11.2±0.4^{b}	10.8±1.1^{b}	4.0±1.1^{c}	5.8±0.5^{d}
Pn3Glc	21.1±0.6^{a}	15.5±0.9^{b}	15.6±1.5^{b}	7.0±0.0^{c}	8.1±0.5^{d}
Mv3Glc	186.2±11.9^{a}	116.1±10.4^{b}	123.1±7.1^{b}	50.5±2.8^{c}	59.9±4.0^{d}
Df3AcGlc	9.4±0.6^{a}	7.4±0.8^{b}	7.6±0.6^{b}	4.0±0.4^{c}	4.5±0.2^{c}
Pt3AcGlc	3.7±0.7^{a}	3.7±0.6^{a}	3.2±0.4^{ab}	1.6±0.4^{c}	2.4±0.1b^{c}
Pn3AcGlc	13.3±1.9^{a}	7.6±0.4^{b}	8.3±0.5^{b}	4.5±0.4^{c}	4.1±0.6^{c}
Mv3acglc	13.7±1.0^{a}	7.9±0.2^{b}	7.8±0.8^{b}	3.1±1.0^{c}	3.3±1.5^{c}
Pn3CmGlc	10.6±2.1^{a}	7.6±0.9^{ab}	7.8±1.9^{ab}	3.6±0.9^{c}	6.1±1.0^{b}
Mv3CmGlc	9.5±0.2^{a}	6.6±0.9^{b}	7.2±1.9^{b}	3.5±0.6^{c}	5.8±1.0^{b}
Total (mg/L)	294.2±17.2^{a}	191.1±8.0^{b}	200.7±9.5^{b}	85.1±1.6^{c}	104.4±7.0^{d}

mean ± standard deviation of triplicates. Different letters within each row represent significant difference according to the Tukey test ($p \leq 0.05$).

For all storage conditions, significant decreases were observed in the amount of monomeric anthocyanins during the storage period. The amount of each individual anthocyanin was found to decrease during this period, however with different statistical importance. All these changes were enhanced with the increase of the temperature.

For wines stored at 5 ºC, the amount of monomeric anthocyanins of W5 and W5P decreased 35 and 32% when compared with WT0. In the case of wine samples stored at 30 ºC, (W30 and W30P samples), decreases of 71 and 65%, respectively, of the total monomeric anthocyanins occurred when compared with WT0. For this storage temperature, the addition of polysaccharide-rich fraction prevented significantly the decrease of anthocyanins. Accordingly, decreases of 37% and 20% on total monomeric anthocyanins was described to occur in wines during a 3 months period at 15 ºC (García-Falcón *et al.*, 2007).

The total monomeric anthocyanins, estimated by HPLC, represented in WT0 71% of the total anthocyanins, estimated colorimetrically. After 3 months, this percentage was 58 and 48%, for wines stored at 5 and 30 ºC, respectively. This might indicate that the decrease in the concentrations of the monomeric anthocyanin structures was due to their participation in reactions of polymerization

(Guadalupe & Ayestarán, 2008a). In agreement, Tsai *et al*., (2004), described a decrease from 97 to 76% of the percentage of monomeric antochyanins and a increase from 3 to 23% of the polymeric anthocyanins after 3 months of storage of a red wine at 20ºC.

Regarding the individual behavior, the rate of loss of the different anthocyanins during aging did not depend on their initial concentration. For example, a similar rate of loss was observed for Mv3Glc than Pt3Glc, whereas Mv3Glc was initially present in tenfold higher concentration than Pt3Glc.

On average, the variation in the content of the different anthocyanin families (anthocyanins 3-Glc, anthocyanins 3-AcGlc, and anthocyanins 3-CmGlc) at 5 ºC was similar, ranging from 71–75% of those initially present (Figure 3.20). No significant effect was possible to observe neither on total amount, neither on individual anthocyanins due the addition of polysaccharides-rich material. In accordance, the addition of commercial mannoproteins had no effect on total red wine monomeric anthocyanins composition during 45 days of oak aging (Guadalupe & Ayestarán, 2008b). Otherwise, Gualalupe and Ayestarán (2008a), reported that the acetylated anthocyanins were the most resistant to degradation during 24 months of botlle storage at 14 ºC.

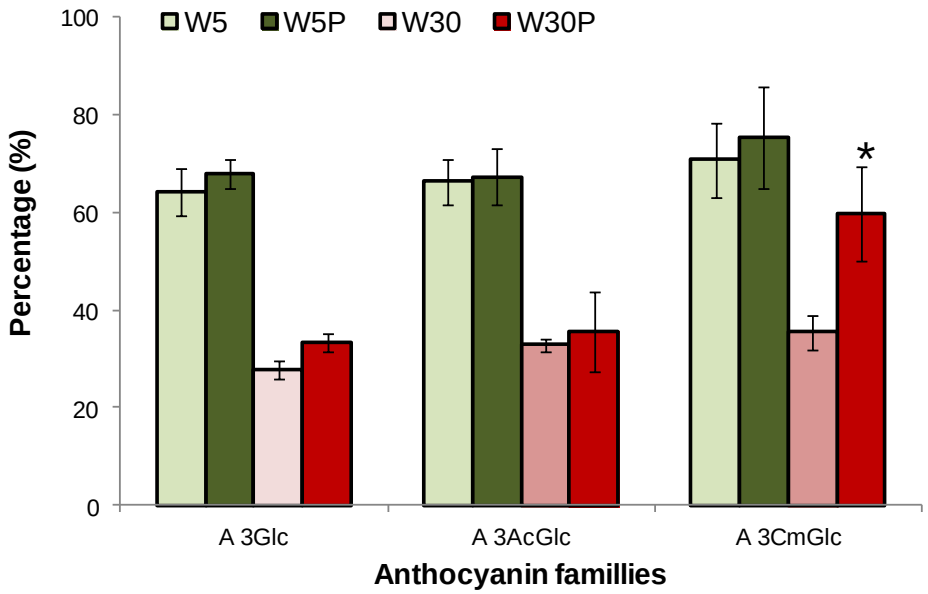


Figure 3.20: Maintenance (%) of anthocyanin families after storage. A 3-Glc- anthocyanin 3-glucosides; A3-AcGlc- anthocyanins 3-(acetyl)glucosides; A3-CmGlc c) anthocyanins 3-(coumaroyl)glucosides. * statistically different from W30 ($p<0.05$).

The total amount of monomeric anthocyanins quantified in W30P was 23% higher than W30. The HPLC analysis of individual anthocyanins of wine samples stored at 30 ºC showed significant differences among W30 and W30P for the anthocyanins 3-Glc and anthocyanins 3-CmGlc. The average value obtained for anthocyanins 3-CmGlc and for anthocyanins 3-Glc in W30P was 68 and 20% higher than that observed for W30. In general, W30P contained slightly higher average amounts of anthocyanins 3-AcGlc, however not statistically different. It can be concluded, that in the presence of polysaccharides-rich material, the monomeric anthocyanins exhibited more resistance to degradation. These results suggested a preferencial interaction of the polysaccharides-rich material with coumaroylated anthocyanins, reinforcing the previous results. Accordingly with results of previous section, this interaction may be due to the presence of arabinogalactans and mannoproteins.

The value of antioxidant activity of WT0, measured by ABTS and DPPH assays were 9.3 and 8.5 mM of TE, respectivelly. Similar values of antioxidant activity were obtained for the wines stored at 5 ºC. These results are in good agreement with those described by Sun *et al.* (2011), that verified no significant alterations on antioxidant activity of red wines after 6 months of storage at room temperature. The wines stored at 30 ºC presented significant lower values of antioxidant activity, representing 73 and 76% of the initial value.

In order to evaluate the contribution of the different phenolic classes of composunds to the antioxidant activity of wines, the amount total phenols, tannins, anthocyanins and total monomeric anthocyanins was plotted against the respective antioxidant activy determined by ABTS and DPPH assays Figure 3.21. The correlation coefficient (r^2) was determined for all phenolic parameters and antioxidant activity for both methods.

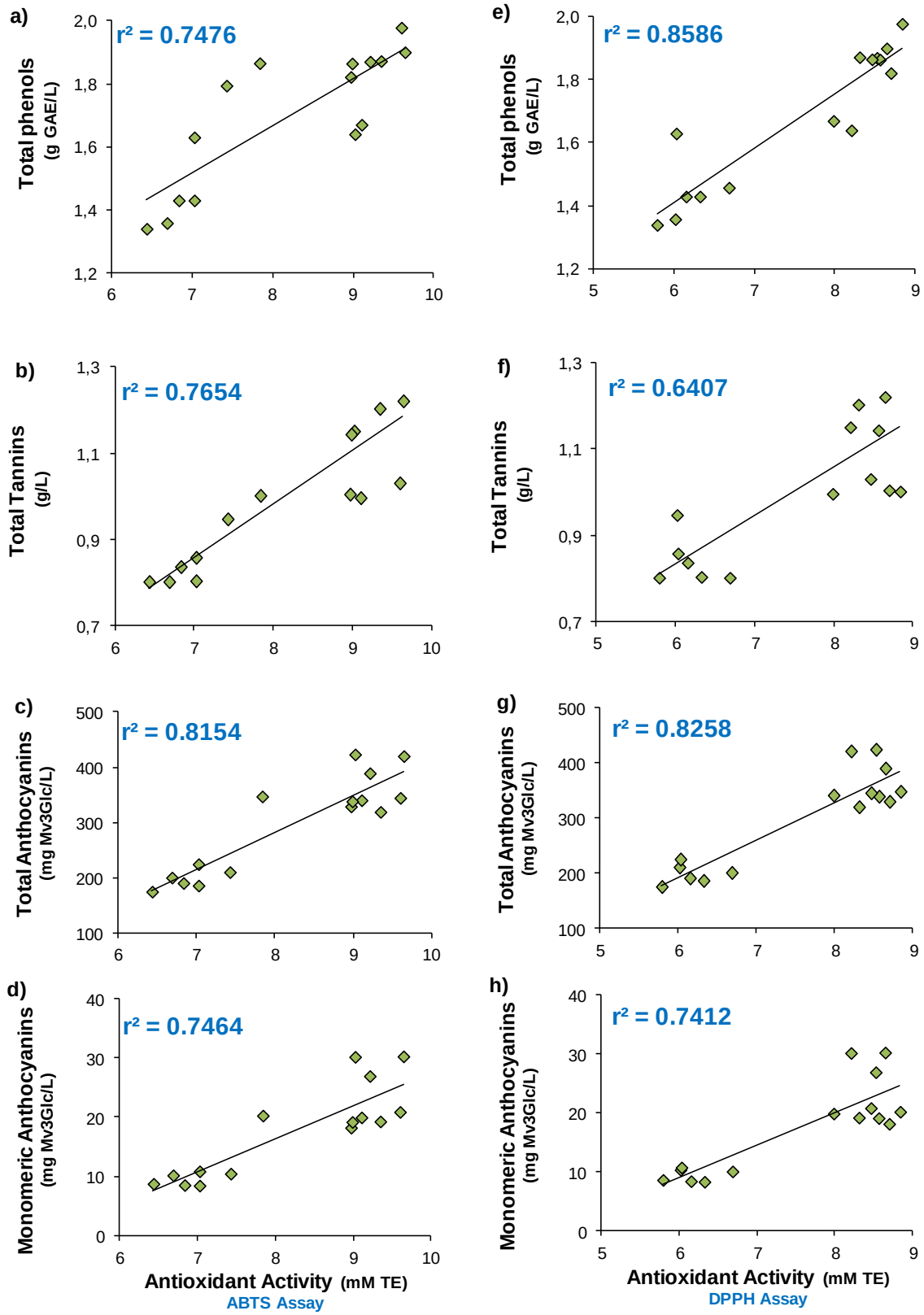


Figure 3.21: Linear relationship and correlation coefficient (r^2) between total phenols, tannins, total anthocyanins and monomeric anthocyanins and antioxidant activity of wine samples. a-d) ABTS assay; e-h) DPPH assay.

For ABTS, the best correlation was observed with total anthocyanins (r^2= 0.8154), indicating a good contribution for antioxidant activity. For DPPH, the antioxidant capacity seemed strongly correlated with total phenols (r^2= 0.8586), in accordance with results reported in literature (Katalinic *et al.*, 2004; Villano *et al.*, 2006). The changes on phenolic compounds, namely anthocyanins, with formation of new compounds, led to the decrease of wine antioxidant activity. For example, the formation of cyclic-adducts led to a loss of an active OH group in the meta-position of the A-ring from the flavylium cation mainly responsible for the loss in the antiradical activity (Rice-Evans *et al*., 1996).

In order to try to check the possible relationship between the amount of the individual monomeric anthocyanins and the antioxidant activity of wines, it was plotted their linear relationship, Figure 3.22 for ABTS assay and Figure 3.23 for DPPH assay. The antioxidant activity of flavonoids is dependent of the position and degree of hydroxylation of B ring. In particular, the presence of *o*-dihydroxylation (3', 4' hydroxyl) in the B ring is fundamental to the antioxidant activity of anthocyanins. This substitution in the B ring is important for stabilizing the resulting free radical form.

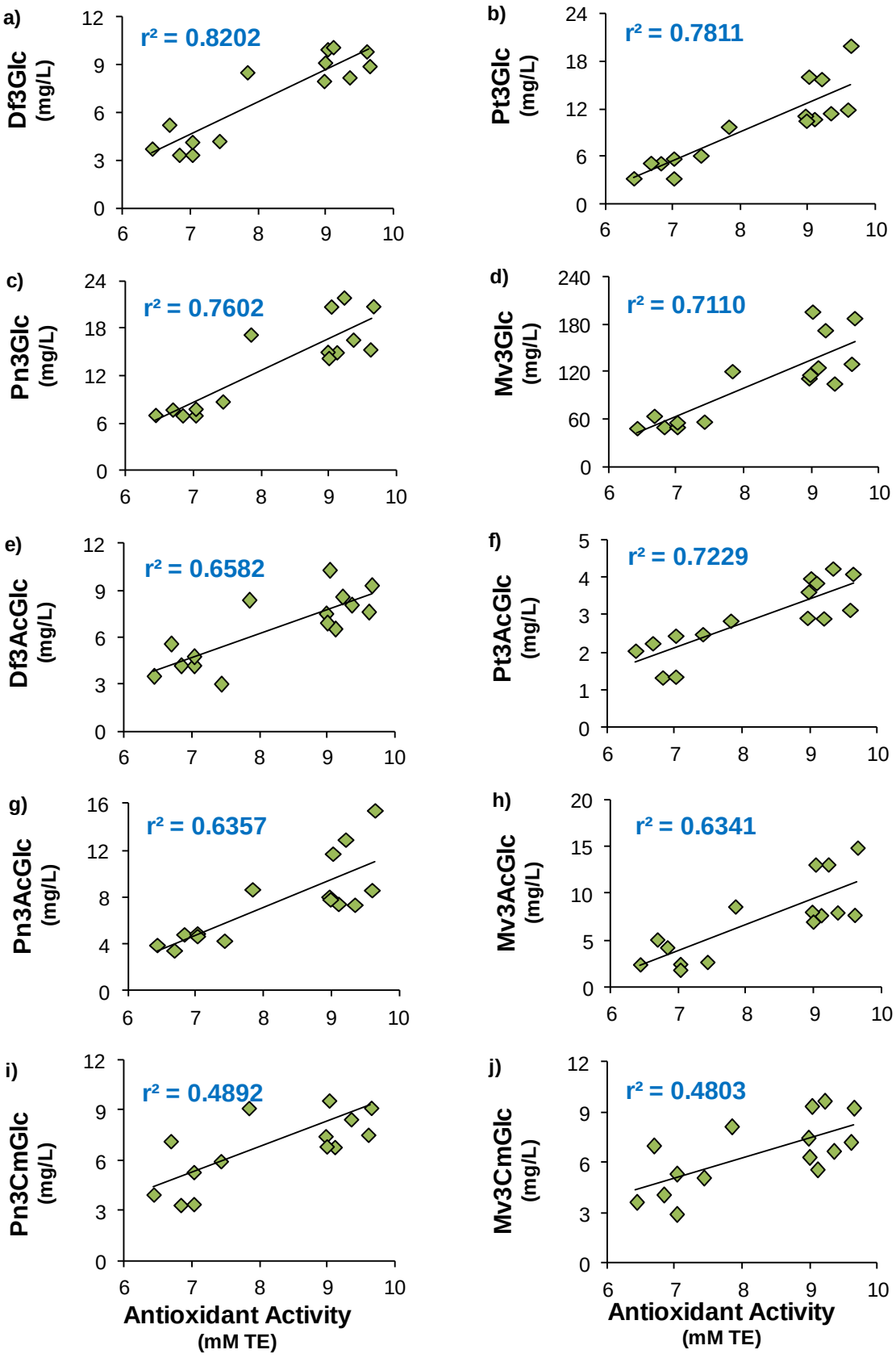


Figure 3.22: Linear relationship and correlation values (r^2) between individual monomeric anthocyanins and antioxidant activity values of wine (ABTS assay).

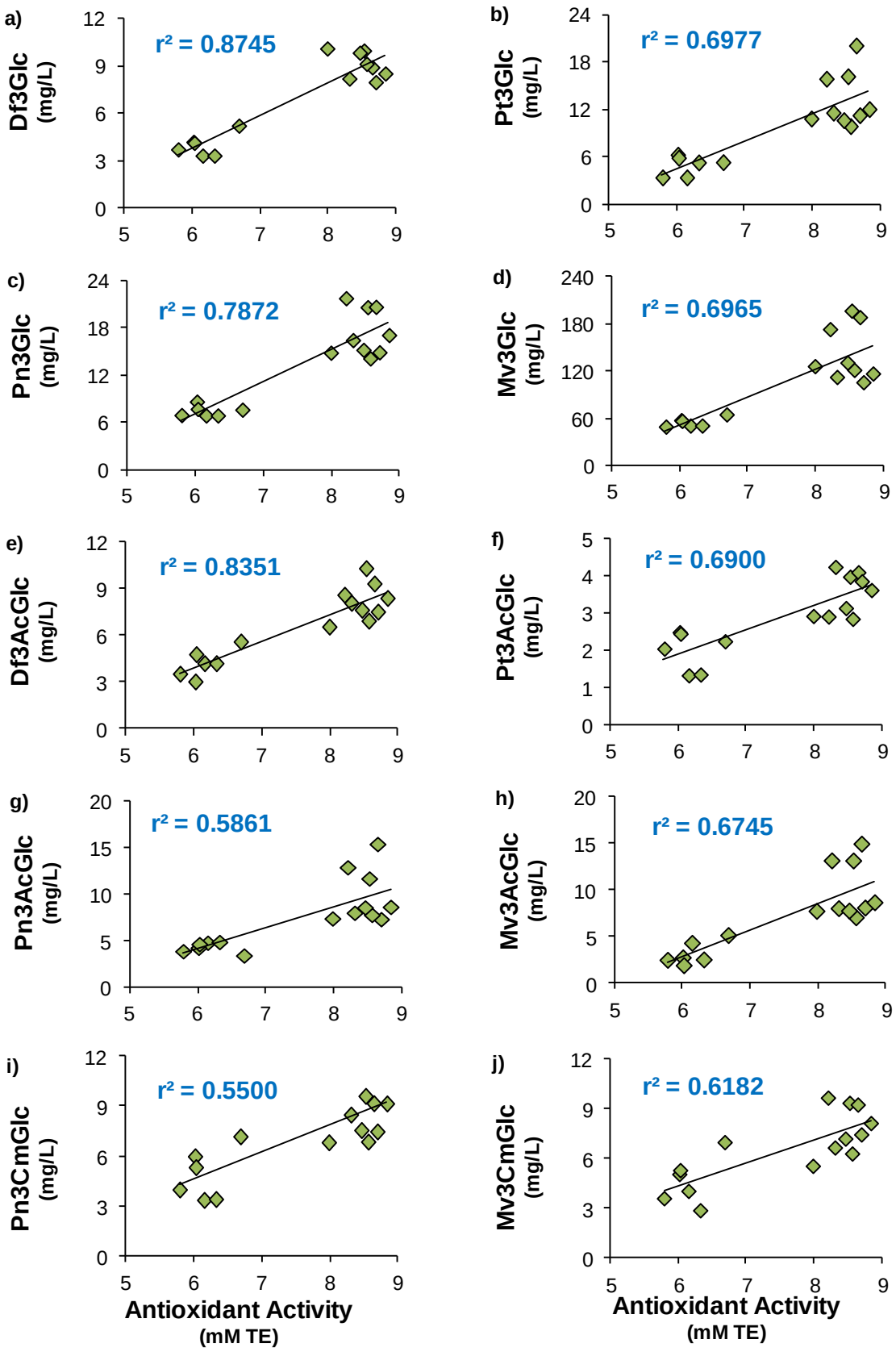


Figure 3.23:Linear relationship and correlation values (r^2) between individual monomeric anthocyanins and antioxidant activity values of wine samples (DPPH assay).

The correlation coefficients values of linear relationship between monomeric anthocyanins and antioxidant activity are showed in Figure 3.22 and Figure 3.23. A very close relationship between the amount of Df3Glc and antioxidant activity of wines was obtained for both, ABTS (r^2 = 0.8202) and DPPH (r^2 = 0.8745) assays. Also, strong correlation with the acetyl derivative of delphinidin (Df3AcGlc) was observed for DPPH (r^2= 0.8351). This is in accordance with their structural features of B ring, which possess three hydroxyl groups in the B ring.

Mv3Glc, the main individual anthocyanin present in all wines, showed correlation coefficients of 0.7110 and 0.6965 for ABTS and DPPH assays, respectively. As observed to ABTS assay, also to DPPH assay, the coumaroyl derivatives exhibited low correlation coefficients (Figure 3.23). In accordance, Rice-Evans *et al*. (1996) described values of antioxidant activity for delphinidin twofold higher than malvidin. In general, the acylated derivatives showed lower correlation coefficients than non-acylated. The correlation coefficients obtained were higher than those reported by Di Majo *et al.* (2008) (ranging from 0.29 to 0.54) for some individual anthocyanins (cyanidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside) with the antioxidant activity of red wines.

The results obtained for a storage temperature of 30 ºC showed that the addition of wine polysaccharides may be a form to preserve anthocyanin levels during 3 months.

3.3.4 Final remarks

The results obtained allowed to conclude that:

- The presence of a nitrogen atmosphere allows the preservation of antioxidant activity of phenolic compounds.
- The antioxidant activity was best preserved when the phenolic extracts were stored in the dark, inside a glass desiccator under a nitrogen atmosphere. In these conditions, 95% of the antioxidant activity was preserved after 342 days of storage at room temperature.
- The total phenolic compounds, total tannins, and antioxidant activity did not change significantly when the wines were stored at 5 ºC during 3 months. However, a significant decrease in the amount of total and individual monomeric anthocyanin content occured.
- The wines stored at 30 ºC present lower values of total phenolic compounds, tannins, and anthocyanins than those stored at 5 ºC. Also, the antioxidant activity exhibited the same tendency.
- The addition of polysaccharides promoted protection to the anthocyanin content of wines stored at 30 ºC.

3.4 Study of Biological Effects

3.4.1 Overview

The results obtained previously showed that different wine fractions possess antioxidant activity evaluated by ABTS and DPPH. However, this type of assays did not provide evidence that these fractions have an antioxidant activity in cells. The potential biological effects depend on bioavailability and bioaccessibility of the bioactive compounds. Also, bioaccessibility of phenolic compounds is dependent of their stability during the gastrointestinal tract. To examine the stability of the dealcoholized wine phenolic compounds along an *in vitro* model of the digestive tract, it was evaluated the phenolic composition and antioxidant activity after each phase simulating the composition of mouth, stomach, and small intestine.

The results obtained previously showed that different wine fractions possess antioxidant activity evaluated by ABTS and DPPH assays. However, this type of assays did not provide evidence that these fractions have an antioxidant activity in cells. Thus, the objective of this section was to evaluate the effect of these compounds on cells viability and their protective action using *in vitro* cell systems. In the first experiment, the phenolic fractions (F1, F2, and F3) obtained from C18 sep-pak cartridge separation were tested in mammary epithelial (HC11) and human colon carcinoma (HT-29) cell lines to evaluate the cell viability by the WST-1 assay. Also, the cells were exposed to two oxidative agents (UV radiation and H_2O_2) in order to evaluate the potential protective effect of these compounds against these damage agents. This exposure was performed in the presence and in the absence of phenolic compounds. After the exposure to the damage agent, the medium was replaced by newly solutions of phenolic compounds and incubated during 48 h. After that, it was evaluated the effect on cells viability.

The objective of a second set of experiments was to evaluate the anti-inflammatory activity of wine extracts. For that, lipopolysaccharide (LPS)-stimulated Raw 264.7 macrophages were used as an *in vitro* cell model. The

macrophages were exposed to an inflammatory stimulus in the presence and in the absence of the extracts. The effect on the production of nitric oxide, a pro-inflammatory mediator, originated by the addition of extracts, was evaluated by the Griess reaction. The fractions tested were the wine phenolic compounds (WFC), wine polysaccharides fraction (WPS), phenolic acids extract (PA-E), procyanidins rich extract (PR-E), and the anthocyanin rich fraction (APP-E), obtained from DW2. The selection of the concentrations used was based on the value of the EC_{50} of each extract, using the ABTS assay. Also, the citotoxicity of the extracts in the concentrations tested was evaluated by the MTT assay.

In the third set of experiments, with the goal of elucidating the antioxidant properties of dealcoholized wine extracts in human cells, red blood cells (RBC) were selected as a metabolically simplified model system. The anti-hemolytic efficacy of extracts were tested as inhibitors of hydrogen peroxide (H_2O_2)- and 2,2´-Azo-bis(2-amidinopropane) dihydrochloride (AAPH)-induced RBC hemolysis. The fractions tested were the wine polymeric material (WPM), wine polysaccharides fraction (WPS), wine phenolic compounds (WFC), phenolic acids extract (PA-E), procyanidins rich extract (PR-E), and the anthocyanin rich fraction (APP-E), obtained from DW2.

3.4.2 Stability of phenolic compounds under *in vitro* simulated conditions of digestive tract

The aim of the present study was to evaluate the stability of wine phenolic compounds in the digestive tract. For that, the dealcoholized red wines (DW1 and DW2) were passed through an *in vitro* model that simulated the composition of mouth, stomach and small intestine juices. The method consists of three sequential phases: an initial phase with α-amylase during 2 min to simulate mouth conditions, followed by pepsin/HCl digestion for 2 h to simulate gastric conditions and finally the digestion with bile salts/pancreatin for 2 h to simulate small intestine conditions. All phases were carried out at 37 ºC mimmicking body temperature. The amount of phenolic compounds (by the Folin-Ciocalteu reagent) and the antioxidant activity (by DPPH assay) was determined before and along simulation.

The concentration of phenolic compounds initially determined was 933 mg/L and 2659 mg/L, for RW1 and RW2, respectively. Figure 3.24 shows the results obtained for phenolic compounds of red wines after each step, expressed as percentage of remaining phenolic compounds. In both wines, there was a progressive decrease in the amount of phenolic compounds present as they moved through the simulated phases.

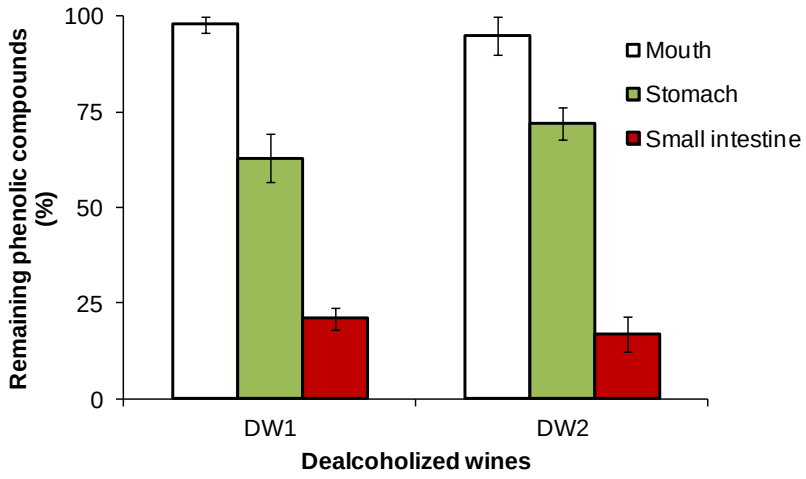


Figure 3.24: Recovery of total phenolic compounds (%) after simulated phase of digestive tract for dealcoholized wine 1 (DW1) and dealcoholized wine 2 (DW2).

It can be seen from Figure 3.24 that the amount of phenolic compounds determined after the exposure to the mouth conditions was similar to that before digestion. On average, the percentage of phenolic compounds after the exposure to gastric phase represented 63% for DW1 and 72% for DW2 of the initially quantified, in accordance with the 66% of recovery of phenols after the gastric digestion, assayed in the red wine described by McDougall *et al*. (2005). A decrease in the amount of total phenolic compounds was observed in the small intestine conditions when compared to the gastric ones. After duodene stage, the amount of phenolic compounds was 80 and 205 mg GAE/L, which means that only 21 and 17 % of the initial amount of phenolic compounds was potentially accessible for absortion.

The changes in antioxidant activity of the samples in the *in vitro* digestive tract model are shown in Figure 3.25. As for phenolic compounds, the antioxidant activity decreased along the different phases of digestion. In general, both dealcoholized wines had a similar behavior in the different steps of the simulated model. A higher decrease of total phenolic compounds was verified to occur in intestinal phase when compared with gastric and mouth phases. After crossing gastrointestinal simulated conditions, the antioxidant activity represented around 20 % of its initial value.

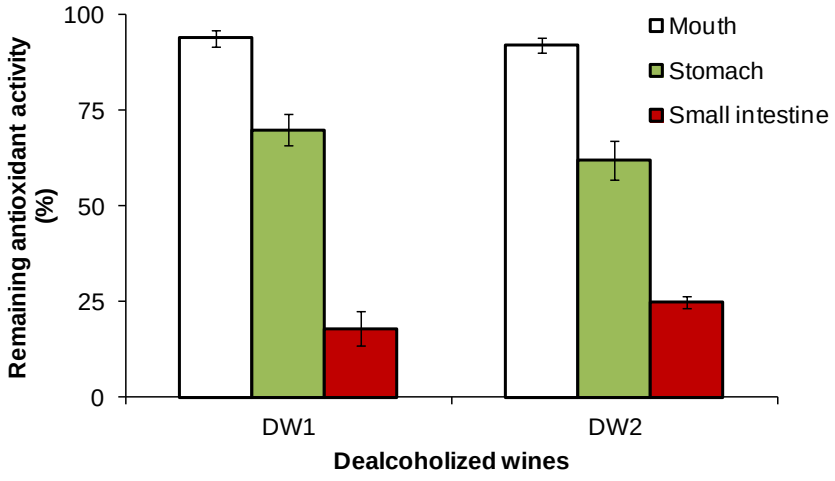


Figure 3.25: Antioxidant activity (%) after each simulated phase of digestive tract for dealcoholized wine 1 (DW1) and dealcoholized wine 2 (DW2).

The bioaccessibility of a phenolic compound differs greatly from one to another and is dependent upon its release from the food matrix (Manach *et al*., 2005; Ortuno *et al*., 2010). Thus, the impact on the antioxidant activity that occurs for the different classes of phenolic compounds as they pass through the gastrointestinal system was evaluated. For that, the fractions F1 (phenolic acids), F2 (catechins and oligomeric procyanidins) and F3 (anthocyanins and polymeric procyanidins) of phenolic compounds obtained by C18 sep-pak cartridge, were submitted to *in vitro* gastrointestinal conditions.

Figure 3.26. shows the relative amount of phenolic compounds of each fraction after the simulated digestion. In general, the tendency observed for DW1 and DW2 was also observed for the fractions, allowing to conclude that accordingly to the model used, the digestion decreased in comparable proportions the amount of all classes of phenolic compounds. No statistically differences were found between the behavior of the three fractions.

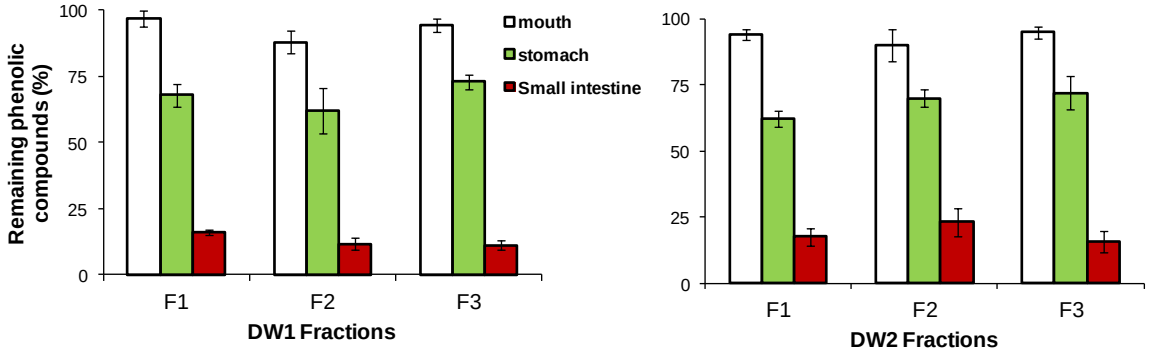


Figure 3.26: Recovery of total phenolic compounds (%) after each simulated phase of digestive tract for F1, F2 and F3 of dealcoholized red wine 1 (DW1) and dealcoholized wine 2 (DW2).

After mouth simulation, the amount of compounds present in F1 fraction was approached the initial amount. The percentage of recovery of phenolic acids of F1 was 68 and 62%, for DW1 and DW2, after gastric digestion, decreasing to 16 and 12 % after of the small intestine phase, respectively. Accordingly, Tagliazucchi *et al.* (2010), reported the degradation of 25% and 43% of caffeic and gallic acid solutions, respectively, during the incubation in the small intestinal

environment. However, the data obtained by these authors showed high stability (> 95%) of gallic and caffeic acids in stomach step.

The relative amount of fraction F2 was slightly lower than of F1, what could be due to interactions of procyanidins preventing their reaction with the Folin-Ciocalteu reagent. For F2 fraction, containing catechins and procyanidins, the higher reduction on the amount was observed during small intestine phase. Concerning this class of polyphenols, contradictory data are available in literature about their behavior along the digestive tract. For example, in accordance with the results obtained for DW1 and DW2, Bouayead *et al.* (2012) reported the degradation of the catechin obtained from apple intestine digestion, On the contrary, data obtained by Tagliazucchi *et al*. (2010) showed high stability of a solution of catechin during this phase. Furthermore, some studies (Bouayed *et al*., 2012; Tourino *et al*., 2011) suggested that oligomeric procyanidins are cleaved during the gastrointestinal track transit, while other authors (Tsang *et al*., 2005) suggested that procyanidins are not depolymerized.

The amount of phenolic compounds of anthocyanin and polymeric procyanidins-rich fraction (F3) did not change after mouth simulation. The percentage of recovery of F3 was 72% after the gastric phase, a lower value when compared with the almost 100% of recovery of anthocyanins of a red wine reported by McDougall *et al*. (2005). The incubation with small intestine juices decreased the bio-accessibility of F3 fraction to 11 and 16%, in DW1 and DW2, respectively. In agreement, McDougall *et al.* (2005) reported the decrease (~60 %) of anthocyanins quantified after *in vitro* digestion. Also, 50% of the amount of grape anthocyanins were lost after intestinal digestion (Tagliazucchi *et al*., 2010). The behavior of anthocyanins during digestion may be explained by the different stability of their forms with pH changes. The stability under gastric digestion anthocyanins is due to acid stability of the predominant flavylium cation form of anthocyanins at pH 2. When pH increases to neutral or slightly alkaline pH, the chalcone pseudobase begins to dominate originating the destruction of the anthocyanin structure (McDougall *et al*., 2005; Tagliazucchi *et al*., 2010).

As performed to total phenols, the antioxidant activity of these fractions was determined following the simulated digestive tract steps (Figure 3.27).

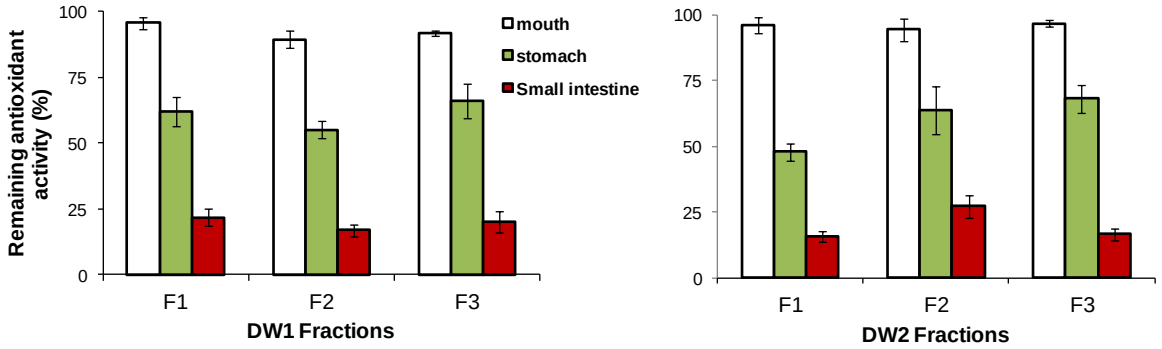


Figure 3.27: Recovery of antioxidant activity (%) after each simulated phase of digestive tract for F1, F2 and F3 of dealcoholized red wine 1 (DW1) and dealcoholized red wine 2 (DW2).

The profile of antioxidant activity, obtained by DPPH assay, was comparable to those observed to phenolic compounds. A similar behavior was observed for all fractions, where the higher decreases in the amount of phenolic compounds and antioxidant activity occurred in the small intestine conditions. The results obtained, showed that the amount of phenolic compounds and antioxidant activity after *in vitro* digestion model represented only 20 % of the initial values before pass throught the simulated phases of digestive tract.

3.4.3 Effect of phenolic extracts on viability of HC11 and HT-29 cells

Epidemiological studies have shown that polyphenols isolated from red wine inhibited the growth of some tumor cells *in vitro* (Gomez-Cordoves *et al.*, 2001; Iijima *et al.*, 2000). Thus, the effect of phenolic fractions (F1, F2 and F3), isolated from red wine 2, on the growth of human normal and tumor cells *in vitro* lines was investigated. F1, F2 and F3 fractions were obtained by C18 sep-pak cartridge eluted with phosphate buffer pH 7.0, ethyl acetate and methanol. To avoid cell toxicity, the organic solvents were eliminated and the samples were resuspended in an aqueous solution with 0.1% of ethanol, that is not toxic at this concentration. F1, F2 and F3 were mainly composed by phenolic acids, procyanidins and anthocyanins, respectively.

The WST-1 reagent (water-soluble tetrazolium; 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) is a ready-to-use substrate which measures the metabolic activity of viable cells. The cells were grown in a 96-well tissue culture plate. The reagent WST-1 was added and the cells were incubated for variable periods (0.5 to 4 h). The cleavage of the WST-1 reagent by plasma membrane enzymes of live cells yields soluble formazan salt (Berridge *et al.*, 2005; Ishiyama *et al.*, 1996). This reduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. WST-1 reaction product can be quantified colorimetrically. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

3.4.3.1 Effect of phenolic compounds on cell viability

The effect on cell viability of phenolic compounds was performed in mammary epithelial (HC11) and human colon carcinoma (HT-29) cells. In the first approach, HC11 cells were incubated during 48 h in the absence (control) or in the presence of the phenolic compounds. The tested doses (Table 3.23) ranged from 90, 500 and 300 mg GAE/L, in dilution $1x10^{-3}$ ($d1x10^{-3}$) to 0.090, 0.500 and 0.300 mg GAE/L, in dilution $1x10^{-6}$ ($d1x10^{-6}$) for F1, F2 and F3 fractions, respectively.

Table 3.23 : Total phenols (mg GAE/L) present in the different dilutions of the phenolic fractions

	Phenolic fraction		
Dilution	F1	F2	F3
$d10^{-3}$	90	500	300
$d10^{-4}$	9.0	50.0	30.0
$d10^{-5}$	0.90	5.00	3.00
$d10^{-6}$	0.09	0.50	0.30

Phenolic fractions obtained by C18 sep-pak cartridges. F1- eluted with pH 7.0 phosphate buffer; F2- eluted with ethyl acetate; F3- eluted with acidic methanol.

The results obtained, represented as cell survival rate, considering 100% the control, after 1 h of WST-1 incubation, for the addition of the phenolic solutions to the cells (HC11), are shown in Figure 3.28a. The cell survival rate ranged from 105 to 128 % for F1, 90 to 110 % for F2 and 91 to 97 for F3, showing not significant differences by the addition of phenolic compounds.

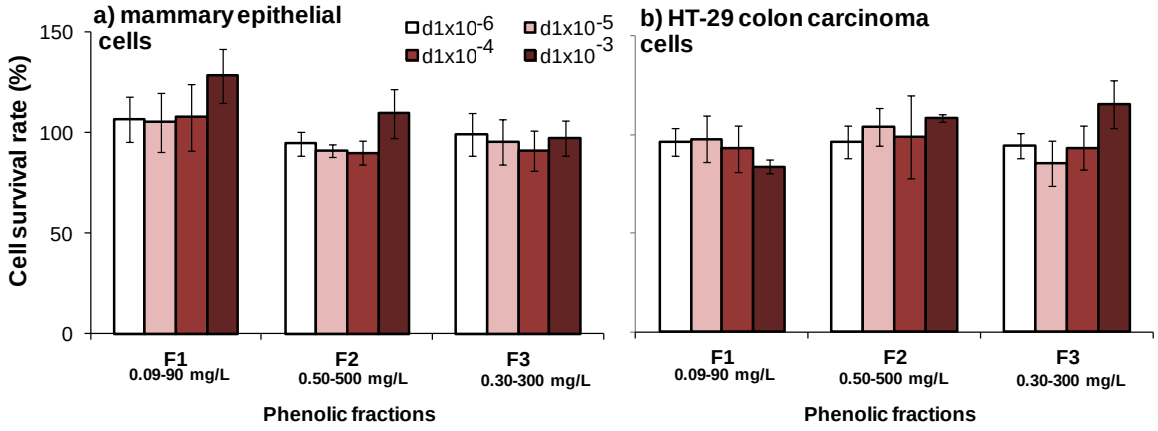


Figure 3.28 : Effect of addition of the diferent dealcoholized wine fractions obtained by C18 fractionation on cell survival rate after 1 h of WST-1 incubation. a) HC11 cells; b) HT-29 cells. F1- fraction eluted with pH 7.0 phosphate buffer; F2- eluted with ethyl acetate; F3- eluted with acidic methanol.

The results obtained for HT-29 colon carcinoma cells (Figure 3.28b) were similar to those obtained for HC-11 cells, whereas no significant effect was observed by the addition of phenolic compounds to the cells. However, some authors reported an antiproliferative activity of red wine and of phenolic compounds on HT-29 cells. The results obtained by Briviba *et al.* (2002) indicated that phenolic compounds of red wine, in concentration of 16.2 mg GAE/L, inhibited the proliferation of HT-29 carcinoma cells by modulating mitogene-activated protein kinases intracellular signal transduction pathways. Yun *et al.* (2010) reported the antiproliferative activity on HT-29 cells of an anthocyanins fraction isolated from fruits of *Vitis coignetiae Pulliat*., composed mainly by Df3Glc, Pt3Glc, Pn3Glc and Mv3Glc; the concentration used ranged from 10 to 400 mg/L, however the anti-proliferative effect was observed upper to 100 mg/L, in a dose-dependent.

3.4.3.2 Effect of phenolic compounds against H_2O_2-induced damage on cell viability

The HT-29 colon carcinoma cell line was chosen because the free radical production has been shown by inflammatory cells in intestinal mucosa (Ruan *et al.*, 1997). In addition, the phenolic fractions can cross the gastrointestinal tract and reach colon, and interact with these cells potentially exerting a health benefit in human body due their antioxidant activity. The effect of phenolic compounds is dependent of their concentration and also vary according to the physiology of the tested cells. For example, Glavas-Obrovac *et al.* (2006) obtained different growth inhibitory effects on HT-29 and Caco-2 cells testing four red wines.

The protective effect of the phenolic fractions against H_2O_2 oxidative agent was evaluated. For that 0.5 mM of H_2O_2 was added to the cells in the presence of the different phenolic compounds. The cell survival rate (CSR) of each fraction was compared with the cells not exposed to H_2O_2 (control) and expressed as percentage. To evaluate the potential protective effect of the phenolic compounds, the CSR of cells in presence of phenolic frations was compared with the CSR of

cells exposed to H_2O_2 in absence of phenolic compounds (C0.5). The results obtained after 1 h of WST-1 incubation are shown in Figure 3.29.

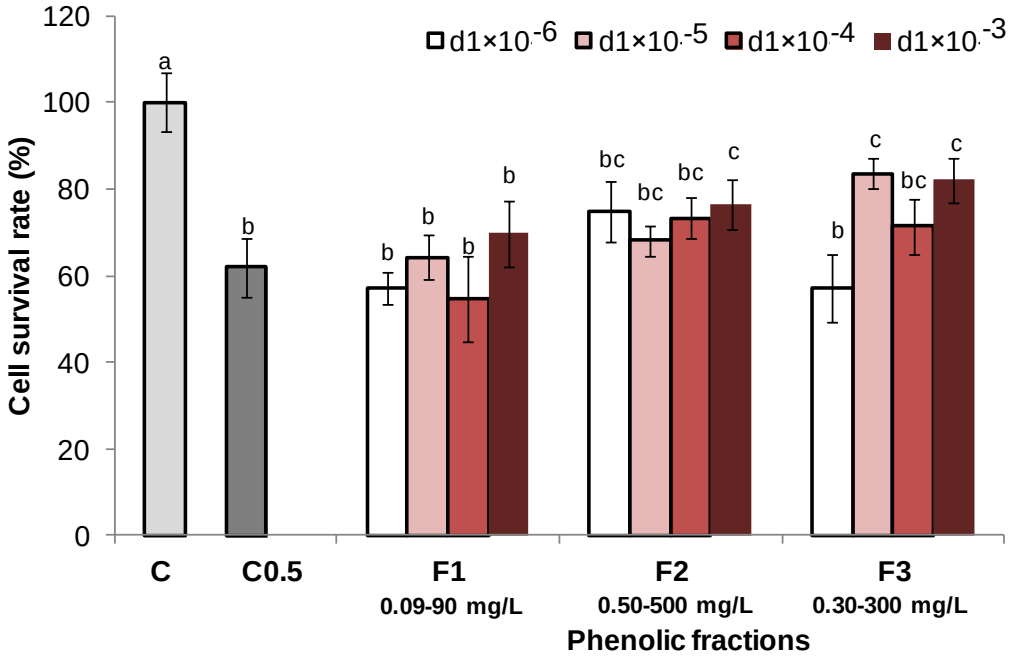


Figure 3.29: Effect of presence of phenolic fractions on cell survival rate after addition of 0.5 mM H_2O_2. Phenolic fractions obtained by C18 sep-pak cartridges. F1- eluted with pH 7.0 phosphate buffer; F2- eluted with ethyl acetate; F3- eluted with acidic methanol. Bars showing the same letter are not significantly different according to the Dunnett test followed by Tukey test ($p \leq 0.05$)

The CSR of cells exposed to 0.50 mM of H_2O_2 was 62% when compared with the CSR of control cells. When the addition of H_2O_2 was performed in presence phenolic acids (F1) were not observed significance variations on the CSR, comparing with C0.5. Regarding F2 fraction, a tendency to an increase on the CSR (68–77%) was observed, however the difference only was statistically significance in case of the highest concentration of procyanidins (500 mg GAE/L). When the addition of 0.5 mM H_2O_2 was performed in the presence of F3 higher CSR were verified to occur, with statistical significance in the case of concentrations 3 and 300 mg GAE/L ($d10^{-5}$ and $d10^{-3}$, respectivelly, Figure 3.29) reaching 84 and 82 % of CSR.

As the results obtained for F2 and F3 fractions reached significant protection on cells, their protective effect was evaluated by addition of 0.25 and

1.00 mM of H_2O_2. For this study they were tested the lowest and the highest concentrations of F2 and F3 fractions.

All additions of H_2O_2 were performed in presence of the phenolic compounds. To C0.25, C0.50 and C1.00, it was added 0.25, 0.50 and 1.00 mM of H_2O_2 in absence of phenolic fractions, and used as control for the effect of those amounts of H_2O_2 in the CSR. The results obtained are shown in Figure 3.30.

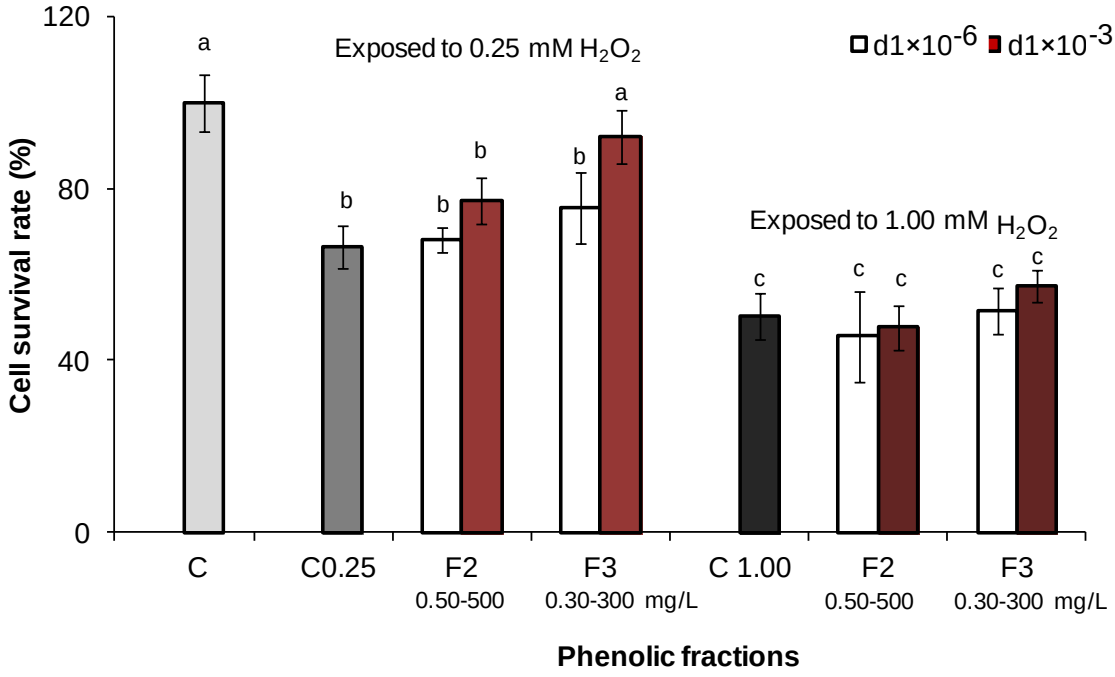


Figure 3.30: Effect of presence of phenolic fractions on cell survival rate after addition of 0.25 and 1.0 mM H2O2. Phenolic fractions obtained by C18 sep-pak cartridges. F2- eluted with ethyl acetate; F3- eluted with acidic methanol. Bars showing the same letter are not significantly different according to the Dunnett test followed by Tukey test ($p \leq 0.05$).

To C0.25 and C1.0 cells, it was added 0.25 and 1.0 mM of H_2O_2, respectively, in absence of phenolic compounds, and used as control for the effect of those amounts of H_2O_2 in the CSR. In case of addition of 0.25 mM of H_2O_2, the CSR was 66%, a statistically similar value when compared with the 68 and 76% of CSR observed for F2 fractions. Otherwise, the presence of 300 mg GAE/L of anthocyanins ($d10^{-3}$) led to a significant higher CSR (92%). When it was used the highest concentration of H_2O_2 (1.0 mM), no significant effect was observed by addition of any phenolic compounds.

3.4.3.3 Effect of phenolic compounds against UV-induced damage on cell viability

The protective effect of phenolic fractions was evaluated in cells exposed to 10 min of UV radiation. This exposure followed two methodologies: in the first case, the exposure to the irradiation was performed in the presence of the compounds, while in the second case, the compounds were added after the exposure to UV light. The results obtained when the exposure was performed in presence of phenolic fractions are shown in Figure 3.31.

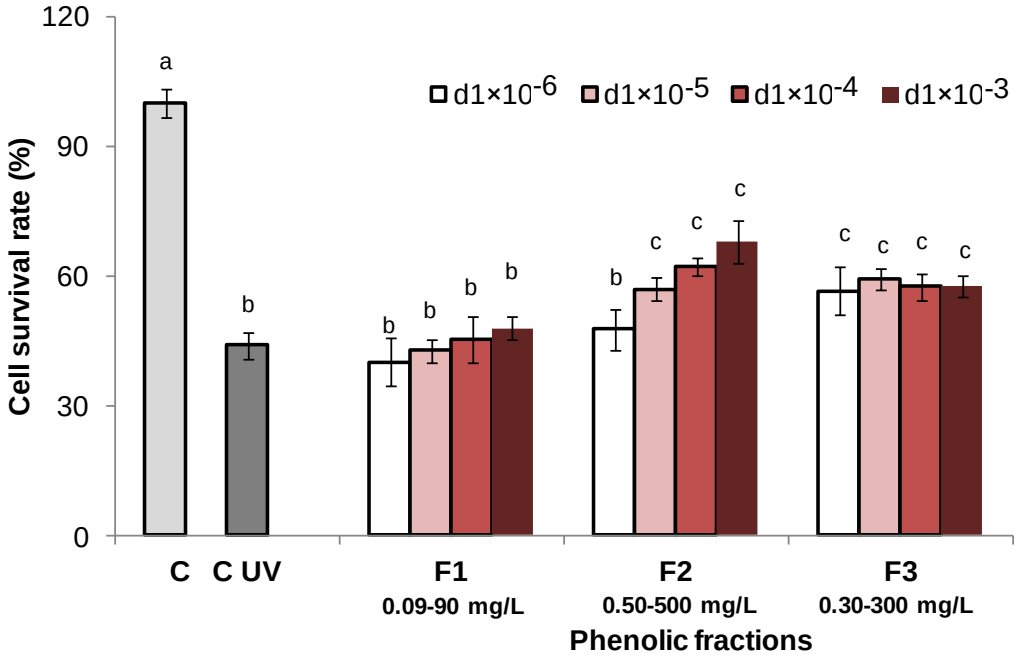


Figure 3.31: Effect of exposure (10 min) to UV light in presence of different dealcoholized wine fractions obtained by C18 fractionation on cell survival rate after 1 h of WST-1 incubation. F2- eluted with ethyl acetate; F3- eluted with acidic methanol. Bars showing the same letter are not significantly different according to the Dunnet test ($p \leq 0.05$).

When the cells were exposed to the UV radiation (C UV), the cell survival rate (CSR) observed was 44%, compared with the not exposed control cells (C-cells). For F1 (phenolic acids-rich fraction) it was not possible to observe a significant effect on CSR. In case of addition of F2 (procyanidins-rich fraction), a significant higher survival rate was verified to occur in dilution $1x10^{-5}$, wich represented a concentration of 5.00 mg GAE/L of phenolic compounds. In case of the highest dose ($d1x10^{-3}$, 500 mg/L), the CSR was 68%, which represented more 14% than UV-cells. Regarding F3 (anthocyanins and polymeric procyanidins-rich fraction), a significant higher survival rate was verified to occur for all concentratios testet (0.300–300 mg GAE/L of phenolic compounds), when compared to the C UV cells. In this case, the CSR ranged from 57 to 59%, compared with control cells. However no dose response was possible to observe.

When the compounds were added to cells after cell irradiation no significant effect was verified to occur (Figure 3.32) compared with the cell survival rate of cells exposed to UV radiation (C UV).

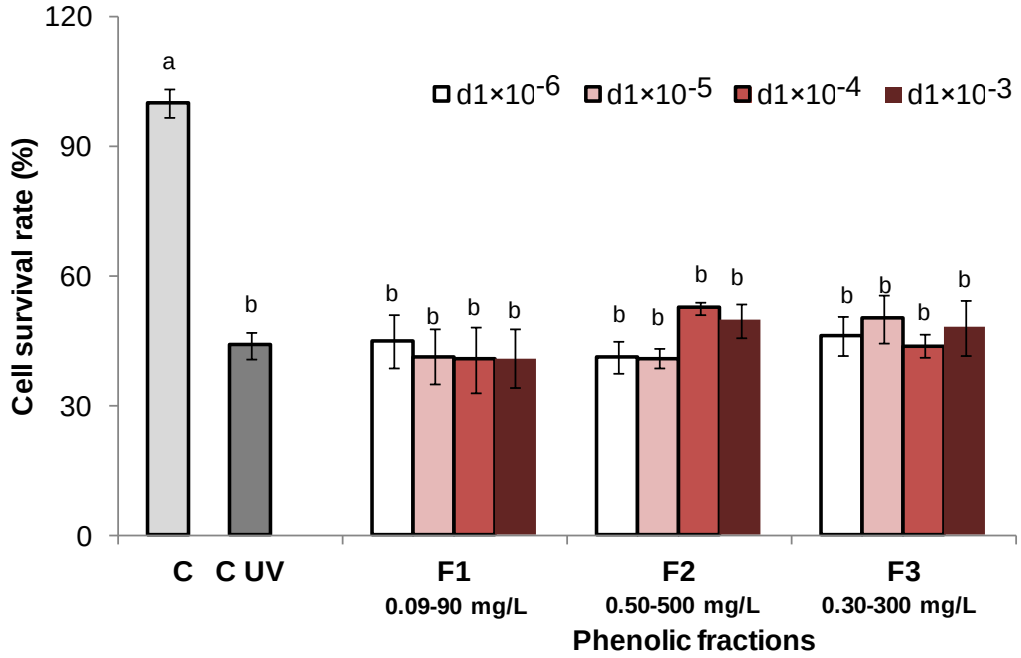


Figure 3.32: Effect of exposure (10 min) to UV light in absence of different dealcoholized wine fractions obtained by C18 fractionation on cell survival rate after 1 h of WST-1 incubation. F2- eluted with ethyl acetate; F3- eluted with acidic methanol. Bars showing the same letter are not significantly different according to the Dunnet test ($p \leq 0.05$).

The protection exerted when the phenolic compounds are present during irradiation, may induce that they undergo on oxidation reaction, preferentially to cell components.

3.4.4 Effect of wine extracts on nitric oxide production by macrophages

In the inflammatory process, macrophages have a key role in providing an immediate defense against foreign agents. When exposed to an inflammatory stimulus, such as a lipopolysaccharide (LPS), macrophages produce nitric oxide (NO) (Geller & Billiar, 1998). The Griess diazotization reaction provides a simple and rapid method for determining indirectly the production of NO in aqueous medium, by measuring the accumulation of nitrite (NO_2), a stable end product of NO in the presence of oxygen (Dirsch *et al*., 1998).

The samples used for evaluate the capacity of inhibit NO production were the wine polysaccharide-rich fraction (WPS), wine phenolic compounds-rich fraction (WFC), and the freeze-dryed extracts obtained from lyophilisation of F1, F2 and F3 solutions, assigned as phenolic acids extract (PA-E), procyanidins-rich extract (PR-E) and anthocyanins and procyanidins-rich extract (APP-E), respectivelly. The extract concentrations used for this study were selected based on EC_{50} determined by the ABTS assay (section 3.1). The highest concentration corresponded to the EC_{50} value, whereas the lowest concentrations corresponded to 80 % of the EC_{50}.To have the appropriate concentrations the extracts were dissolved with phosphate buffer solution pH 7. The Table 3.24 shows the extract concentrations (mg/mL) used in this study.

Table 3.24: Concentration (mg/mL) of wine extracts and their effect on macrophage cell viability.

	Concentration (mg/mL)	
	0.8 EC_{50}	EC_{50}
WPS	0.24	0.30
WPC	0.26	0.33
PA-E	1.33	1.66
PR-E	0.17	0.21
APP-E	0.15	0.19

Each value of cell viability represents the mean±Standard deviation from 3 independent experiments. WPS, wine polysaccharides; WPC, wine phenolic compounds; PA-E, phenolic acids-rich extract; PR-E, procyanidins-rich extract; APP-E,– anthocyanins and polymeric procyanidins-rich extract. 0.8EC_{50} - lowest concentration; EC_{50} - highest concentration tested. Different letters indicate samples that were significantly different ($p < 0.05$).

To evaluate the possibility the possible cytotoxicity of the wine extracts, the cells were incubated in the presence of the extracts during 24 h, and then RAW 264.7 cell viability was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 3.33). The results showed cell viability approaching 100%, which allowed to infer that the wine extracts at the concentrations used in the experiments did not caused cytotoxicity. Thus, any inhibitory activity on NO production can be attributed to the extracts.

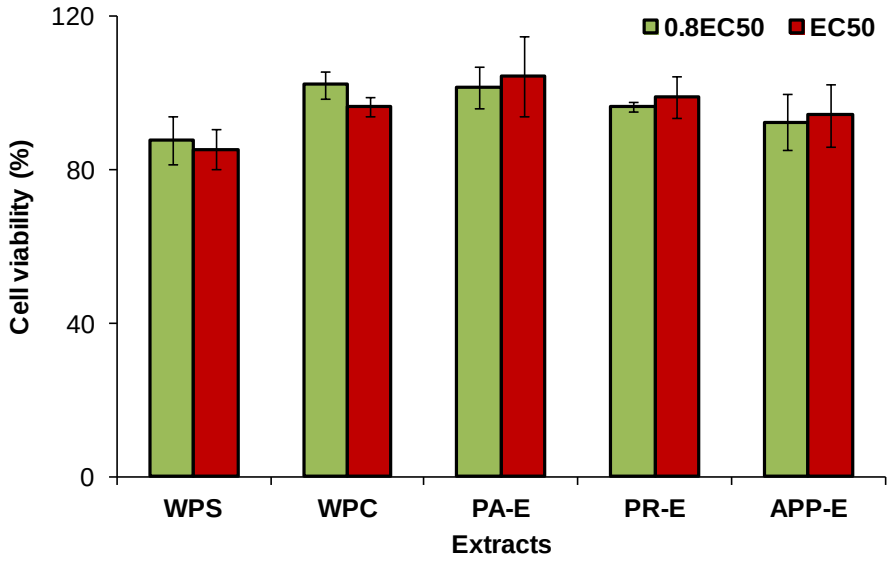


Figure 3.33: Viability (%) of cells incubated in presence of extracts. WPS, wine polysaccharides; WPC, wine phenolic compounds; PA-E, phenolic acids-rich extract; PR-E, procyanidins-rich extract; APP-E,– anthocyanins and polymeric procyanidins-rich extract. $0.8EC_{50}$ - lowest concentration; EC_{50} - highest concentration tested. Different letters indicate samples that were significantly different ($p < 0.05$).

The NO production induced by LPS (0.001–10 μg/mL) in RAW 264.7 cells was found to be concentration-dependent (Kumar-Roiné *et al*., 2009). In our study, the amount of NO production by LPS-activated cell was 14.6±3.6 μM (data not shown). The effect of extracts on LPS-induced NO production was analyzed by measuring the accumulation of nitrite in the culture medium. The results were expressed as percentage of cells stimulated with LPS alone. The lower the production of NO, it was observed higher anti-inflamatory activity. The percentage of NO production in the presence of WPS extract, was, for both concentration,

62% (Figure 3.34), which corresponded to 38% of inhibition of the nitrite production.

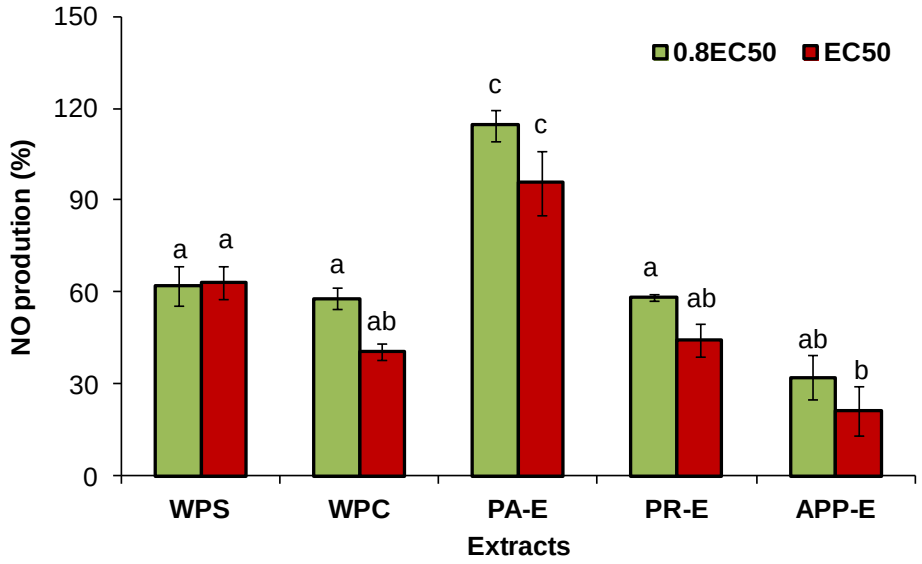


Figure 3.34: Effect of wine extracts on LPS-induced NO production (% of LPS treatment). Each value represents mean ± standard deviation from 3 independent experiments. WPS, wine polysaccharides; WPC, wine phenolic compounds; PA-E, phenolic acids-rich extract; PR-E, procyanidins-rich extract; APP-E,– anthocyanins and polymeric procyanidins-rich extract. $0.8EC_{50}$ - lowest concentration; EC_{50} - highest concentration tested. Different letters indicate samples that were significantly different ($p < 0.05$).

The WPC extracts containing the phenolic compounds obtained from C18 sep-pak cartridge with acidic methanol solution, conferred 59 and 42% of inhibition, respectively for WPC 2 (0.33 mg/mL) and WPC 1 (0.26 mg/mL), indicating a strong anti-inflammatory activity. Also, phenolic extracts of black raspberry fruits (Jeong *et al*., 2010) and *Cymbopogon citratus* (1.115 mg/mL) (Francisco *et al*., 2011) have been showed to suppress nitric oxide production in LPS-stimulated RAW 264.7 cells. Kumar *et al*., (2009) also verified a NO inhibitory capacity ranging from 12 to 45% of 12 plant extracts (0.25 mg/mL) in RAW 264.7 cells exposed to 10 μg/mL LPS.

The contribution of each wine phenolic fraction, namely phenolic acids extract (PA-E), procyanidins-rich extract (PR-E), and anthocyanins and polymeric procyanidins-rich extract (APP-E) to the anti-inflammatory activity of WPC was

evaluated. The LPS-induced nitrite production was strongly reduced by APP-E extract to 24 and 21% of LPS, for concentrations of 0.15 and 0.19 mg/mL, respectively. The PR-E extract also exhibited a reduction of NO production, reaching 58% with 0.17 mg/mL and 44% with 0.21 mg/mL. These results indicated that APP-E and PR-E extracts are partially responsible for the anti-inflammatory properties of the WPC extract by inhibition of NO production, being the APP-E extracts the more active one. On the contrary, the PA-E did not affect the NO production. WPC, PR-E and APP-E extracts showed a higher inhibitory effect for the highest concentration tested, however no significant differences were observed.

All together, the anti.inflmatory properties studied, demonstrated that WPS, WPC, PR-E and APP-E extracts decreased the LPS-induced NO production in RAW 264.7 macrophage cells. The APP-E extract exhibited the highest NO inhibitory capacity. However more studies are necessary to find out the properties and mechanisms of action of the compounds present in these extracts.

3.4.5 Effect of wine extracts on prevention of human red blood cells cytotoxity

In order to further elucidate the antioxidant properties of wine extracts in human cells, red blood cells (RBC) were selected as a metabolically simplified model system. Erythrocytes are responsible for providing oxygen to organs, thus they are constantly exposed to reactive oxygen species (ROS) during blood circulation. ROS are generated in biological systems through metabolic processes and exogenous sources such as food components, drugs, ultraviolet light, ionizing radiation, and pollution (Samuni *et al*., 1983). ROS have been implicated in an oxidative mechanism of damage to RBC (Scott *et al*., 1993), including the membrane and hemoglobin (Hb), which may lead to hemolysis.

Hemolysis assay is one of the important *in vitro* methods to evaluate erythrocyte resistance to oxidation. The conditions used can be considered an *in vivo* situation since this method proposes the influence of an antioxidant contribution on red blood cells near to an oxidising stress state. The rate of cell lysis can be regarded as an *in vitro* marker of the oxidative damage. The susceptibility of the red blood cells could be determined by their exposure to an oxidative system that produces ROS and by measuring at regular lap time the rate of hemolysis.

Over last years, there is an increasing evidence of the protective effect of phenolic compounds against oxidative damage. In particular, lyophilized extracts of red wines have been shown to possess anti-hemolytic capacity (Tedesco *et al*., 2000). Oxidative damage of RBC membrane generally involves lipid peroxidation, which may alter membrane structure, receptor functions and fluidity, causing its malfunctioning leading to RBCs Hemolysis involved in cell injury and death.

The efficacy of extracts as inhibitors of H_2O_2- and AAPH-induced RBC hemolysis was tested. H_2O_2 can easily cross the cellular membranes and thus oxidize a number of compounds and structures inside the cell. In contrast, AAPH-radicals attack the membrane but do not has the capacity to cross membranes (Colado Simao *et al.*, 2006). The hemolysis induced by AAPH provides a good approach for studying free radical-induced membrane damages (Dai *et al*., 2006).

3.4.5.1 Protective effect against H_2O_2 induced damage

In this study, wine polymeric material (WPM2), wine polysaccharides-rich extract (WPS), extract containing wine phenolic compounds (WPC), procyanidins-rich extract (PR-E) and anthocyanins and polymeric procyanidins-rich extract (APP-E) were used in concentrations ranging from 0.05 to 0.50 mg/mL. The chemical characterization of these fractions was present in section 3.1.

The effect against H_2O_2 induced cytotoxicity in RBC of the extracts was shown in Figure 3.35. The results were calculated relative to the value of hemolysis obtained with the oxidant agent devoid of extracts (which was taken as 100%) and presented as percentage of hemolysis inhibition. The higher percentage of inhibition corresponds to the higher antioxidant activity.

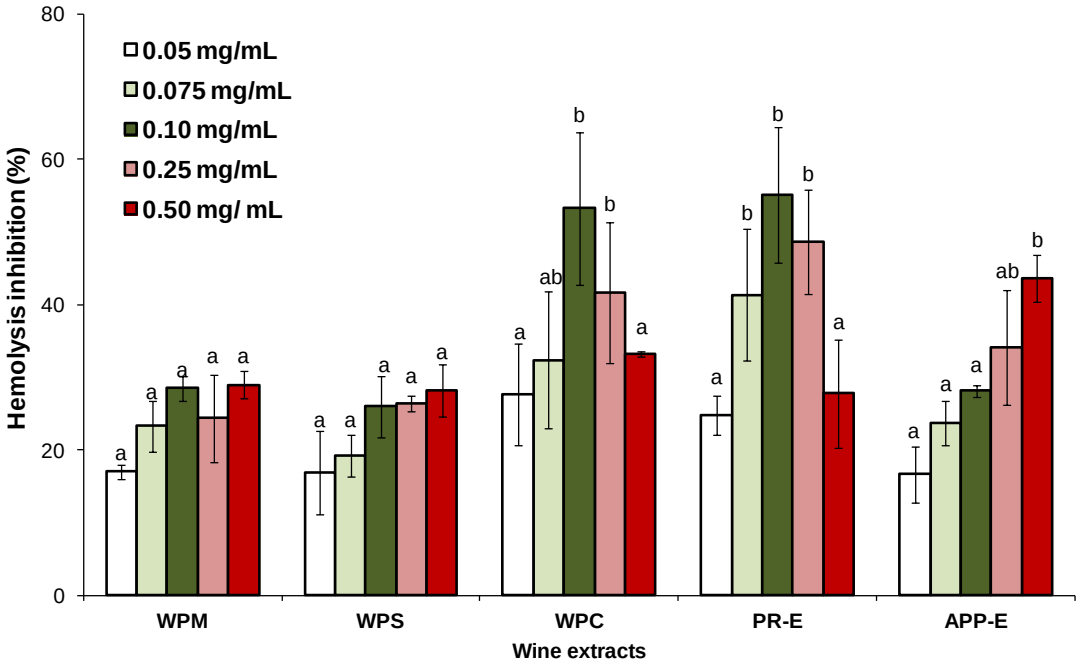


Figure 3.35: Percentage of inhibition of lysis of RBCs at 2% hematocrit incubated for 3 h with 7.5 mM of H_2O_2 and exctracts at different concentrations. Mean (error bars represent standard deviation) of four determinations for each duplicate. Different letters within a concentration indicate samples that were significantly different ($p < 0.05$). WPM2, wine polymeric material 2; WPS, wine polysaccharides; WPC, wine phenolic compounds; PR-E, procyanidins-rich extract; APP-E, anthocyanins and polymeric procyanidins-rich extract.

For the range of concentrations between 0.05 and 0.10 mg/mL, a good concentration-dependent response ($0.764 < r^2 < 0.982$) effect was verified to occur. WPC and PR-E extracts, at 0.10 mg/mL, showed a significant better protective activity (53 and 55%) when compared with WPM2, WPS and APP-E (26–29%). For a extract concentration of 0.075 mg/mL, the same tendency was observed, but only PR-E presented a significant diference. The percentage of hemolysis of RBCs pretreated with extracts at 0.05 mg/mL ranged from 17 to 28%, a lower value than those (ca. 35-75%) described for phenolic extracts of Quercus species at same concentration, containing 26 to 39% of phenolic compounds in GAE (Şöhretoğlu *et al*., 2012). In general, wine phenolic compounds (WPC) and procyanidins (PR-E) extracts were the most efficient on the hemolysis inhibition, except at highest extract concentration (0.5 mg/mL), whereas APP-E exhibited the highest performance.

For the highest concentrations used (0.25 and 0.50 mg/mL), different behaviors were observed for the extracts understudy. The antioxidant activity of a molecule in the cell depends of its scavenging capacity as well as of its localization, its access to different cell components or of its interaction with cell membrane. The more non-polar compounds interact with the hydrophobic interior of the membrane, while the more hydrophilic can establish hydrogen bonds with the polar head groups of lipids at the membrane interface. The WPM2 and WPS extracts, constituted by high molecular weight compounds, exhibited a similar percentage of hemolysis inhibition compared to 0.10 mg/mL. APP-E exhibited a dose-response tendency, reaching 44% of inhibition for 0.50 mg/mL. In contrast, WPC and PR-E showed lower protective effect at increasing concentrations. A possible explaination can be their hemolytic effect (Şöhretoğlu *et al*., 2012). Furthermore, prooxidant effect of phenolic compounds under certain conditions has been reported. For instance, Green tea catechin, also present in WPC and PR-E, has been reported to promote H_2O_2 generation in the presence of transition metal ions.

3.4.5.2 Protective effect against AAPH induced damage

Thermal decomposition of AAPH (2,2´-Azo-bis(2-amidinopropane) dihydrochloride) in the aqueous dispersion of RBC produces hydrophilic radicals which attack RBC from the outside of membrane, causing lipid peroxidation and protein damage, promoting hemolysis proportionally to its concentration (Colado Simao *et al.*, 2006; Ko *et al.*, 1997). Some standard solutions of phenolic compounds usually present in wine, such as anthocyanins (Cy3Glc and Df3Glc), flavanols (catechin, epicatechin epigallocatechin and gallocatechin) have been showed to delay AAPH-induced hemolysis of RBCs (Tabart *et al*., 2009). In addition, Dai *et al*. (2006), comparing the protective effect of flavonols, showed that the presence of an *ortho*-dihydroxyl function on molecule structure enhanced their antihemolytic efficiency.

The results obtained on inhibition of hemolysis by AAPH in the presence of wine extracts are shown in Figure 3.36.

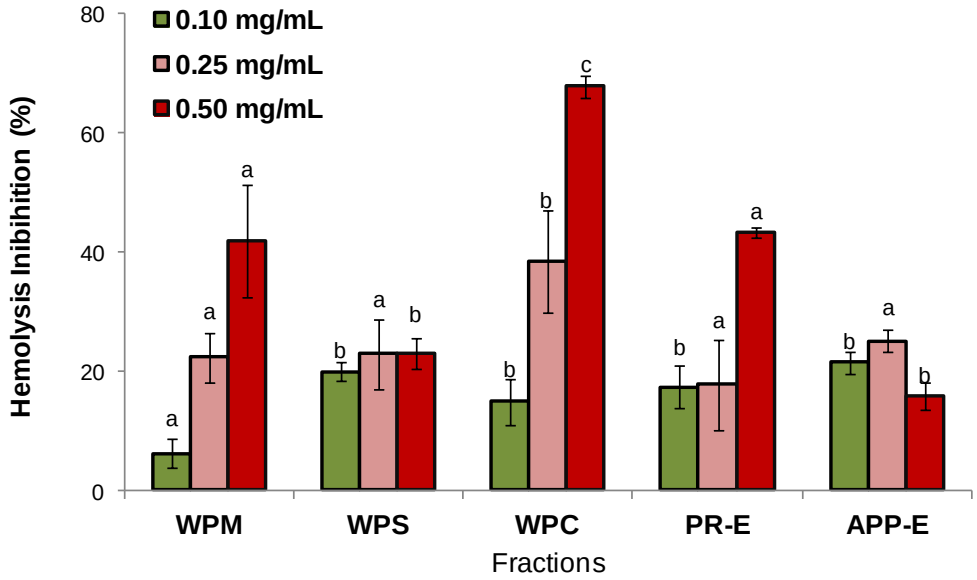


Figure 3.36: Percentage of inhibition of lysis of RBCs at 2% hematocrit incubated for 3 h with 60 mM of AAPH and wine extracts at different concentrations. Mean (error bars represent standard deviation) of four determinations for each duplicate. Different letters within a concentration indicate samples that were significantly different ($p< 0.05$). WPM2, wine polymeric material 2; WPS, wine polysaccharides; WPC, wine phenolic compounds; PR-E, procyanidins-rich extract; APP-E, anthocyanins and polymeric procyanidins-rich extract.

For the highest tested concentration, the WPC extract exhibited an important protection, with 70% of hemolysis inhibition. For this concentration, the hemolysis inhibition capacity showed significant differences in the order WPC> PR-E, WPM2 (42 %) > WPS, APP-E (16-23%). Also, for a concentration of 0.25 mg/mL, the WPC extract exhibited an inhibition value (38%) significantly higher than the values (18-25 %) obtained for the other extracts. For the lowest tested concentration (0.10 mg/mL), the highest inhibition (21%) was conferred by APP-E, although statistically similar to other extracts, except to WPM extract. In general, the concentration was lower a decrease in protection was verified. This can be observed in WPM2 and WPC extracts, which showed a good dose-response factor, r = 0.996 and 0.997, respectively. In the presence of AAPH, all the extracts (0.50-0.10 mg/mL), protected RBCs from oxidative-induced hemolysis. The capacity of one compound to inhibit hemolysis depends not only of its hability to directly interact with AAPH in the medium, but also on its interaction with cell membranes (Alvarez-Suarez *et al.*, 2012). Therefore, the more hydrophobic phenolic compounds can act as chain-breaking antioxidants located in lipophilic regions of the membranes. Otherwise, the more hydrophilic compounds can establish hydrogen bounds at the surface of bilayers reducing the access of oxidants agents, thus protecting them from external damage. Comparing the effect as inhibitors of H_2O_2- and AAPH-induced RBC hemolysis, it was possible to observe different behaviors of extracts. In general, for extracts concentration range of 0.50–0.10 mg/mL, a higher inhibition was conferred to H_2O_2-induced RBC hemolysis. The exception was observed for WPM2, WPC and PR-E, all at 0.50 mg/mL. Since H_2O_2 can easily cross the cellular membranes, whereas AAPH does not, possible the ability of extracts to cross membrane may rule partially their anti-hemolitic effect.

Figure 3.37 Ilustrates on the left, the intact RBC, as negative control and on the right, the cells obtained after exposure to AAPH. From optical microscopic evaluation, it is possible to see a lower number of erythrocyte after AAPH addition.

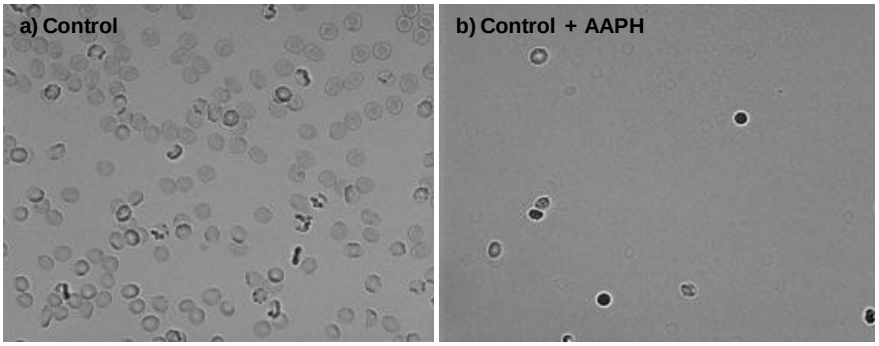


Figure 3.37: Optical microscopic evaluation of the erythrocytecells. a) Control only with RBCs. b) Control with RBCs plus AAPH.

The effect of addition of WPM2, WPC and PR-E (at 0.1 and 0.5 mg/mL) on the erythrocyte cells number and morphology, after 4 h of incubation, evaluated by optical microscopic is shown in Figure 3.38.

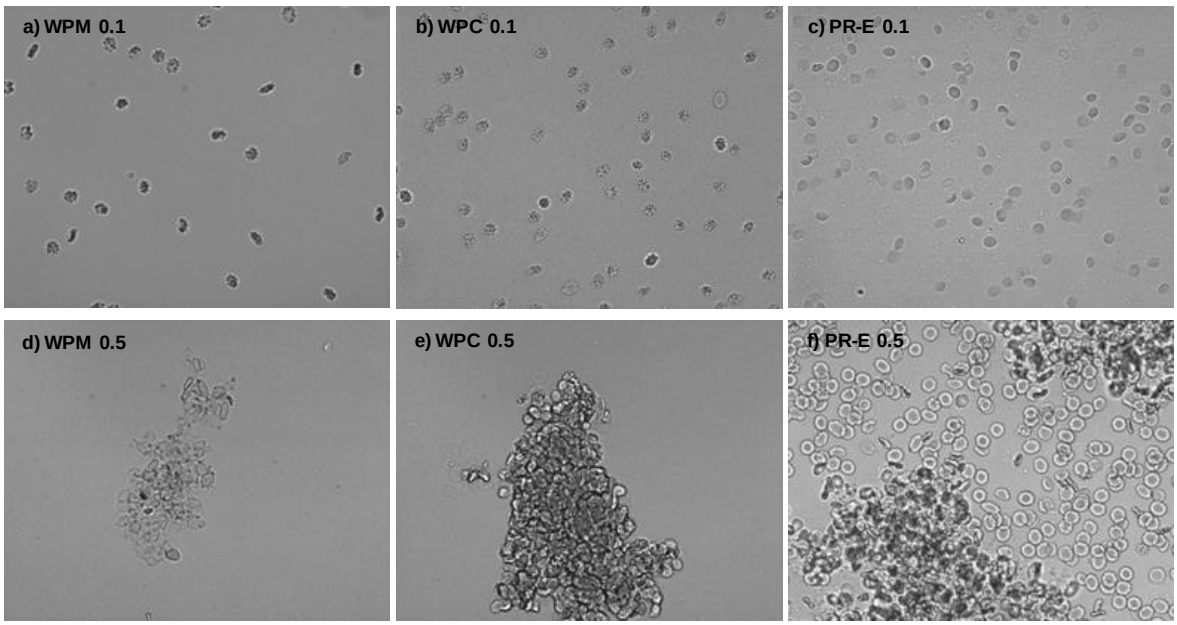


Figure 3.38: Optical microscopic evaluation of the erythrocyte cells. a-c)- RBCs plus extracts (WPM2, WPC and PR-E) at 0.1 mg/mL and AAPH after 4 h of incubation; d-f)- RBCs plus extracts (WPM2, WPC and PR-E) at 0.5 mg/mL and AAPH after 4 h of incubation. WPM2- wine polymeric material 2; WPC- wine phenolic compounds; PR-E- procyanidin-rich fraction.

For the concentration of 0.10 mg/mL, it was possible to observe a higher number RBC, when compared with the RBC exposed to AAPH alone (Figure 3.37).

A higher reduction was observed for WPM, which was in accordance with the lower hemolysis inhibition determined colorimetrically, compared with WPC and PR-E. For the highest concentration, it was possible to observe the formation of aggregates between RBC and all the compounds.

The visible spectra (450-650 nm) of RBC lysates (obtained after 3 h of incubation at 37 ºC) in the presence of AAPH with WPM2, WFC and PR-E, at 0.10, 0.25 and 0.50 mg/mL is shown in Figure 3.39. For comparison purposes, a negative control, only with RBC in PBS and spectra of RBC with AAPH is also shown.

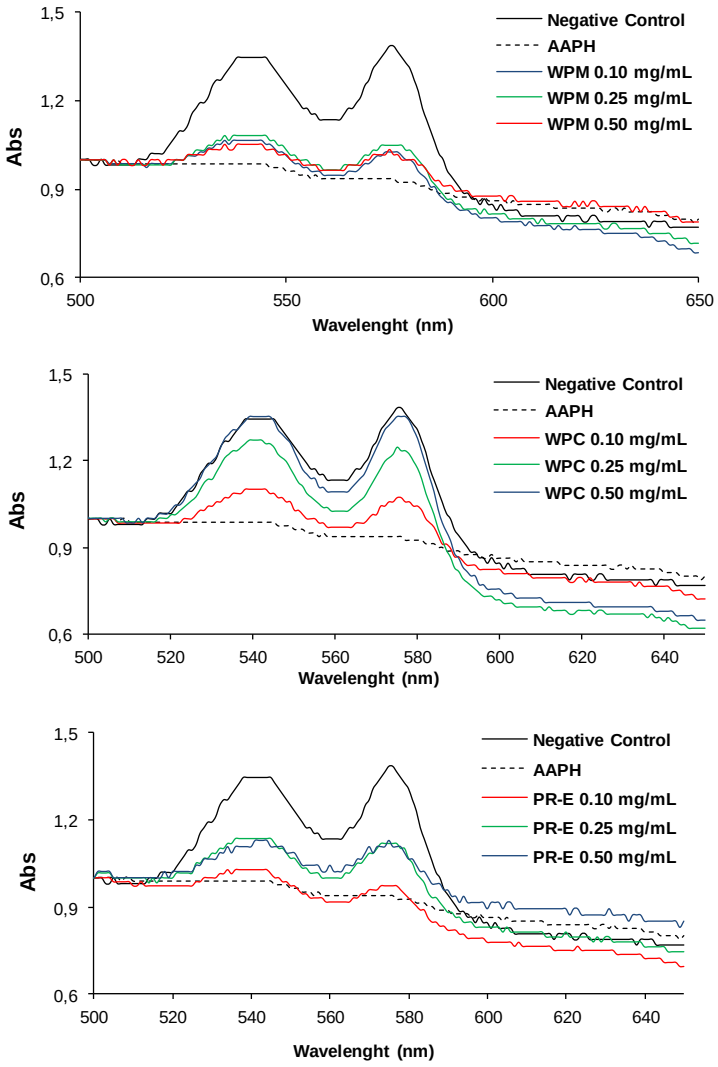


Figure 3.39: Visible spectra (450-650 nm) of RBC lysates (obtained after 3 h of incubation at 37 ºC) in the presence of a) WPM, b) WPC, c) PR-E, in different concentrations.
WPM- wine polymeric material; WPC- wine phenolic compounds; PR-E- procyanidin-rich fraction.

In case of control is possible to see clearly the peaks (540 and 578 nm) corresponding to oxy-hemoglobin, which is formed when oxygen binds to the heme component of the protein hemoglobin in RBC. When AAPH was added, these peaks almost disappear, indicative of hemoglobin oxidation. The addition of any one of the compounds reduced the size of the oxy-hemoglobin peaks as compared with control. In case of WPM2, the concentration did not change significantly the spectra profile, in contrast with de percentage of inhibition obtained previously. Regarding WPC, the decrease of peaks was higher for lower concentration of compounds. As verified for the highest percentage of inhibition of hemolysis, it is possible to observe a higher similarity of spectra of WPC at 0.50 mg/mL with control. In case of PR-E at 0.1 mg/mL slightly differences were observed when compared with the spectra of RBCs with AAPH alone.

The results obtained reinforce the idea that phenolic compounds of wine may exert a beneficial role on cell, contributing for cell defenses against oxidative radical agents.

3.4.6 Final remarks

The results obtained from the biological effects of wine extracts allowed to conclude that:

- The *in vitro* digestive tract model used decreased in comparable proportions the amount of all classes of phenolic compounds for DW1, DW2 and the phenolic fractions.

- The highest decrease on the amount of phenolic compounds and antioxidant activity was observed in the during small intestine conditions. After gastrointestinal track, the amount of phenolic compounds and antioxidant activity represented around 20% of those before digestion.

- On the conditions tested, the addition of phenolic compounds had no significant differences on the cell survival rate (CSR) of mammary epithelial (HC-11) and on the colon carcinoma (HT-29) cells.

- The procyanidins-rich fraction (500 mg/L) and the anthocyanins-rich fraction (3.00 and 300 mg GAE/L) increased significantly the CSR of HT-29 cells exposed to H_2O_2. Also, protected the cells that were exposed to 10 min of UV radiation, in concentration ranging from 5.00 to 500 mg/L for the procyanidins-rich fraction and from 0.300 to 300 mg GAE/L for the anthocyanins-rich fraction.

- The concentrations used of wine extracts in the experiments did not caused cytotoxicity to RAW 264.7 macrophage cells. In addition, the WPS, WPC, PR-E and APP-E extracts decreased the LPS-induced NO production in RAW 264.7 macrophage cells, showing anti- inflammatory properties, where the APP-E extract exhibited the highest capacity.

- In general, wine phenolic compounds (WPC) and procyanidin (PR-E) extracts were the most efficient on the hemolysis inhibition, except at highest extract concentration (0.5 mg/mL), whereas APP-E exhibited the highest performance.

- WPM2, WPS, WPC, PR-E and APP-E extracts in concentrations ranging from (0.05 to 0.50 mg/mL), showed a reduction on H_2O_2-induced RBC hemolysis. Also, for AAHP-induced RBC hemolysis, an inhibition of the hemolysis occurred

for the concentrations tested (0.10 to 0.50 mg/mL). In general, the wine phenolic compounds (WPC) showed the best protective effect.

- The results obtained reinforce the idea that phenolic compounds of dealcoholized wine may exert a beneficial role on cells, contributing for cell defenses against oxidative radical agents.

-

CHAPTER IV - Conclusions

In this thesis, it was shown that the phenolic compounds and their antioxidant activity are preserved despite the compounds have been submitted to a distillation process. A total amount of 1.3 and 3.1 g/L of phenolic compounds, expressed as gallic acid equivalents, 1.2 and 1.6 g/L of tannins, and 0.24 and 0.41 g/L of anthocyanins, expressed as malvidin 3-glucoside, could be recovered from the two wines studied, RW1 and RW2, respectively. Also, the distillation process allows to obtain a composition of monomeric anthocyanins similar to those present in wine, where malvidin 3-glucoside is the most abundant, representing 60% of all monomeric anthocyanidins. Furthermore, 28.6 mg GAE/L of hydroxybenzoic acids, 46.9 mg CAE/L of hydroxycinnamic acids, 37.8 mg Cat/L of catechins, 83.7 mg/L of procyanidins, and 375 mg Mw3Glc/L of monomeric anthocyanins were present. These compounds occur in similar amounts to those described in literature for red wines.

The total antioxidant activity of the compounds present in the material remaining in the aqueous liquid solution after red wine distillation was mainly attributed to the contribution of the anthocyanins-rich fraction.

Together with the phenolic compounds, polymeric material (polysaccharides and proteins) were also present in the dealcoolized solution. The wine polymeric material of the wines understudy (WPM1 and WPM2) accounted for 1.1 and 1.3 g/L of the solid material, respectively, of which 31 and 36% were polysaccharides and 10 and 12% were proteins. The arabinogalactans (106-137 mg/g) and mannoproteins (77-96 mg/g) were the main polysaccharides present. Wine phenolic compounds (18-28%) were shown to be associated with the WPM. The extraction of WPM with acidic methanol showed that part of these phenolic compounds was tightly bounded to the WPM. To evaluate the strength of these interactions, a dialysis experiment was developed, and the anthocyanins released were characterized by HPLC. The rate of the diffusion of the phenolic compounds through the dialysis membrane was determined using solutions of phenolic compounds in presence and in absence of the polymeric material and under different concentrations and temperatures. This allowed to estimate the activation energy (E_a) for the release of phenolic compounds from WPM and PMi fractions as 25 and 61 kJ/mol, respectively.

The interactions that can occur between phenolic compounds, namely anthocyanins, and wine polymeric material were also studied by performing dialysis of phenolic compounds in the presence and in the absence of the WPM. The WPM had a capacity to interact with the phenolic compounds, partially retaining (~33%) their passage through a dialysis membrane. In particular, all monomeric anthocyanins were retained by the WPM, although in different extents. The higher retention capacity (RC from 0.77 to 0.87) was observed for acetylglucosilated anthocyanins whereas the glucosilated anthocyanins present RC values ranging from 0.47 to 0.52, suggesting weaker interactions. The dialyses of phenolic compounds in the presence of mannoprotein-rich (MP) and arabinogalactan-rich (AG) fractions allowed to observe a retention of different anthocyanins by both, MP and AG polysaccharides. The higher RC obtained for AG rather than for MP fractions allowed to conclude for a higher contribution of AG than MP for the retention capacity of wine polymeric material. This difference was especially observed for acetylglucosilated anthocyanins, where the RC ranged from 0.61 to 0.63 for AG, and from 0.29 to 0.39 for MP fraction. The higher RC obtained for acylated anthocyanins allowed suggesting that the association of anthocyanins with wine polymeric material is mainly ruled by hydrophobic interactions.

It was evaluated the effect of the polysaccharide-rich fraction addition on the phenolic composition of a wine after 3 months of storage. This allowed to conclude that the addition of polysaccharides significantly reduce the anthocyanins degradation, especially when the wine solution was stored at 30ºC. The more significant effect was observed on coumaroylated anthocyanins also suggesting the hydrophobic interaction between these different types of compounds. Otherwise, the addition of polysaccharides had no significant effect on the antioxidant activity of the wines. The 95% of the initial antioxidant activity of a wine phenolic extract (powder) stored under light and oxygen controlled conditions, during 342 days, was preserved.

Several *in vitro* assays were performed to evaluate potential biological properties of the fractions obtained from DW. Firstly, after the passage through the *in vitro* digestive tract model used, 20% of the phenolic compounds were potentially available. The highest decreasing on their stability was observed in small intestine conditions. Furthermore, the DW1, DW2 and the phenolic compounds fractions exhibited a similar behavior. The different *in vitro* cellular systems showed that the phenolic compounds exerted a beneficial role on cells, contributing to their defenses against oxidative radical agents. These experiments allowed to conclude that the DW contained compounds with potential biological effects. The results obtained with HT-29 cells showed that the anthocyanins-rich fraction (0.300–300 mg GAE/L), as well as the procyanidins-rich fraction (5.00-500 mg GAE/L), had protective effect against H_2O_2 and UV damage agents, promoting cell viability if present during the damage exposure. These fractions, in concentrations ranging from 50 to 500 mg/L, exhibited anti-hemolytic properties, namely the anthocyanins and polymeric procyanidins-rich fraction which exhibited the highest capacity. In addition, the anthocyanins and polymeric procyanidins-rich fraction (150–190 mg/L), and procyanidins-rich fraction (170–210 mg/L) showed anti-inflammatory properties. Also, the wine polysaccharides and the wine phenolic compounds exhibited anti-inflammatory and anti-hemolytic properties.

In conclusion, the results presented in this thesis showed that dealcoholized wines may be used as a source of phenolic compounds with biological activity. The interaction of wine phenolic compounds with the polymeric material allows to predict a continuous and gradual dosage of red wine anthocyanins upon ingestion, contributing for a longer period of their exposure and, as a consequence, of their potential health benefits. The valuation of the distillation process is possible through the recovery of the phenolic compounds as well as the polymeric material. They can be incorporated in novel food ingredients with functional activities, namely antioxidant and dietary fiber. Also aplications of their anti-inflamatory and anti-hemolytic properties should be exploited.

References

Adam, A., Crespy, V., Levrat-Verny, M. A., Leenhardt, F., Leuillet, M., Demigne, C., & Remesy, C. (2002). The bioavailability of ferulic acid is governed primarily by the food matrix rather than its metabolism in intestine and liver in rats. *Journal of Nutrition, 132*(7), 1962-1968.

Aguirre, M. J., Chen, Y. Y., Isaacs, M., Matsuhiro, B., Mendoza, L., & Torres, S. (2010). Electrochemical behaviour and antioxidant capacity of anthocyanins from Chilean red wine, grape and raspberry. *Food Chemistry, 121*(1), 44-48.

Aguirre, M. J., Isaacs, M., Matsuhiro, B., Mendoza, L., & Zuniga, E. A. (2009). Characterization of a neutral polysaccharide with antioxidant capacity from red wine. *Carbohydrate Research, 344*(9), 1095-1101.

Alcalde-Eon, C., Escribano-Bailon, M. T., Santos-Buelga, C., & Rivas-Gonzalo, J. C. (2006). Changes in the detailed pigment composition of red wine during maturity and ageing - A comprehensive study. *Analytica Chimica Acta, 563*(1-2), 238-254.

Alén-Ruiz, F., García-Falcón, M. S., Pérez-Lamela, M. C., Martínez-Carballo, E., & Simal-Gándara, J. (2009). Influence of major polyphenols on antioxidant activity in Mencía and Brancellao red wines. *Food Chemistry, 113*(1), 53-60.

Alvarez-Suarez, J. M., Giampieri, F., González-Paramás, A. M., Damiani, E., Astolfi, P., Martinez-Sanchez, G., Bompadre, S., Quiles, J. L., Santos-Buelga, C., & Battino, M. (2012). Phenolics from monofloral honeys protect human erythrocyte membranes against oxidative damage. *Food and Chemical Toxicology, 50*(5), 1508-1516.

Anson, N. M., van den Berg, R., Havenaar, R., Bast, A., & Haenen, G. R. M. M. (2009). Bioavailability of ferulic acid is determined by its bioaccessibility. *Journal of Cereal Science, 49*(2), 296-300.

Argyri, K., Komaitis, M., & Kapsokefalou, M. (2006). Iron decreases the antioxidant capacity of red wine under conditions of in vitro digestion. *Food Chemistry, 96*(2), 281-289.

Armand, M., Borel, P., Dubois, C., Senft, M., Peyrot, J., Salducci, J., Lafont, H., & Lairon, D. (1994). Characterization of emulsions and lipolysis of dietary lipids in the human stomach. *American Journal of Physiology, 266*(3), G372-G381.

Arnold, R. A., Noble, A. C., & Singleton, V. L. (1980). Bitterness and astringency of phenolic fractions in wine. *Journal of Agricultural and Food Chemistry, 28*(3), 675-678.

Arnous, A., Makris, D. P., & Kefalas, P. (2001). Effect of principal polyphenolic components in relation to antioxidant characteristics of aged red wines. *Journal of Agricultural and Food Chemistry, 49*, 5736-5742.

Arnous, A., & Meyer, A. S. (2009). Quantitative prediction of cell wall polysaccharide composition in Grape (*Vitis vinifera* L.) and Apple (*Malus domestica*) skins from acid hydrolysis monosaccharide profiles. *Journal of Agricultural and Food Chemistry, 57*(9), 3611-3619.

Atanasova, V., Fulcrand, H., Cheynier, V., & Moutounet, M. (2002). Effect of oxygenation on polyphenol changes occurring in the course of wine-making. *Analytica Chimica Acta, 458*(1), 15-27.

Auger, C., Chaabi, M., Anselm, E., Lobstein, A., & Schini-Kerth, V. B. (2010). The red wine extract-induced activation of endothelial nitric oxide synthase is mediated by a great variety of polyphenolic compounds. *Molecular Nutrition & Food Research, 54*, S171-S183.

Ayestarán, B., Guadalupe, Z., & León, D. (2004). Quantification of major grape polysaccharides (*Tempranillo* v.) released by maceration enzymes during the fermentation process. *Analytica Chimica Acta, 513*(1), 29-39.

Bacon, J. R., & Rhodes, M. J. C. (2000). Binding affinity of hydrolyzable tannins to parotid saliva and to proline-rich proteins derived from it. *Journal of Agricultural and Food Chemistry, 48*(3), 838-843.

Bakker, J., & Timberlake, C. F. (1985). The distribution of anthocyanins in grape skin extracts of port wine cultivars as determined by high performance liquid chromatography. *Journal of the Science of Food and Agriculture, 36*(12), 1315-1324.

Bakker, J., & Timberlake, C. F. (1997). Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines. *Journal of Agricultural and Food Chemistry, 45*(1), 35-43.

Bakowska-Barczak, A. M., & Kolodziejczyk, P. P. (2011). Black currant polyphenols: Their storage stability and microencapsulation. *Industrial Crops and Products, 34*(2), 1301-1309.

Bakowska, A., Kucharska, A. Z., & Oszmianski, J. (2003). The effects of heating, UV irradiation, and storage on stability of the anthocyanin-polyphenol copigment complex. *Food Chemistry, 81*(3), 349-355.

Baldi, A., Romani A, Mulinacci N, Vincieri F, & B, C. (1995). HPLC/MS application to anthocyanins of *Vitis vinifera* L. *Journal of Agricultural and Food Chemistry, 43*, 21042109.

Baptista, J. A. B., Tavares, J. F. d. P., & Carvalho, R. C. B. (2001). Comparison of polyphenols and aroma in red wines from Portuguese mainland versus Azores Islands. *Food Research International, 34*(4), 345-355.

Baranac, J. M., Petranovic, N. A., & DimitricMarkovic, J. M. (1996). Spectrophotometric study of anthocyan copigmentation reactions. *Journal of Agricultural and Food Chemistry, 44*(5), 1333-1336.

Beecher, G. R. (2003). Overview of dietary flavonoids: Nomenclature, occurrence and intake. *Journal of Nutrition, 133*(10), 3248S-3254S.

Belisario-Sanchez, Y. Y., Taboada-Rodriguez, A., Marin-Iniesta, F., & Lopez-Gomez, A. (2009). Dealcoholized wines by spinning cone column distillation: phenolic compounds and antioxidant activity measured by the 1,1-Diphenyl-2-picrylhydrazyl method. *Journal of Agricultural and Food Chemistry, 57*(15), 6770-6778.

Belleville, M. P., Williams, P., & Brillouet, J. M. (1993). A linear arabinan from a red wine. *Phytochemistry, 33*(1), 227-229.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “Antioxidant Power”: The FRAP assay. *Analytical Biochemistry, 239*(1), 70-76.

Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnology Annual Review, 11*, 127-152.

Biehler, E., & Bohn, T. (2010). Methods for assessing aspects of carotenoid bioavailability. *Current Nutrition & Food Science, 6*, 44–69.

Birt, D. F., Hendrich, S., & Wang, W. (2001). Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacology & Therapeutics, 90*(2–3), 157-177.

Blanco-Colio, L. M., Valderrama, M., Alvarez-Sala, L. A., Bustos, C., Ortego, M., Hernandez-Presa, M. A., Cancelas, P., Gomez-Gerique, J., Millan, J., & Egido, J. (2000). Red wine intake prevents nuclear factor-kappa B activation in peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia. *Circulation, 102*(9), 1020-1026.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54*(2), 484-489.

Bogianchini, M., Cerezo, A. B., Gomis, A., Lopez, E., & Garcia-Parrilla, M. C. (2011). Stability, antioxidant activity and phenolic composition of commercial and reverse osmosis obtained dealcoholised wines. *Lwt-Food Science and Technology, 44*(6), 1369-1375.

Bohn, T., Tian, Q., Chitchumroonchokchai, C., Failla, M. L., Schwartz, S. J., Cotter, R., & Waksman, J. A. (2007). Supplementation of test meals with fat-free phytosterol products can reduce cholesterol micellarization during simulated digestion and cholesterol accumulation by Caco-2 cells. *Journal of Agricultural and Food Chemistry, 55*(2), 267-272.

Boido, E., Alcalde-Eon, C., Carrau, F., Dellacassa, E., & Rivas-Gonzalo, J. C. (2006). Aging effect on the pigment composition and color of *Vitis vinifera* L. cv. tannat wines. Contribution of the main pigment families to wine color. *Journal of Agricultural and Food Chemistry, 54*(18), 6692-6704.

Bouayed, J., Deusser, H., Hoffmann, L., & Bohn, T. (2012). Bioaccessible and dialysable polyphenols in selected apple varieties following in vitro digestion vs. their native patterns. *Food Chemistry, 131*(4), 1466-1472.

Boulet, J. C., Williams, P., & Doco, T. (2007). A Fourier transform infrared spectroscopy study of wine polysaccharides. *Carbohydrate Polymers, 69*(1), 79-85.

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *American Journal of Enology and Viticulture, 52*(2), 67-87.

Bourne, L., Paganga, G., Baxter, D., Hughes, P., & Rice-Evans, C. (2000). Absorption of ferulic acid from low-alcohol beer. *Free Radical Research, 32*(3), 273-280.

Bralley, E. E., Hargrove, J. L., Greenspan, P., & Hartle, D. K. (2007). Topical anti-inflammatory activities of Vitis rotundifolia (muscadine grape) extracts in the

tetradecanoylphorbol acetate model of ear inflammation. *Journal of Medicinal Food, 10*(4), 636-642.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology, 28*(1), 25-30.

Bravo, L., Abia, R., & Sauracalixto, F. (1994). Polyphenols as dietary fiber associated compounds. Comparative study on *in vivo* and *in vitro* properties. *Journal of Agricultural and Food Chemistry, 42*(7), 1481-1487.

Brillouet, J. M., Bosso, C., & Moutounet, M. (1990). Isolation, purification, and characterization of an arabinogalactan from a red wine. *American Journal of Enology and Viticulture, 41*(1), 29-36.

Briviba, K., Pan, L., & Rechkemmer, G. (2002). Red wine polyphenols inhibit the growth of colon carcinoma cells and modulate the activation pattern of mitogen-activated protein kinases. *Journal of Nutrition, 132*(9), 2814-2818.

Brouillard, R. (1981). The chemistry of anthocyanin pigments .Origin of the exceptional color stability of the zebrina anthocyanin. *Phytochemistry, 20*(1), 143-145.

Brouns, F. (2002). Soya isoflavones: a new and promising ingredient for the health foods sector. *Food Research International, 35*(2-3), 187-193.

Burns, J., Gardner, P. T., O'Neil, J., Crawford, S., Morecroft, I., McPhail, D. B., Lister, C., Matthews, D., MacLean, M. R., Lean, M. E. J., Duthie, G. G., & Crozier, A. (2000). Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines. *Journal of Agricultural and Food Chemistry, 48*(2), 220-230.

Cai, H., Marczylo, T. H., Teller, N., Brown, K., Steward, W. P., Marko, D., & Gescher, A. J. (2010). Anthocyanin-rich red grape extract impedes adenoma development in the Apc(Min) mouse: Pharmacodynamic changes and anthocyanin levels in the murine biophase. *European Journal of Cancer, 46*(4), 811-817.

Cao, G. H., Russell, R. M., Lischner, N., & Prior, R. L. (1998). Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *Journal of Nutrition, 128*(12), 2383-2390.

Cardoso, S. M., Silva, A. M. S., & Coimbra, M. A. (2002). Structural characterisation of the olive pomace pectic polysaccharide arabinan side chains. *Carbohydrate Research, 337*(10), 917-924.

Carvalho, E., Mateus, N., Plet, B., Pianet, I., Dufourc, E., & De Freitas, V. (2006). Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins. *Journal of Agricultural and Food Chemistry, 54*(23), 8936-8944.

Cavalcanti, R. N., Santos, D. T., & Meireles, M. A. A. (2011). Non-thermal stabilization mechanisms of anthocyanins in model and food systems-An overview. *Food Research International, 44*(2), 499-509.

Chacón, M. R., Ceperuelo-Mallafré, V., Maymó-Masip, E., Mateo-Sanz, J. M., Arola, L., Guitiérrez, C., Fernandez-Real, J. M., Ardèvol, A., Simón, I., & Vendrell, J. (2009).

Grape-seed procyanidins modulate inflammation on human differentiated adipocytes in vitro. *Cytokine, 47*(2), 137-142.

Chalier, P., Angot, B., Delteil, D., Doco, T., & Gunata, Z. (2007). Interactions between aroma compounds and whole mannoprotein isolated from Saccharomyces cerevisiae strains. *Food Chemistry, 100*(1), 22-30.

Chen, H., Zhang, M., Qu, Z., & Xie, B. (2008). Antioxidant activities of different fractions of polysaccharide conjugates from green tea (Camellia Sinensis). *Food Chemistry, 106*(2), 559-563.

Cheynier, V., Rigaud, J., Souquet, J. M., Barillere, J. M., & Moutounet, M. (1989). Effect of pomace contact and hyperoxidation on the phenolic composition and quality of grenache and chardonnay wines. *American Journal of Enology and Viticulture, 40*(1), 36-42.

Cheynier, V., Souquet, J. M., Kontek, A., & Moutounet, M. (1994). Anthocyanin degradation oxidizing grape musts. *Journal of the Science of Food and Agriculture, 66*(3), 283-288.

Chira, K., Jourdes, M., & Teissedre, P.-L. (2012). Cabernet sauvignon red wine astringency quality control by tannin characterization and polymerization during storage. *Eur Food Res Technol, 234*, 253–261.

Cilla, A., Gonzalez-Sarrias, A., Tomas-Barberan, F. A., Espin, J. C., & Barbera, R. (2009). Availability of polyphenols in fruit beverages subjected to in vitro gastrointestinal digestion and their effects on proliferation, cell-cycle and apoptosis in human colon cancer Caco-2 cells. *Food Chemistry, 114*(3), 813-820.

Coelho, E., Rocha, S. M., & Coimbra, M. A. (2011). Foamability and foam stability of molecular reconstituted model sparkling wines. *Journal of Agricultural and Food Chemistry, 59*(16), 8770-8778.

Coimbra, M. A., Barros, A. S., Coelho, E., Goncalves, F., Rocha, S. M., & Delgadillo, I. (2005). Quantification of polymeric mannose in wine extracts by FT-IR spectroscopy and OSC-PLS1 regression. *Carbohydrate Polymers, 61*(4), 434-440.

Coimbra, M. A., & Delgadillo, I. (1997) “Heteroglicanas - Polissacarídeos das Plantas” *In* Bioquímica, Edição Revista, M.J. Halpern, Ed., 18, 185-194, LIDEL Edições Técnicas, Lisboa.

Coimbra, M. A., Delgadillo, I., Waldron, K. W., & Selvendran, R. R. (1996). Isolation and analysis of cell wall polymers from olive pulp. *Modern Methods of Plant Analysis, 17*, 19-44.

Coimbra, M. A., Goncalves, F., Barros, A. S., & Delgadillo, I. (2002). Fourier transform infrared spectroscopy and chemometric analysis of white wine polysaccharide extracts. *Journal of Agricultural and Food Chemistry, 50*(12), 3405-3411.

Colado Simao, A. N., Suzukawa, A. A., Casado, M. F., Oliveira, R. D., Guarnier, F. A., & Cecchini, R. (2006). Genistein abrogates pre-hemolytic and oxidative stress damage induced by 2,2'-Azobis (Amidinopropane). *Life sciences, 78*(11), 1202-1210.

Cosme, F., Ricardo-Da-Silva, J. M., & Laureano, O. (2009). Tannin profiles of *Vitis vinifera* L. cv. red grapes growing in Lisbon and from their monovarietal wines. *Food Chemistry, 112*(1), 197-204.

Cruz, L., Teixeira, N., Silva, A. M. S., Mateus, N., Borges, J., & de Freitas, V. (2008). Role of Vinylcatechin in the Formation of Pyranomalvidin-3-glucoside-(+)-Catechin. *Journal of Agricultural and Food Chemistry, 56*(22), 10980-10987.

Dai, F., Miao, Q., Zhou, B., Yang, L., & Liu, Z. L. (2006). Protective effects of flavonols and their glycosides against free radical-induced oxidative hemolysis of red blood cells. *Life sciences, 78*(21), 2488-2493.

Dallas, C., & Laureano, O. (1994). Effcts of pH, sulfur-dioxide, alcohol content, temperature and storage time on color composition of a young portuguese red table wine. *Journal of the Science of Food and Agriculture, 65*(4), 477-485.

Dallas, C., Ricardo-da-Silva, J. M., & Laureano, O. (1996). Products formed in model wine solutions involving anthocyanins, procyanidin B-2, and acetaldehyde. *Journal of Agricultural and Food Chemistry, 44*(8), 2402-2407.

De Freitas, V., Carvalho, E., & Mateus, N. (2003). Study of carbohydrate influence on protein-tannin aggregation by nephelometry. *Food Chemistry, 81*(4), 503-509.

De Freitas, V. A. P., & Glories, Y. (1999). Concentration and compositional changes of procyanidins in grape seeds and skin of white *Vitis vinifera* varieties. *Journal of the Science of Food and Agriculture, 79*(12), 1601-1606.

De Freitas , V. A. P., Glories, Y., Bourgeois, G., & Vitry, C. (1998). Characterisation of oligomeric and polymeric procyanidins from grape seeds by liquid secondary ion mass spectrometry. *Phytochemistry, 49*(5), 1435-1441.

Degenhardt, A., Knapp, H., & Winterhalter, P. (2000). Separation and purification of anthocyanins by high-speed countercurrent chromatography and screening for antioxidant activity. *Journal of Agricultural and Food Chemistry, 48*(2), 338-343.

Deng, Y., Wu, Y., & Li, Y. F. (2005). Effects of high O_2 levels on post-harvest quality and shelf life of table grapes during long-term storage. *European Food Research and Technology, 221*(3-4), 392-397.

Dinkova-Kostova, A. T., & Talalay, P. (2008). Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Molecular Nutrition & Food Research, 52*, S128-S138.

Doco, T., & Brillouet, J. M. (1993). Isolation and characterization of a rhamnogalacturonan-II from red wine. *Carbohydrate Research, 243*(2), 333-343.

Doco, T., Lecas, M., Pellerin, P., Brillouet, J. M., & Moutounet, M. (1995). Les polysaccharides pectiques de la pulpe et de la pellicule de raisin. *Revue Francaise Oenologie, 153*, 16-23.

Doco, T., O'Neill, M. A., & Pellerin, P. (2001). Determination of the neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC-EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydrate Polymers, 46*(3), 249-259.

Doco, T., Quellec, N., Moutounet, M., & Pellerin, P. (1999). Polysaccharide patterns during the aging of Carignan noir red wines. *American Journal of Enology and Viticulture, 50*(1), 25-32.

Doco, T., Vuchot, P., Cheynier, V., & Moutounet, M. (2003). Structural modification of wine arabinogalactans during aging on lees. *American Journal of Enology and Viticulture, 54*(3), 150-157.

Doco, T., Williams, P., & Cheynier, V. (2007). Effect of flash release and pectinolytic enzyme treatments on wine polysaccharide composition. *Journal of Agricultural and Food Chemistry, 55*(16), 6643-6649.

Dolara, P., Luceri, C., De Filippo, C., Femia, A. P., Giovannelli, L., Caderni, G., Cecchini, C., Silvi, S., Orpianesi, C., & Cresci, A. (2005). Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 591*(1-2), 237-246.

Dubourdieu, D., Ribereaugayon, P., & Fournet, B. (1981). Struture of the extracellular beta-D-glucan from *botrytis-cinerea*. *Carbohydrate Research, 93*(2), 294-299.

Ducasse, M.-A., Williams, P., Meudec, E., Cheynier, V., & Doco, T. (2010). Isolation of Carignan and Merlot red wine oligosaccharides and their characterization by ESI-MS. *Carbohydrate Polymers, 79*(3), 747-754.

Dumitriu, S. (1998). Polysaccharides, structural diversity and functionality. In *Ed. Marcel Dekker, N. Y.*).

Eiro, M. J., & Heinonen, M. (2002). Anthocyanin color behavior and stability during storage: Effect of intermolecular copigmentation. *Journal of Agricultural and Food Chemistry, 50*(25), 7461-7466.

Ersus, S., & Yurdagel, U. (2007). Microencapsulation of anthocyanin pigments of black carrot (Daucus carota L.) by spray drier. *Journal of Food Engineering, 80*(3), 805-812.

Escot, S., Feuillat, M., Dulau, L., & Charpentier, C. (2001). Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. *Australian Journal of Grape and Wine Research, 7*(3), 153-159.

Estruch, R., Sacanella, E., Badia, E., Antúnez, E., Nicolás, J. M., Fernández-Solá, J., Rotilio, D., de Gaetano, G., Rubin, E., & Urbano-Márquez, A. (2004). Different effects of red wine and gin consumption on inflammatory biomarkers of atherosclerosis: a prospective randomized crossover trial: Effects of wine on inflammatory markers. *Atherosclerosis, 175*(1), 117-123.

Fave, G., Coste, T. C., & Armand, M. (2004). Physicochemical properties of lipids: New strategies to manage fatty acid bioavailability. *Cellular and Molecular Biology, 50*(7), 815-831.

Felgines, C., Talavera, S., Gonthier, M. P., Texier, O., Scalbert, A., Lamaison, J. L., & Remesy, C. (2003). Strawberry Anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans. *Journal of Nutrition, 133*(5), 1296-1301.

Fernandes, J. C., Eaton, P., Nascimento, H., Belo, L., Rocha, S., Vitorino, R., Amado, F., Gomes, J., Santos-Silva, A., Pintado, M. E., & Malcata, F. X. (2008). Effects of chitooligosaccharides on human red blood cell morphology and membrane protein structure. *Biomacromolecules, 9*(12), 3346-3352.

Fernandez-Pachon, M. S., Villano, D., Garcia-Parrilla, M. C., & Troncoso, A. M. (2004). Antioxidant activity of wines and relation with their polyphenolic composition. *Analytica Chimica Acta, 513*(1), 113-118.

Ferreira, R. B., Piçarra-Pereira, M. A., Monteiro, S., Loureiro, V. l. B., & Teixeira, A. R. (2001). The wine proteins. *Trends in Food Science & Technology, 12*(7), 230-239.

Feuillat, M. (1998). Autolyse de levures In Oenologie: Fondements scientifiques et technologiques. *Flancy, C., Ed.; Lavoisiser: Paris, France.*, 444-454.

Feuillat, M. (2003). Yeast macromolecules: Origin, composition, and enological interest. *American Journal of Enology and Viticulture, 54*(3), 211-213.

Filip, V., Plockova, M., Smidrkal, J., Spickova, Z., Melzoch, K., & Schmidt, S. (2003). Resveratrol and its antioxidant and antimicrobial effectiveness. *Food Chemistry, 83*(4), 585-593.

Fleschhut, J., Kratzer, F., Rechkemmer, G., & Kulling, S. E. (2006). Stability and biotransformation of various dietary anthocyanins in vitro. *European Journal of Nutrition, 45*(1), 7-18.

Fogliano, V., Verde, V., Randazzo, G., & Ritieni, A. (1999). Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. *Journal of Agricultural and Food Chemistry, 47*(3), 1035-1040.

Fornairon-Bonnefond, C., Demaretz, V., Rosenfeld, E., & Salmon, J. M. (2002). Oxygen addition and sterol synthesis in Saccharomyces cerevisiae during enological fermentation. *Journal of Bioscience and Bioengineering, 93*(2), 176-182.

Francisco, V., Figueirinha, A., Neves, B. M., García-Rodríguez, C., Lopes, M. C., Cruz, M. T., & Batista, M. T. (2011). Cymbopogon citratus as source of new and safe anti-inflammatory drugs: Bio-guided assay using lipopolysaccharide-stimulated macrophages. *Journal of Ethnopharmacology, 133*(2), 818-827.

Frankel, E. N., German, J. B., Kinsella, J. E., Parks, E., & Kanner, J. (1993). Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *The Lancet, 341*(8843), 454-457.

Frankel, E. N., Waterhouse, A. L., & Teissedre, P. L. (1995). Principal phenolic phytochemicals in selected california wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins *Journal of Agricultural and Food Chemistry, 43*(4), 890-894.

Fulcrand, H., dosSantos, P. J. C., SarniManchado, P., Cheynier, V., & FavreBonvin, J. (1996). Structure of new anthocyanin-derived wine pigments. *Journal of the Chemical Society-Perkin Transactions 1*(7), 735-739.

Fuleki, T., & Ricardo-da-Silva, J. M. (1997). Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. *Journal of Agricultural and Food Chemistry, 45*(4), 1156-1160.

García-Falcón, M. S., Perez-Lamela, C., Martinez-Carballo, E., & Simal-Gandara, J. (2007). Determination of phenolic compounds in wines: Influence of bottle storage of young red wines on their evolution. *Food Chemistry, 105*(1), 248-259.

Gaspar, Y., Johnson, K. L., McKenna, J. A., Bacic, A., & Schultz, C. J. (2001). The complex structures of arabinogalactan-proteins and the journey towards understanding function. *Plant Molecular Biology, 47*(1-2), 161-176.

Geller, D. A., & Billiar, T. R. (1998). Molecular biology of nitric oxide synthases. *Cancer and Metastasis Reviews, 17*(1), 7-23.

Ghiselli, A., Nardini, M., Baldi, A., & Scaccini, C. (1998). Antioxidant activity of different phenolic fractions separated from an Italian red wine. *Journal of Agricultural and Food Chemistry, 46*(2), 361-367.

Ginjom, I., D'Arcy, B., Caffin, N., & Gidley, M. (2011). Phenolic compound profiles in selected Queensland red wines at all stages of the wine-making process. *Food Chemistry, 125*(3), 823-834.

Glavas-Obrovac, L., Opacak, T., Bares, V., Subaric, D., Babic, J., Ilakovac, V., & Karner, I. (2006). Effects of dealcoholized red and white wines on human tumour and normal cells proliferation. *Acta Alimentaria, 35*(2), 153-162.

Gomez-Cordoves, C., Bartolome, B., Vieira, W., & Virador, V. M. (2001). Effects of wine phenolics and sorghum tannins on tyrosinase activity and growth of melanoma cells. *Journal of Agricultural and Food Chemistry, 49*(3), 1620-1624.

Gómez-Plaza, E., Gil-Muñoz, R., López-Roca, J. M., Martínez-Cutillas, A., & Fernández-Fernández, J. I. (2002). Maintenance of colour composition of a red wine during storage. Influence of prefermentative practices, maceration time and storage. *LWT - Food Science and Technology, 35*(1), 46-53.

Goncalves, F. J., & Jordao, A. M. (2009). Changes in antioxidant activity and proanthocyanidin fraction of red wine aged in contact with portuguese (*Quercus pyrenaica* Willd.) and american (*Quercus alba* L.) oak wood chips. *Italian Journal of Food Science, 21*(1), 51-64.

Gonzalez-Manzano, S., Rivas-Gonzalo, J. C., & Santos-Buelga, C. (2004). Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. *Analytica Chimica Acta, 513*(1), 283-289.

Gonzalez-Manzano, S., Santos-Buelga, C., Duenas, M., Rivas-Gonzalo, J. C., & Escribano-Bailon, T. (2008). Colour implications of self-association processes of wine anthocyanins. *European Food Research and Technology, 226*(3), 483-490.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and N-15-labeled nitrate in biological-fluids. *Analytical Biochemistry, 126*(1), 131-138.

Gris, E. F., Ferreira, E. A., Falcao, L. D., & Bordignon-Luiz, M. T. (2007). Caffeic acid copigmentation of anthocyanins from Cabernet Sauvignon grape extracts in model systems. *Food Chemistry, 100*(3), 1289-1296.

Gryglewski, R. J., Korbut, R., Robak, J., & Swies, J. (1987). On the mecanism of antithrombotic action of flavonoids. *Biochemical Pharmacology, 36*(3), 317-322.

Gu, L. W., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., & Prior, R. L. (2003). Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *Journal of Agricultural and Food Chemistry, 51*(25), 7513-7521.

Guadalupe, Z., & Ayestarán, B. (2007). Polysaccharide profile and content during the vinification and aging of Tempranillo red wines. *Journal of Agricultural and Food Chemistry, 55*(26), 10720–10728.

Guadalupe, Z., & Ayestarán, B. (2008a). Changes in the color components and phenolic content of red wines from *Vitis vinifera* L. Cv. "Tempranillo" during vinification and aging. *European Food Research and Technology, 228*(1), 29-38.

Guadalupe, Z., & Ayestarán, B. (2008b). Effect of commercial mannoprotein addition on polysaccharide, polyphenolic, and color composition in red wines. *Journal of Agricultural and Food Chemistry, 56*(19), 9022-9029.

Guadalupe, Z., Martínez-Pinilla, O., Garrido, Á., Carrillo, J. D., & Ayestarán, B. (2012). Quantitative determination of wine polysaccharides by gas chromatography–mass spectrometry (GC–MS) and size exclusion chromatography (SEC). *Food Chemistry, 131*(1), 367-374.

Guadalupe, Z., Martinez, L., & Ayestarán, B. (2010). Yeast mannoproteins in red winemaking: effect on polysaccharide, polyphenolic, and color composition. *American Journal of Enology and Viticulture, 61*(2), 191-200.

Guadalupe, Z., Palacios, A., & Ayestarán, B. (2007). Maceration enzymes and mannoproteins: A possible strategy to increase colloidal stability and color extraction in red wines. *Journal of Agricultural and Food Chemistry, 55*(12), 4854-4862.

Hager, A., Howard, L. R., Prior, R. L., & Brownmiller, C. (2008). Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed black raspberry products. *Journal of Food Science, 73*(6), H134-H140.

Hakimuddin, F., Tiwari, K., Paliyath, G., & Meckling, K. (2008). Grape and wine polyphenols down-regulate the expression of signal transduction genes and inhibit the growth of estrogen receptor-negative MDA-MB231 tumors in nu/nu mouse xenografts. *Nutrition Research, 28*(10), 702-713.

Halliwell, B., Rafter, J., & Jenner, A. (2005). Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *American Journal of Clinical Nutrition, 81*(1), 268S-276S.

Hashimoto, M., Hossain, M. S., Shimada, T., Yamasaki, H., Fujii, Y., & Shido, O. (2001). Effects of docosahexaenoic acid on annular lipid fluidity of the rat bile canalicular plasma membrane. *Journal of Lipid Research, 42*(7), 1160-1168.

He, J. R., Oliveira, J., Silva, A. M. S., Mateus, N., & De Freitas, V. (2010). Oxovitisins: a new class of neutral pyranone-anthocyanin derivatives in red wines. *Journal of Agricultural and Food Chemistry, 58*(15), 8814-8819.

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry, 13*(10), 572-584.

Hendrich, A. B. (2006). Flavonoid-membrane interactions: possible consequences for biological effects of some polyphenolic compounds. *Acta Pharmacologica Sinica, 27*(1), 27-40.

Hensel, A., & Meier, I. (1999). Pectins and xyloglucans exhibit antimutagenic activities against nitroaromatic compounds. *Planta Medica, 65*(5), 395-399.

Hollman, P. C. H., Cassidy, A., Comte, B., Heinonen, M., Richelle, M., Richling, E., Serafini, M., Scalbert, A., Sies, H., & Vidry, S. (2011). The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans Is not established. *Journal of Nutrition, 141*(5), 989S-1009S.

Hou, D. X. (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Current Molecular Medicine, 3*(2), 149-159.

Huang, D. J., Ou, B. X., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*(6), 1841-1856.

Hwang, Y. P., Choi, J. H., Yun, H. J., Han, E. H., Kim, H. G., Kim, J. Y., Park, B. H., Khanal, T., Choi, J. M., Chung, Y. C., & Jeong, H. G. (2011). Anthocyanins from purple sweet potato attenuate dimethylnitrosamine-induced liver injury in rats by inducing Nrf2-mediated antioxidant enzymes and reducing COX-2 and iNOS expression. *Food and Chemical Toxicology, 49*(1), 93-99.

Iacobucci, G. A., & Sweeny, J. G. (1983). The chemistry of anthocyanins, anthocyanidins and related flavylium salts. *Tetrahedron, 39*(19), 3005-3038.

Iijima, K., Yoshizumi, M., Hashimoto, M., Kim, S., Eto, M., Ako, J., Liang, Y. Q., Sudoh, N., Hosoda, K., Nakahara, K., Toba, K., & Ouchi, Y. (2000). Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and downregulate expression of cyclin A gene. *Circulation, 101*(7), 805-811.

Ishii, T., & Matsunaga, T. (1996). Isolation and characterization of a boron-rhamnogalacturonan-II complex from cell walls of sugar beet pulp. *Carbohydrate Research, 284*(1), 1-9.

Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K., Ohkura, Y., & Ueno, K. (1996). A combines assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biological & Pharmaceutical Bulletin, 19*(11), 1518-1520.

Ivanova, V., Dornyei, A., Mark, L., Vojnoski, B., Stafilov, T., Stefova, M., & Kilar, F. (2011). Polyphenolic content of Vranec wines produced by different vinification conditions. *Food Chemistry, 124*(1), 316-325.

Jeong, J.-H., Jung, H., Lee, S.-R., Lee, H.-J., Hwang, K. T., & Kim, T.-Y. (2010). Anti-oxidant, anti-proliferative and anti-inflammatory activities of the extracts from black raspberry fruits and wine. *Food Chemistry, 123*(2), 338-344.

Jigami, Y., & Odani, T. (1999). Mannosylphosphate transfer to yeast mannan. *Biochimica Et Biophysica Acta-General Subjects, 1426*(2), 335-345.

Jimenez, A., Lisa-Santamaria, P., Garcia-Marino, M., Escribano-Bailon, M. T., Rivas-Gonzalo, J. C., & Revuelta, J. L. (2010). The biological activity of the wine anthocyanins delphinidin and petunidin is mediated through Msn2 and Msn4 in Saccharomyces cerevisiae. *Fems Yeast Research, 10*(7), 858-869.

Jordão, A. M., Ricardo-da-Silva, J. M., & Laureano, O. (2001). Evolution of catechin and procyanidin composition during grape maturation of two varieties (*Vitis vinifera* L.) Castelão Francês and Touriga Francesa. *American Journal of Enology and Viticulture, 52*, 230-234.

Kader, F., Rovel, B., Girardin, M., & Metche, M. (1997). Mechanism of browning in fresh highbush blueberry fruit (Vaccinium corymbosum L). Role of blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. *Journal of the Science of Food and Agriculture, 74*(1), 31-34.

Kallithraka, S., Mohdaly, A. A.-A., Makris, D. P., & Kefalas, P. (2005). Determination of major anthocyanin pigments in Hellenic native grape varieties (*Vitis vinifera* sp.): association with antiradical activity. *Journal of Food Composition and Analysis, 18*(5), 375-386.

Kandra, L., Gyemant, G., Zajacz, A., & Batta, G. (2004). Inhibitory effects of tannin on human salivary alpha-amylase. *Biochemical and Biophysical Research Communications, 319*(4), 1265-1271.

Katalinic, V., Milos, M., Modun, D., Music, I., & Boban, M. (2004). Antioxidant effectiveness of selected wines in comparison with (+)-catechin. *Food Chemistry, 86*(4), 593-600.

Kim, G.-N., Shin, J.-G., & H-D., J. (2009). Antioxidant and antidiabetic activity of Dangyuja (*Citrus grandis Osbeck*) extract treated with *Aspergillus saitoi*. *Food Chemistry, 117*, 35–41.

Kirca, A., & Cemeroglu, B. (2003). Degradation kinetics of anthocyanins in blood orange juice and concentrate. *Food Chemistry, 81*(4), 583-587.

Kirca, A., Ozkan, M., & Cemeroglu, B. (2007). Storage stability of strawberry jam color enhanced with black carrot juice concentrate. *Journal of Food Processing and Preservation, 31*(5), 531-545.

Klis, F. M., Mol, P., Hellingwerf, K., & Brul, S. (2002). Dynamics of cell wall structure in Saccharomyces cerevisiae. *Fems Microbiology Reviews, 26*(3), 239-256.

Ko, F. N., Hsiao, G., & Kuo, Y. H. (1997). Protection of oxidative hemolysis by demethyldiisoeugenol in normal and beta-thalassemic red blood cells. *Free Radical Biology and Medicine, 22*(1-2), 215-222.

Kogan, G., Stasko, A., Bauerova, K., Polovka, M., Soltes, L., Brezova, V., Navarova, J., & Mihalova, D. (2005). Antioxidant properties of yeast (1 -> 3)-beta-D-glucan studied by electron paramagnetic resonance spectroscopy and its activity in the adjuvant arthritis. *Carbohydrate Polymers, 61*(1), 18-28.

Koli, R., Erlund, I., Jula, A., Marniemi, J., Mattila, P., & Alfthan, G. (2010). Bioavailability of various polyphenols from a diet containing moderate amounts of berries. *Journal of Agricultural and Food Chemistry, 58*(7), 3927-3932.

Konczak-Islam, I., Yoshimoto, M., Hou, D. X., Terahara, N., & Yamakawa, O. (2003). Potential chemopreventive properties of anthocyanin-rich aqueous extracts from in vitro produced tissue of sweetpotato (Ipomoea batatas L.). *Journal of Agricultural and Food Chemistry, 51*(20), 5916-5922.

Konczak, I., & Zhang, W. (2004). Anthocyanins - More than nature's colours. *Journal of Biomedicine and Biotechnology*(5), 239-240.

Kondo, K., Kurihara, M., Fukuhara, K., Tanaka, T., Suzuki, T., Miyata, N., & Toyoda, M. (2000). Conversion of procyanidin B-type (catechin dimer) to A-type: evidence for abstraction of C-2 hydrogen in catechin during radical oxidation. *Tetrahedron Letters, 41*(4), 485-488.

Kong, J. M., Chia, L. S., Goh, N. K., Chia, T. F., & Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry, 64*(5), 923-933.

Kosir, I. J., & Kidric, J. (2002). Use of modern nuclear magnetic resonance spectroscopy in wine analysis: determination of minor compounds. *Analytica Chimica Acta, 458*(1), 77-84.

Kuhnle, G., Spencer, J. P. E., Schroeter, H., Shenoy, B., Debnam, E. S., Srai, S. K. S., Rice-Evans, C., & Hahn, U. (2000). Epicatechin and catechin are O-methylated and glucuronidated in the small intestine. *Biochemical and Biophysical Research Communications, 277*(2), 507-512.

Kulkarni, S. D., Acharya, R., Rajurkar, N. S., & Reddy, A. V. R. (2007). Evaluation of bioaccessibility of some essential elements from wheatgrass (Triticum aestivum L.) by in vitro digestion method. *Food Chemistry, 103*(2), 681-688.

Kumar-Roiné, S., Matsui, M., Reybier, K., Darius, H. T., Chinain, M., Pauillac, S., & Laurent, D. (2009). Ability of certain plant extracts traditionally used to treat ciguatera fish poisoning to inhibit nitric oxide production in RAW 264.7 macrophages. *Journal of Ethnopharmacology, 123*(3), 369-377.

Labarbe, B., Cheynier, V., Brossaud, F., Souquet, J. M., & Moutounet, M. (1999). Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. *Journal of Agricultural and Food Chemistry, 47*, 2719-2723.

Lachman, J., Šulc, M., & Schilla, M. (2007). Comparison of the total antioxidant status of Bohemian wines during the wine-making process. *Food Chemistry, 103*(3), 802-807.

Larrauri, J. A., Sanchez-Moreno, C., Ruperez, P., & Saura-Calixto, F. (1999). Free radical scavenging capacity in the aging of selected red Spanish wines. *Journal of Agricultural and Food Chemistry, 47*(4), 1603-1606.

Le Bourvellec, C., Bouchet, B., & Renard, C. M. G. C. (2005). Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1725*(1), 10-18.

Le Goff, A., Renard, C., Bonnin, E., & Thibault, J. F. (2001). Extraction, purification and chemical characterisation of xylogalacturonans from pea hulls. *Carbohydrate Polymers, 45*(4), 325-334.

Lee, K. W., Kim, Y. J., Kim, D. O., Lee, H. J., & Lee, C. Y. (2003). Major phenolics in apple and their contribution to the total antioxidant capacity. *Journal of Agricultural and Food Chemistry, 51*(22), 6516-6520.

Li, W. G., Zhang, X. Y., Wu, Y. J., & Tian, X. (2001). Anti-inflammatory effect and mechanism of proanthocyanidins from grape seeds. *Acta Pharmacologica Sinica, 22*(12), 1117-1120.

Liao, H., Cai, Y., & Haslam, E. (1992). Polyphenol interactions. 6. Anthocyanin co-pigmentation and colour changes in red wines. *Journal of the Science of Food and Agriculture, 59*, 299–305.

Lotito, S. B., Actis-Goretta, L., Renart, M. L., Caligiuri, M., Rein, D., Schmitz, H. H., Steinberg, F. M., Keen, C. L., & Fraga, C. G. (2000). Influence of oligomer chain length on the antioxidant activity of procyanidins. *Biochemical and Biophysical Research Communications, 276*(3), 945-951.

Luck, G., Liao, H., Murray, N. J., Grimmer, H. R., Warminski, E. E., Williamson, M. P., Lilley, T. H., & Haslam, E. (1994). Polyphenols, astringency and proline-rich ptroteins. *Phytochemistry, 37*(2), 357-371.

Maccarone, E., Maccarrone, A., & Rapisarda, P. (1985). Stabilization of anthocyanins of blood orange fruit juice. *Journal of Food Science, 50*(4), 901-904.

Mackenzi.Sl, & Tenaschu.D. (1974). Gas-liquid-chromatography of n-heptafluorobutyryl isobutyl esters of amino-acids. *Journal of Chromatography, 97*(1), 19-24.

Maier, T., Fromm, M., Schieber, A., Kammerer, D. R., & Carle, R. (2009). Process and storage stability of anthocyanins and non-anthocyanin phenolics in pectin and gelatin gels enriched with grape pomace extracts. *European Food Research and Technology, 229*(6), 949-960.

Malien-Aubert, C., Dangles, O., & Amiot, M. J. (2001). Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra and intermolecular copigmentation. *Journal of Agricultural and Food Chemistry, 49*(1), 170-176.

Malone, M. E., Appelqvist, I. A. M., & Norton, I. T. (2003). Oral behaviour of food hydrocolloids and emulsions. Part 2. Taste and aroma release. *Food Hydrocolloids, 17*(6), 775-784.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: food sources and bioavailability. *American Journal of Clinical Nutrition, 79*(5), 727-747.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition, 81*(1), 230S-242S.

Mazza, G. (1995). Anthocyanins in grapes and grape products. *Critical Reviews in Food Science and Nutrition, 35*(4), 341-371.

Mazza, G., & Brouillard, R. (1990). The mechanism of copigmentation of anthocyanins in aqueous-solutions. *Phytochemistry, 29*(4), 1097-1102.

Mazza, G., Fukumoto, L., Delaquis, P., Girard, B., & Ewert, B. (1999). Anthocyanins, phenolics, and color of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. *Journal of Agricultural and Food Chemistry, 47*(10), 4009-4017.

McDougall, G. J., Fyffe, S., Dobson, P., & Stewart, D. (2005). Anthocyanins from red wine – Their stability under simulated gastrointestinal digestion. *Phytochemistry, 66*(21), 2540-2548.

Miller, N. J., Riceevans, C., Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science, 84*(4), 407-412.

Minussi, R. C., Rossi, M., Bologna, L., Cordi, L. v., Rotilio, D., Pastore, G. M., & Durán, N. (2003). Phenolic compounds and total antioxidant potential of commercial wines. *Food Chemistry, 82*(3), 409-416.

Mirabel, M., Saucier, C., Guerra, C., & Glories, Y. (1999). Copigmentation in model wine solutions: Occurrence and relation to wine aging. *American Journal of Enology and Viticulture, 50*(2), 211-218.

Moine-Ledoux, V., & Dubourdieu, D. (2002). Rôle des mannoproteínes des levures vis à vis de la stabilisation tartrique des vins. . *Bulletin OIV, 75*, 471-482.

Monagas, M., Gomez-Cordoves, C., & Bartolome, B. (2005). Evolution of polyphenols in red wines from *Vitis vinifera* L. during aging in the bottle - I. Anthocyanins and pyranoanthocyanins. *European Food Research and Technology, 220*(5-6), 607-614.

Monagas, M., Gomez-Cordoves, C., Bartolome, B., Laureano, O., & Ricardo-Da-Silva, J. M. (2003). Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from *Vitis vinifera* L. cv. Graciano, Tempranillo, and Cabernet Sauvignon. *Journal of Agricultural and Food Chemistry, 51*(22), 6475-6481.

Monagas., Gomez-Cordoves, C., Bartolome, B., Laureano, O., & Ricardo-Da-Silva, J. M. (2003). Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from Vitis vinifera L. cv. Graciano, Tempranillo, and Cabernet Sauvignon. *Journal of Agricultural and Food Chemistry, 51*(22), 6475-6481.

Monteiro, F. F., & Bisson, L. F. (1991). Amino-acid utilization and urea formation during vinification fermentations. *American Journal of Enology and Viticulture, 42*(3), 199-208.

Moreno-Labanda, J. F., Mallavia, R., Perez-Fons, L., Lizama, V., Saura, D., & Micol, V. (2004). Determination of piceid and resveratrol in Spanish wines deriving from

Monastrell (Vitis vinifera L.) grape variety. *Journal of Agricultural and Food Chemistry, 52*(17), 5396-5403.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival - application to proliferation and cyto-toxicity assays. *Journal of Immunological Methods, 65*(1-2), 55-63.

Mudnic, I., Modun, D., Rastija, V., Vukovic, J., Brizic, I., Katalinic, V., Kozina, B., Medic-Saric, M., & Boban, M. (2010). Antioxidative and vasodilatory effects of phenolic acids in wine. *Food Chemistry, 119*(3), 1205-1210.

Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A, 1054*(1-2), 95-111.

Ngamwongsatit, P., Banada, P. P., Panbangred, W., & Bhunia, A. K. (2008). WST-1-based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic Bacillus species using CHO cell line. *Journal of Microbiological Methods, 73*(3), 211-215.

Nunes, C., Saraiva, J. A., & Coimbra, M. A. (2008). Effect of candying on cell wall polysaccharides of plums (*Prunus domestica* L.) and influence of cell wall enzymes. *Food Chemistry, 111*(3), 538-548.

O' Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G., & Albersheim, P. (1996). Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester - In vitro conditions for the formation and hydrolysis of the dimer. *Journal of Biological Chemistry, 271*(37), 22923-22930.

O´Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G., & Albersheim, P. (1996). Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester - In vitro conditions for the formation and hydrolysis of the dimer. *Journal of Biological Chemistry, 271*(37), 22923-22930.

Oliveira, J., Azevedo, J., Silva, A. M. S., Teixeira, N., Cruz, L., Mateus, N., & de Freitas, V. (2010). Pyranoanthocyanin dimers: a new family of turquoise blue anthocyanin-derived pigments found in Port Wine. *Journal of Agricultural and Food Chemistry, 58*(8), 5154-5159.

Ollila, F., Halling, K., Vuorela, P., Vuorela, H., & Slotte, J. P. (2002). Characterization of flavonoid-biomembrane interactions. *Archives of Biochemistry and Biophysics, 399*(1), 103-108.

Ortega-Regules, A., Romero-Cascales, I., Ros-Garcia, J. M., Lopez-Roca, J. M., & Gomez-Plaza, E. (2006). A first approach towards the relationship between grape skin cell-wall composition and anthocyanin extractability. *Analytica Chimica Acta, 563*(1-2), 26-32.

Ortuno, J., Covas, M.-I., Farre, M., Pujadas, M., Fito, M., Khymenets, O., Andres-Lacueva, C., Roset, P., Joglar, J., Lamuela-Raventos, R. M., & de la Torre, R. (2010). Matrix effects on the bioavailability of resveratrol in humans. *Food Chemistry, 120*(4), 1123-1130.

Oteiza, P. I., Erlejman, A. G., Verstraeten, S. V., Keen, C. L., & Fraga, C. G. (2005). Flavonoid-membrane interactions: a protective role of flavonoids at the membrane surface? *Clinical & developmental immunology, 12*(1), 19-25.

Ou, K., Percival, S. S., Zou, T., Khoo, C., & Gu, L. (2012). Transport of cranberry A-Type procyanidin dimers, trimers, and tetramers across monolayers of Human Intestinal epithelial Caco-2 Cells. *Journal of Agricultural and Food Chemistry, 60*(6), 1390-1396.

Ozawa, T., Lilley, T. H., & Haslam, E. (1987). Polyphenols interactions - astringengy and the loss of astringency in ripening fruit. *Phytochemistry, 26*(11), 2937-2942.

Pafumi, Y., Lairon, D., de la Porte, P. L., Juhel, C., Storch, J., Hamosh, M., & Armand, M. (2002). Mechanisms of inhibition of triacylglycerol hydrolysis by human gastric lipase. *Journal of Biological Chemistry, 277*(31), 28070-28079.

Palma, M., Pineiro, Z., & Barroso, C. G. (2001). Stability of phenolic compounds during extraction with superheated solvents. *Journal of Chromatography A, 921*(2), 169-174.

Panico, A. M., Cardile, V., Avondo, S., Garufi, F., Gentile, B., Puglia, C., Bonina, F., Santagati, N. A., & Ronsisvalle, G. (2006). The in vitro effect of a lyophilized extract of wine obtained from Jacquez grapes on human chondrocytes. *Phytomedicine, 13*(7), 522-526.

Paulo, L., Domingues, F., Queiroz, J. A., & Gallardo, E. (2011). Development and Validation of an Analytical Method for the Determination of trans- and cis-Resveratrol in Wine: Analysis of Its Contents in 186 Portuguese Red Wines. *Journal of Agricultural and Food Chemistry, 59*(6), 2157-2168.

Pedersen, A. M., Bardow, A., Jensen, S. B., & Nauntofte, B. (2002). Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. *Oral Diseases, 8*(3), 117-129.

Pellerin, P., Doco, T., Vidal, S., Williams, P., Brillouet, J. M., & O'Neill, M. A. (1996). Structural characterization of red wine rhamnogalacturonan II. *Carbohydrate Research, 290*(2), 183-197.

Pellerin, P., Vidal, S., Williams, P., & Brillouet, J. M. (1995). Characterization of 5 type-II arabinogalactan-protein fractions from red wine of increasing uronic-acid content. *Carbohydrate Research, 277*(1), 135-143.

Pellerin, P., Waters, E., & Brillouet, J. M. (1993). Characterization of 2 arabinogalactan-proteins from red wine. *Carbohydrate Polymers, 22*(3), 187-192.

Perez-Jimenez, J., Serrano, J., Tabernero, M., Arranz, S., Diaz-Rubio, M. E., Garcia-Diz, L., Goni, I., & Saura-Calixto, F. (2009). Bioavailability of phenolic antioxidants associated with dietary fiber: plasma antioxidant capacity after acute and long-term intake in humans. *Plant Foods for Human Nutrition, 64*(2), 102-107.

Pérez, S., Rodríguez-Carvajal, M. A., & Doco, T. (2003). A complex plant cell wall polysaccharide: rhamnogalacturonan II. A structure in quest of a function. *Biochimie, 85*(1-2), 109-121.

Peterson, J., & Dwyer, J. (1998). Flavonoids: Dietary occurrence and biochemical activity. *Nutrition Research, 18*(12), 1995-2018.

Plaami, S. P. (1997). Content of dietary fiber in foods and its physiological effects. *Food Reviews International, 13*(1), 29-76.

Poncet-Legrand, C., Doco, T., Williams, P., & Vernhet, A. (2007). Inhibition of grape seed tannin aggregation by wine mannoproteins: Effect of polysaccharide molecular weight. *American Journal of Enology and Viticulture, 58*(1), 87-91.

Puvanesarajah, V., Darvill, A. G., & Albersheim, P. (1991). Structural characterization of 2 oligosaccharide fragments formed by the selective cleavage of rhamnogalacturonan-2 - evidence for the anomeric configuration and attachment sites of apiose and 3-deoxy-2-heptulosaric acid. *Carbohydrate Research, 218*, 211-222.

Queiroz, F., Oliveira, C., Pinho, O., & Ferreira, I. M. P. L. V. O. (2009). Degradation of anthocyanins and anthocyanidins in Blueberry Jams/Stuffed Fish. *Journal of Agricultural and Food Chemistry, 57*(22), 10712-10717.

Quinn, M. K., & Singleton, V. L. (1985). Isolation and identification of ellagitannins from white oak and an estimation of their roles in wine. *American Journal of Enology and Viticulture, 36*(2), 148-155.

Radovanovic, B., & Radovanovic, A. (2010). Free radical scavenging activity and anthocyanin profile of cabernet sauvignon wines from the Balkan region. *Molecules, 15*(6), 4213-4226.

Recamales, Á. F., Sayago, A., González-Miret, M. L., & Hernanz, D. (2006). The effect of time and storage conditions on the phenolic composition and colour of white wine. *Food Research International, 39*(2), 220-229.

Rein, M. J., Ollilainen, V., Vahermo, M., Yli-Kauhaluoma, J., & Heinonen, M. (2005). Identification of novel pyranoanthocyanins in berry juices. *European Food Research and Technology, 220*(3-4), 239-244.

Renaud, S., & Delorgeril, M. (1992). Wine, alcohol, platelets, and the french paradox for coronary heart-disease. *Lancet, 339*(8808), 1523-1526.

Ribereau-gayon, P., Glories, y., Maujean, A., & Dubourdie, D. (1998). Traite d'Oenologie. *Paris: Dumon*, 224–225

Ribereau-Gayon, P., & Stonestreet, E. (1966). Dosage des tanins du vin rouge et determination de leur structure. *Chimie Analytique, 48*, 188-196.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*(7), 933-956.

Riou, V., Vernhet, A., Doco, T., & Moutounet, M. (2002). Aggregation of grape seed tannins in model wine—effect of wine polysaccharides. *Food Hydrocolloids, 16*(1), 17-23.

Rivero-Pérez, M. D., Muñiz, P., & González-Sanjosé, M. L. (2008). Contribution of anthocyanin fraction to the antioxidant properties of wine. *Food and Chemical Toxicology, 46*(8), 2815-2822.

Robak, J., & Gryglewski, R. J. (1988). Flavonoids are scanvengers of superoxide anions. *Biochemical Pharmacology, 37*(5), 837-841.

Robards, K., Prenzler, P. D., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry, 66*(4), 401-436.

Rocculi, P., Romani, S., & Dalla Rosa, M. (2004). Evaluation of physico-chemical parameters of minimally processed apples packed in non-conventional modified atmosphere. *Food Research International, 37*(4), 329-335.

Rocha, S. M., Coutinho, P., Delgadillo, I., & Coimbra, M. A. (2007). Headspace-solid phase microextraction-gas chromatography as a tool to define an index that establishes the retention capacity of the wine polymeric fraction towards ethyl esters. *Journal of Chromatography A, 1150*(1-2), 155-161.

Rodriguez-Diaz, R. C., Aguilar-Caballos, M. P., & Gomez-Hens, A. (2006). Determination of some hydroxybenzoic acids and catechins in white wine samples by liquid chromatography with luminescence detection. *Journal of Separation Science, 29*(18), 2772-2779.

Roussis, I. G., Lambropoulos, I., Tzimas, P., Gkoulioti, A., Marinos, V., Tsoupeis, D., & Boutaris, I. (2008). Antioxidant activities of some Greek wines and wine phenolic extracts. *Journal of Food Composition and Analysis, 21*(8), 614-621.

Ruan, E. A., Rao, S., Burdick, J. S., Stryker, S. J., Telford, G. L., Otterson, M. F., Opara, E. C., & Koch, T. R. (1997). Glutathione levels in chronic inflammatory disorders of the human colon. *Nutrition Research, 17*(3), 463-473.

Sabolovic, N., Humbert, A. C., Radominska-Pandya, A., & Magdalou, J. (2006). Resveratrol is efficiently glucuronidated by UDP-glucuronosyltransferases in the human gastrointestinal tract and in Caco-2 cells. *Biopharmaceutics & Drug Disposition, 27*(4), 181-189.

Saleem, A., Husheem, M., Harkonen, P., & Pihlaja, K. (2002). Inhibition of cancer cell growth by crude extract and the phenolics of Terminalia chebula Retz. fruit. *Journal of Ethnopharmacology, 81*(3), 327-336.

Sanchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture, 76*(2), 270-276.

Sanchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1999). Free radical scavenging capacity of selected red, rose and white wines. *Journal of the Science of Food and Agriculture, 79*(10), 1301-1304.

Santos-Buelga, C., Franciaaricha, E. M., & Escribanobailon, M. T. (1995). Comparative flavan-3-ol composition od seeds from different grape varieties. *Food Chemistry, 53*(2), 197-201.

Sanz, T., & Luyten, H. (2006). Effect of thickening agent in the in vitro mouth, stomach and intestine release of tyrosol from enriched custards. *Food Hydrocolloids, 20*(5), 703-711.

Saucier, C., Mirabel, M., Daviaud, F., Longieras, A., & Glories, Y. (2001). Rapid fractionation of grape seed proanthocyanidins. *Journal of Agricultural and Food Chemistry, 49*(12), 5732-5735.

Saura-Calixto, F., & Díaz-Rubio, M. E. (2007). Polyphenols associated with dietary fibre in wine: A wine Polyphenols gap? *Food Research International, 40*(5), 613-619.

Schwarz, M., Quast, P., Von Baer, D., & Winterhalter, P. (2003). Vitisin a content in Chilean wines from *Vitis vinifera* Cv. Cabernet Sauvignon and contribution to the color of aged red wines. *Journal of Agricultural and Food Chemistry, 51*(21), 6261-6267.

Scott, M. D., Vandenberg, J. J. M., Repka, T., Rouyerfessard, P., Hebbel, R. P., Beuzard, Y., & Lubin, B. H. (1993). Effect of excess alpha-hemoglobin chains on cellular and membrane oxidation in model beta-thalassemic erythrocytes. *Journal of Clinical Investigation, 91*(4), 1706-1712.

Segarra, I., Lao, C., López-Tamames, E., & Torre-Boronat, M. C. (1995). Spectrophotometric methods. *Am J Enol Vitic, 46*(4), 564-570.

Selvendran, R. R., March, J. F., & Ring, S. G. (1979). Determination of aldoses and uronic-acid content of vegetable fiber. *Analytical Biochemistry, 96*(2), 282-292.

Serra, A., Macia, A., Romero, M.-P., Valls, J., Blade, C., Arola, L., & Motilva, M.-J. (2010). Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. *British Journal of Nutrition, 103*(7), 944-952.

Shahidi, F., Janitha, P. K., & Wanasundara, P. D. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition, 32*(1), 67-103.

Shenoy, V. R. (1993). Anthocyanins - prospective food colors. *Current Science, 64*(8), 575-579.

Shi, J., & Le Maguer, M. (2000). Lycopene in tomatoes: Chemical and physical properties affected by food processing. *Critical Reviews in Food Science and Nutrition, 40*(1), 1-42.

Simonetti, P., Pietta, P., & Testolin, G. (1997). Polyphenol content and total antioxidant potential of selected Italian wines. *Journal of Agricultural and Food Chemistry, 45*(4), 1152-1155.

Simonsen, H. T., Nielsen, M. S., Christensen, N. J., Christensen, U., La Cour, T. V., Motawia, M. S., Jespersen, B. P. A., Engelsen, S. B., & Moller, B. L. (2009). Molecular interactions between Barley and Oat beta-glucans and phenolic derivatives. *Journal of Agricultural and Food Chemistry, 57*(5), 2056-2064.

Singleton, V. L., & Rossi, A. (1965). Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144-158.

Şöhretoğlu, D., Sabuncuoğlu, S., & Harput, Ü. Ş. (2012). Evaluation of antioxidative, protective effect against H_2O_2 induced cytotoxicity, and cytotoxic activities of three different Quercus species. *Food and Chemical Toxicology, 50*(2), 141-146.

Somboonkaew, N., & Terry, L. A. (2010). Physiological and biochemical profiles of imported litchi fruit under modified atmosphere packaging. *Postharvest Biology and Technology, 56*(3), 246-253.

Somers, T. C., & Evans, M. E. (1986). Evolution of red wines.1. Ambient influences on color composition during early maturation. *Vitis, 25*(1), 31-39.

Souquet, J. M., Cheynier, V., Brossaud, F., & Moutounet, M. (1996). Polymeric proanthocyanidins from grape skins. *Phytochemistry, 43*, 509-512.

Stahl, W., van den Berg, H., Arthur, J., Bast, A., Dainty, J., Faulks, R. M., Gartner, C., Haenen, G., Hollman, P., Holst, B., Kelly, F. J., Polidori, M. C., Rice-Evans, C., Southon, S., van Vliet, T., Vina-Ribes, J., Williamson, G., & Astley, S. B. (2002). Bioavailability and metabolism. *Molecular aspects of medicine, 23*(1-3), 39-100.

Sun , B. H., Spranger, I., Roque-do-Vale, F., Leandro, C., & Belchior, P. (2001). Effect of different winemaking technologies on phenolic composition in Tinta Miuda red wines. *Journal of Agricultural and Food Chemistry, 49*(12), 5809-5816.

Sun, B. S., Fernandes, T. A., & Spranger, M. I. (2010). A new class of anthocyanin-procyanidin condensation products detected in red wine by electrospray ionization multi-stage mass spectrometry analysis. *Rapid Communications in Mass Spectrometry, 24*(3), 254-260.

Sun, B. S., Leandro, M. C., de Freitas, V., & Spranger, M. I. (2006). Fractionation of red wine polyphenols by solid-phase extraction and liquid chromatography. *Journal of Chromatography A, 1128*(1-2), 27-38.

Sun, B. S., Neves, A. C., Fernandes, T. A., Fernandes, A. L., Mateus, N., De Freitas, V., Leandro, C., & Spranger, M. I. (2011). Evolution of phenolic composition of red wine during vinification and storage and its contribution to wine sensory properties and antioxidant activity. *Journal of Agricultural and Food Chemistry, 59*(12), 6550-6557.

Sun, B. S., Pinto, T., Leandro, M. C., Ricardo-Da-Silva, J. M., & Spranger, M. I. (1999). Transfer of catechins and proanthocyanidins from solid parts of the grape cluster into wine. *American Journal of Enology and Viticulture, 50*(2), 179-184.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J. O., & Dommes, J. (2009). Comparative antioxidant capacities of phenolic compounds measured by various tests. *Food Chemistry, 113*(4), 1226-1233.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., & Conte, A. (2010). In vitro bio-accessibility and antioxidant activity of grape polyphenols. *Food Chemistry, 120*(2), 599-606.

Tapiero, H., Tew, K. D., Ba, G. N., & Mathe, G. (2002). Polyphenols: do they play a role in the prevention of human pathologies? *Biomedicine & Pharmacotherapy, 56*(4), 200-207.

Tarahovsky, Y. S., Muzafarov, E. N., & Kim, Y. A. (2008). Rafts making and rafts braking: how plant flavonoids may control membrane heterogeneity. *Molecular and Cellular Biochemistry, 314*(1-2), 65-71.

Tedeschi, C., Clement, V., Rouvet, M., & Valles-Pamies, B. (2009). Dissolution tests as a tool for predicting bioaccessibility of nutrients during digestion. *Food Hydrocolloids, 23*(4), 1228-1235.

Tedesco, I., Russo, G. L., Nazzaro, F., Russo, M., & Palumbo, R. (2001). Antioxidant effect of red wine anthocyanins in normal and catalase-inactive human erythrocytes. *Journal of Nutritional Biochemistry, 12*(9), 505-511.

Tedesco, I., Russo, M., Russo, P., Iacomino, G., Russo, G. L., Carraturo, A., Faruolo, C., Moio, L., & Palumbo, R. (2000). Antioxidant effect of red wine polyphenols on red blood cells. *Journal of Nutritional Biochemistry, 11*(2), 114-119.

Teissedre, P. L., & Landrault, T. (2000). Wine phenolics: contribution to dietary intake and bioavailability. *Food Research International, 33*(6), 461-467.

Tourino, S., Perez-Jimenez, J., Luisa Mateos-Martin, M., Fuguet, E., Pilar Vinardell, M., Cascante, M., & Lluis Torres, J. (2011). Metabolites in contact with the rat digestive tract after ingestion of a phenolic-rich dietary fiber matrix. *Journal of Agricultural and Food Chemistry, 59*(11), 5955-5963.

Tsai, P. J., & Huang, H. P. (2004). Effect of polymerization on the antioxidant capacity of anthocyanins in Roselle. *Food Research International, 37*(4), 313-318.

Tsai, P. J., Huang, H. P., & Huang, T. C. (2004). Relationship between anthocyanin patterns and antioxidant capacity in mulberry wine during storage. *Journal of Food Quality, 27*(6), 497-505.

Tsang, C., Auger, C., Mullen, W., Bornet, A., Rouanet, J. M., Crozier, A., & Teissedre, P. L. (2005). The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *British Journal of Nutrition, 94*(2), 170-181.

Tseng, K.-C., Chang, H.-M., & Wu, J. S.-B. (2006). Degradation kinetics of anthocyanin in ethanolic solutions. *Journal of Food Processing and Preservation, 30*(5), 503-514.

Tseng, Y.-H., Yang, J.-H., & Mau, J.-L. (2008). Antioxidant properties of polysaccharides from Ganoderma tsugae. *Food Chemistry, 107*(2), 732-738.

Tsuchiya, H., Nagayama, M., Tanaka, T., Furusawa, M., Kashimata, M., & Takeuchi, H. (2002). Membrane-rigidifying effects of anti-cancer dietary factors. *Biofactors, 16*(3-4), 45-56.

Valls, J., Millan, S., Pilar Marti, M., Borras, E., & Arola, L. (2009). Advanced separation methods of food anthocyanins, isoflavones and flavanols. *Journal of Chromatography A, 1216*(43), 7143-7172.

Van Campen, D. R., & Glahn, R. P. (1999). Micronutrient bioavailability techniques: Accuracy, problems and limitations. *Field Crops Research, 60*(1-2), 93-113.

Vandenbussche, S., Diaz, D., Carmen Fernandez-Alonso, M., Pan, W., Vincent, S. P., Cuevas, G., Javier Canada, F., Jimenez-Barbero, J., & Bartik, K. (2008). Aromatic-carbohydrate interactions: An NMR and computational study of model systems. *Chemistry-a European Journal, 14*(25), 7570-7578.

Vasserot, Y., Caillet, S., & Maujean, A. (1997). Study of anthocyanin adsorption by yeast lees. Effect of some physicochemical parameters. *American Journal of Enology and Viticulture, 48*(4), 433-437.

Vernhet, A., Pellerin, P., Belleville, M. P., Planque, J., & Moutounet, M. (1999). Relative impact of major wine polysaccharides on the performances of an organic microfiltration membrane. *American Journal of Enology and Viticulture, 50*(1), 51-56.

Vernhet, A., Pellerin, P., Prieur, C., Osmianski, J., & Moutounet, M. (1996). Charge properties of some grape and wine polysaccharide and polyphenolic fractions. *American Journal of Enology and Viticulture, 47*(1), 25-30.

Verstraeten, S. V., & Oteiza, P. I. (2000). Effects of Al^{3+} and related metals on membrane phase state and hydration: Correlation with lipid oxidation. *Archives of Biochemistry and Biophysics, 375*(2), 340-346.

Vidal, Doco, T., Williams, P., Pellerin, P., York, W. S., O'Neill, M. A., Glushka, J., Darvill, A. G., & Albersheim, P. (2000). Structural characterization of the pectic polysaccharide rhamnogalacturonan II: evidence for the backbone location of the aceric acid-containing oligoglycosyl side chain. *Carbohydrate Research, 326*(4), 277-294.

Vidal, Francis, L., Williams, P., Kwiatkowski, M., Gawel, R., Cheynier, W., & Waters, E. (2004). The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. *Food Chemistry, 85*(4), 519-525.

Vidal, Williams, P., Doco, T., Moutounet, M., & Pellerin, P. (2003). The polysaccharides of red wine: total fractionation and characterization. *Carbohydrate Polymers, 54*(4), 439-447.

Vidal, Williams, P., O'Neill, M. A., & Pellerin, P. (2001). Polysaccharides from grape berry cell walls. Part I: tissue distribution and structural characterization of the pectic polysaccharides. *Carbohydrate Polymers, 45*(4), 315-323.

Vidal, S., Williams, P., Doco, T., Moutounet, M., & Pellerin, P. (2003). The polysaccharides of red wine: total fractionation and characterization. *Carbohydrate Polymers, 54*(4), 439-447.

Villano, D., Fernandez-Pachon, M. S., Troncoso, A. M., & Garcia-Parrilla, M. C. (2006). Influence of enological practices on the antioxidant activity of wines. *Food Chemistry, 95*(3), 394-404.

Vinas, P., Lopez-Erroz, C., Marin-Hernandez, J. J., & Hernandez-Cordoba, M. (2000). Determination of phenols in wines by liquid chromatography with photodiode array and fluorescence detection. *Journal of Chromatography A, 871*(1-2), 85-93.

Vincken, J. P., Schols, H. A., Oomen, R., McCann, M. C., Ulvskov, P., Voragen, A. G. J., & Visser, R. G. F. (2003). If homogalacturonan were a side chain of

rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology, 132*(4), 1781-1789.

Vitaglione, P., Napolitano, A., & Fogliano, V. (2008). Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. *Trends in Food Science & Technology, 19*(9), 451-463.

Wang, Cao, G. H., & Prior, R. L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry, 45*(2), 304-309.

Wang, C. C., Chu, C. Y., Chu, K. O., Choy, K. W., Khaw, K. S., Rogers, M. S., & Pang, C. P. (2004). Trolox-equivalent antioxidant capacity assay versus oxygen radical absorbance capacity assay in plasma. *Clinical Chemistry, 50*(5), 952-954.

Wang, J., & Mazza, G. (2002). Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor alpha in LPS/IFN-gamma-activated RAW 264.7 macrophages. *Journal of Agricultural and Food Chemistry, 50*(15), 4183-4189.

Waterhouse, A. L., & Laurie, V. F. (2006). Oxidation of wine phenolics: A critical evaluation and hypotheses. *American Journal of Enology and Viticulture, 57*(3), 306-313.

Waters, E. J., Pellerin, P., & Brillouet, J. M. (1994). A saccharomyces mannoprotein that protects wine from protein haze. *Carbohydrate Polymers, 23*(3), 185-191.

Wirth, J., Morel-Salmi, C., Souquet, J. M., Dieval, J. B., Aagaard, O., Vidal, S., Fulcrand, H., & Cheynier, V. (2010). The impact of oxygen exposure before and after bottling on the polyphenolic composition of red wines. *Food Chemistry, 123*(1), 107-116.

Xanthopoulou, M. N., Fragopoulou, E., Kalathara, K., Nomikos, T., Karantonis, H. C., & Antonopoulou, S. (2010). Antioxidant and anti-inflammatory activity of red and white wine extracts. *Food Chemistry, 120*(3), 665-672.

Yoshida, K., Kondo, T., & Goto, T. (1991). Unusually stable monoacylated anthocyanin from purple yam dioscorea-alata. *Tetrahedron Letters, 32*(40), 5579-5580.

Yun, J. W., Lee, W. S., Kim, M. J., Lu, J. N., Kang, M. H., Kim, H. G., Kim, D. C., Choi, E. J., Choi, J. Y., Kim, H. G., Lee, Y.-K., Ryu, C. H., Kim, G., Choi, Y. H., Park, O. J., & Shin, S. C. (2010). Characterization of a profile of the anthocyanins isolated from Vitis coignetiae Pulliat and their anti-invasive activity on HT-29 human colon cancer cells. *Food and Chemical Toxicology, 48*(3), 903-909.

Zafrilla, P., Morillas, J., Mulero, J., Cayuela, J. M., Martinez-Cacha, A., Pardo, F., & Nicolas, J. M. L. (2003). Changes during storage in conventional and ecological wine: Phenolic content and antioxidant activity. *Journal of Agricultural and Food Chemistry, 51*(16), 4694-4700.

Zandleven, J., Beldman, G., Bosveld, M., Schols, H. A., & Voragen, A. G. J. (2006). Enzymatic degradation studies of xylogalacturonans from apple and potato, using xylogalacturonan hydrolase. *Carbohydrate Polymers, 65*(4), 495-503.

Zumwalt, R. W., Desgres, J., Kuo, K. C., Pautz, J. E., & Gehrke, C. W. (1987). Amino-acid-analysis by capillary gas-chromatography. *Journal of the Association of Official Analytical Chemists, 70*(2), 253-262.