Universidade de Aveiro Departamento de Química
2015

MICKAEL DA COSTA SANTOS

ESTUDO DA INFLUÊNCIA DE ALTA PRESSÃO HIDROSTÁTICA NAS CARACTERÍSTICAS QUÍMICAS E SENSORIAIS DE VINHO

STUDY OF THE INFLUENCE OF HIGH HYDROSTATIC PRESSURE ON WINE CHEMICAL AND SENSORIAL CHARACTERISTICS

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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, do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Cláudia Sofia Cordeiro Nunes, Pós-doutoranda do Departamento de Química da Universidade de Aveiro

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Esta dissertação é dedicada aos meus pais, ao meu irmão e à Sónia

"Tira a mão do queixo não penses mais nisso
O que lá vai já deu o que tinha a dar
Quem ganhou ganhou e usou-se disso
Quem perdeu há-de ter mais cartas pra dar
E enquanto alguns fazem figura
Outros sucumbem à batota
Chega a onde tu quiseres
Mas goza bem a tua rota

Enquanto houver estrada pra andar
A gente vai continuar
Enquanto houver estrada pra andar
Enquanto houver ventos e mar
A gente não vai parar
Enquanto houver ventos e mar

Todos nós pagamos por tudo o que usamos
O sistema é antigo e não poupa ninguém
Somos todos escravos do que precisamos
Reduz as necessidades se queres passar bem
Que a dependência é uma besta
Que dá cabo do desejo
A liberdade é uma maluca
Que sabe quanto vale um beijo

Enquanto houver estrada pra andar
A gente vai continuar
Enquanto houver estrada pra andar
Enquanto houver ventos e mar
A gente não vai parar
Enquanto houver ventos e mar"

"A gente vai continuar" by Jorge Palma

o júri

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Professor associado com agregação da Universidade de Aveiro (orientador)

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palavras-chave

Alta pressão hidrostática, vinhos tintos e brancos, propriedades físico-químicas e sensoriais, conservação, soluções modelo de vinho, compostos fenólicos, compostos voláteis, reação de Maillard, envelhecimento do vinho.

resumo

Durante os últimos anos, o uso de alta pressão hidrostática (APH) como tecnologia não-térmica para a preservação ou envelhecimento de vinho tem aumentado substancialmente na comunidade académica. No entanto, os vinhos tratados por APH têm sido analisados após o tratamento de pressão, não havendo referências sobre as suas propriedades durante o armazenamento. Os resultados apresentados nesta tese mostram que a aplicação de tratamentos de APH altera as propriedades químicas e sensoriais de vinhos ao longo do armazenamento.

Os tratamentos de alta pressão hidrostática foram aplicados na vinificação para a preservação de vinho, como alternativa ao dióxido de enxofre, sendo o seu efeito avaliado nas propriedades físico-químicas e sensoriais de vinhos tintos e brancos durante o armazenamento em garrafa. Os tratamentos de alta pressão com 5 min de processamento e pressões de 425 e 500 MPa mostraram influenciar as características físico-químicas e sensoriais de vinhos tintos e brancos. No entanto, o efeito foi apenas percetível após pelo menos 6 meses de armazenamento. As alterações que ocorreram nas características do vinho tinto pressurizado, tais como a cor mais laranja-vermelho, menor atividade antioxidante (menos 15 a 27%), menor conteúdo de compostos fenólicos totais (menos 9%) e menor teor de antocianinas (menos 45-61%), foram devidas a um aumento das reacções de condensação de compostos fenólicos. O aumento destas reações de condensação levou à formação de compostos com maior grau de polimerização que se tornaram insolúveis no vinho ao longo do armazenamento, aumentando consequentemente a quantidade de depósito nos vinhos pressurizados. Em relação ao vinho branco, os vinhos pressurizados mostraram, depois de um ano de armazenamento, uma cor mais acastanhada, menor atividade antioxidante (menos 15%) e menor teor de compostos fenólicos totais (menos 10%) comparando com os vinhos não pressurizados. Estes resultados, juntamente com o baixo teor de aminoácidos livres (menos 15 a 20%) e um maior teor de furanos (até 70% mais) para os vinhos pressurizados após nove meses de armazenamento, levam a propor que os tratamentos de APH aceleraram as reações de Maillard que ocorrem durante o período de armazenamento do vinho.

No entanto, ao contrário dos vinhos tintos pressurizados, os vinho brancos pressurizados não foram considerados adequados para comercialização como vinhos de mesa, visto que apresentavam uma cor acastanhada e um elevado aroma a fruta cozida, características estas de vinhos envelhecidos ou tratados termicamente.

Adicionalmente, foi avaliado o impacto dos tratamentos de APH sobre a composição volátil dos vinhos tintos e brancos sem dióxido de enxofre durante o armazenamento em garrafas. Mais de 160 compostos voláteis, distribuídos por 12 grupos químicos, foram identificados em ambos os vinhos. No final do armazenamento, os vinhos pressurizados apresentaram um teor mais elevado de furanos, aldeídos, cetonas e acetais quando comparados com os vinhos não pressurizados. Estes resultados indicam que os tratamentos de APH influenciam a composição volátil de vinhos brancos e tintos, , sendo mais evidente em longos períodos de armazenamento. As mudanças na composição volátil dos vinhos indicaram que os tratamentos de APH aceleraram as reações de Maillard e também a oxidação de álcoois e ácidos gordos, originando vinhos com uma composição volátil próxima de vinhos com envelhecimento acelerado ou tratados termicamente.

A aceleração das reações de Maillard e de polimerização dos compostos fénolicos causada pelos tratamentos de APH foi também estudada em soluções modelo de vinho (solução hidroalcoólica com pH ácido). Os resultados mostraram que o tratamento de APH acelera a reação de Maillard , sendo este efeito quantificado, apenas, após 6 meses de armazenamento. As soluções modelo de vinho pressurizadas apresentaram concentrações mais elevadas de 2-furfuraldeído, fenilacetaldeído e benzaldeído, em comparação com os controlos. Em termos de polimerização dos compostos fénolicos, as soluções modelo pressurizadas não apresentaram diferenças relevantes, em comparação com os controlos. Por conseguinte, os tratamentos de APH aparentem ter mais impacto em termos de modificações nas cineticas de reação do que na formação de novos compostos.

Por último, a aplicação de tratamentos de APH foi estudada para melhorar as propriedades de vinhos jovens. Para este propósito, o efeito de tratamentos de APH na composição fenólica de um vinho tinto foi estudado e comparado com o efeito de diferentes práticas enológicas. Vinhos pressurizados a 500 MPa durante 5 min e a 600 MPa durante 20 min, a 20 ºC, mostraram depois de 5 meses de armazenamento um menor teor de antocianinas monoméricas (8-14%), ácidos fenólicos (8-11%) e flavonóis (14 -22%), quando comparados com os vinhos não-pressurizados. O vinho pressurizado a 500 MPa apresentou um teor de flavonóis e um grau de polimerização de taninos muito semelhante aos vinhos tratados por processos de envelhecimento tradicionais. Em termos de propriedades sensoriais, os tratamentos de pressão aumentaram o aroma de fruta cozida e diminuiram os aromas florais e frutados, tendo no caso do tratamento de 600 MPa sido verificado também um aumento da amargura. Assim sendo, os tratamentos de APH parecem promover reações que são semelhantes às observadas em vinhos tratados com processos de envelhecimento em madeira.

Em conclusão, os resultados apresentados nesta tese mostram que a aplicação de tratamentos de APH acelera as reações de Maillard e a polimerização dos compostos fenólicos presentes no vinho, ao longo do armazenamento, alterando assim as propriedades químicas e sensoriais dos vinhos. A APH pode ser potencialmente utilizada para preservar ou acelerar o processo de envelhecimento de vinho tinto produzindo vinhos com características agradáveis e distintas.

keywords

High hydrostatic pressure, red and white wines, physicochemical and sensorial properties, preservation, model wine solutions, phenolic compounds, volatile compounds, Maillard reaction, wine aging

abstract

During the last years, the use of high hydrostatic pressure (HHP) as a non-thermal technology for preservation or aging of wine has increased substantially in the academic community. However, HHP treated wine has been only analysed after the pressure treatment, with no knowledge available on the effects of HHP during subsequent storage. The results presented in this thesis showed that HHP treatments influence the chemical and sensorial properties of wine during storage.

The application of high hydrostatic pressure treatments in winemaking for wine preservation, as an alternative to sulphur dioxide, was evaluated studying the effect of HHP in the physicochemical and sensorial properties of red and white wines during bottle storage. High pressure treatments with 5 min of processing time and pressures of 425 and 500 MPa were shown to influence on both red and white wine physicochemical and sensorial characteristics. However, the effects were only perceptible after, at least, 6 months of storage. The alterations that occurred on the pressurized red wine characteristics, such as the more orange-red colour and the lower antioxidant activity (15-27% less), total phenolic content (9% less), and anthocyanins content (45–61% less), were due to an increase of condensation reactions of phenolic compounds. The increase of these condensation reactions lead to the formation of compounds with higher degree of polymerisation that became insoluble along storage, increasing consequently the amount of wine deposits in the pressurized wines. In terms of white wines, pressurized wines showed, after one year of storage, a more brownish colour and a lower antioxidant activity (15% less) and total content of phenolic compounds (10% less) when compared to the unpressurized wines. These results, together with the lower content of free amino acids (15-20% less) and higher content of furans (up to 70% more), present in the pressurized wines after nine months of storage, led to propose an effect of HHP treatments in the acceleration of Maillard reactions that occur during the wine storage period. Therefore, contrary to the pressurized sulphur dioxide-free red wine, the pressurized white wines were not considered suitable for commercialization as table wines due to the higher brownish colour and cooked fruit aroma, characteristics of an aged or thermally treated wine.

Additionally, the impact of the pressure treatments on the volatile composition of sulphur dioxide-free red and white wines, during bottle storage, was evaluated. More than 160 volatile compounds, distributed by 12 chemical groups, were identified in both wines. At the end of storage, the pressurized wines presented a higher content of furans, aldehydes, ketones, and acetals when compared to the unpressurized wines. These results indicate that pressure influences the white and red wine long term volatile composition, being this particularly evident for longer storage periods. The changes on the volatile composition of the pressurized wines, indicated that the HHP treatments accelerate the Maillard reactions, and the oxidation of alcohols and fatty acids, leading to wines with a volatile composition network approaching the characteristic of faster aged and/or thermally treated wines.

The acceleration of Maillard reactions and phenolic compounds condensation by HHP treatments was also studied in model wine solutions (hydro alcoholic solution at acidic pH). The results showed that the high pressure treatment accelerated the Maillard reaction and this effect was quantifiable, mainly, after 6 months of storage. Pressurized model solutions presented higher concentration of 2-furfural, phenylacetaldehyde and benzaldehyde, when compared to the controls. In terms of phenolic compounds condensation reactions, the pressurized model wine solutions showed no relevant differences, when compared to controls. Therefore, it seems that the pressure treatment had a higher impact in terms of kineticks of reactions and in less extent in terms of different compounds formed.

Lastly, the application of HHP treatments in winemaking to improve the properties of young wines was evaluated. For this propose, the effect of HHP treatments in the phenolic composition of a red wine was studied and compared with the effect of different oenological practices. Wines pressurized at 500 MPa for 5 min, and 600 MPa for 20 min, at 20 ºC, showed, after 5 months of storage, a lower monomeric anthocyanins (8-14%), phenolic acids (8-11%) and flavonols (14-22%) content, when compared to the unpressurized ones. The wine pressurized at 500 MPa presented a flavanols content and a degree of polymerization very similar to the wines treated by traditional aging processes. In terms of sensorial properties, the pressure treatments increased the cooked fruit aroma and decreased the floral and fruit odours and, in the case of the 600 MPa treatment, increased the bitterness. Therefore, the HHP treatments seem to promote reactions that are similar to those observed in wines treated with wood aging processes.

In conclusion, the results presented in this thesis showed that HHP treatments accelerated the Maillard reaction and the polymerization reactions between phenolic compounds present in the wine, influencing the chemical and sensorial properties of wine. HHP can be potentially used to preserve or accelerate the wine aging process, producing wines with pleasant and distinct characteristics.

Publications related to this thesis

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Mickael C. Santos, Cláudia Nunes, Jorge A. Saraiva and Manuel A. Coimbra. Chemical and physical methodologies for the replacement/reduction of sulfur dioxide use during winemaking: review of their potentialities and limitations. *European Food Research and Technology*, 2012, 234 (1), 1-12.

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Mickael C. Santos, Cláudia Nunes, Michael Jourdes, Pierre-Louis Teissedre, Osvaldo Amado, Ana Rodrigues, Jorge A. Saraiva, and Manuel A. Coimbra, Impact of high pressure treatment on the phenolic composition of a red wine: comparison with different aging processes, (submitted).

Mickael C. Santos, Cláudia Nunes, Jorge A. Saraiva, and Manuel A. Coimbra, Formation of Maillard volatile compounds in model wine solutions promoted by high pressure treatments, (under preparation).

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Articles in proceedings of national scientific meetings

Mickael C. Santos, M. Angelica M. Rocha, Cláudia Nunes, Jorge A. Saraiva, Sílvia M. Rocha, Manuel A. Coimbra. Impact of high hydrostatic pressure treatments in volatile composition of white wine. 11th Meeting of Food Chemistry, 16-19 September 2012; Bragança, Portugal; P171 CD-ROM.

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GENERAL INDEX

ABREVIATIONS AND SYMBOLS

Wine samples and model wine solution used	
425 MPa	Sample pressurized at 425 MPa during 5 minutes
500 MPa	Sample pressurized at 425 MPa during 5 minutes
600 MPa	Sample pressurized at 600 MPa during 20 minutes
Control	Sample without any aging treatment
Heated	Sample heated at 60 ºC during 5 minutes
Oak barrels	Sample stored in oak barrels
Oak chips	Sample with addition of oak chips
Oak chips + mO_2	Sample with addition of oak chips and micro-oxygenation process
SO_2	Sample with the addition of 40 ppm of sulphur dioxide
Unpressurized	Sample without pressure treatments
Untreated	Sample without any treatment
Amino acids	
Ala	Alanine
Arg	Arginine
Asx	Asparagine + aspartic acid
Gly	Glycine
Glx	Glutamic acid + glutamine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Val	Valine

Anthocyanins

Cy3AcGlc	Cyanidin-3-*O*-(6-*O*-acetyl)-glucoside
Cy3Glc	Cyanidin-3-*O*-glucoside
Dp3AcGlc	Delphinidin-3-*O*-(6-*O*-acetyl)-glucoside,
Dp3Glc	Delphinidin-3-*O*-glucoside
Mv3AcGlc	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside
Mv3CmGlc	Malvidin-3-*O*-(6-*O*-*p*-coumaryl)-glucoside
Mv3Glc	Malvidin-3-*O*-glucoside
Pn3AcGlc	Peonidin -3-*O*-(6-*O*-acetyl)-glucoside
Pn3CmGlc	Peonidin-3-*O*-(6-*O*-*p*-coumaryl)-glucoside
Pn3Glc	Peonidin-3-*O*-glucoside
Pt3AcGlc-	Petunidin-3-*O*-(6-*O*-acetyl)-glucoside
Pt3Glc	Petunidin-3-*O*-glucoside

Other abbreviations used

ΔE*	Colour differences between two samples
ΔV	Volume change of the process ($cm^3.mol^{-1}$)
%P	Percentage of prodelphinidins
%PP	Percentage of polymerized pigments
^1t_R	Retention time for first dimension
^2t_R	Retention time for second dimension
*a**	Red-green value
Anth	Anthocyanins
AOA	Antioxidant activity
a. u.	Arbitrary units
*b**	Yellow-blue value
Bl%	Contribution of blue coloration to the overall colour of wine
BHT	3,5-di-tert-butyl-4-hydroxytoluene

C^*_{ab}	Chroma
CI	Colour intensity
dA%	Coloration produced by free and bound anthocyanins under their flavylium cations form
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
e.g	*exempli gratia*/for example
et al	*et alii*/and others
EGC	(-)-Epigallocatechin
ESI-MS	Electrospray ionization mass spectrometry
ESI-MS^n	Electrospray ionization tandem-mass spectrometry
FID	Flame ionisation detector
GC	Gas chromatography
GC x GC-ToFMS	Comprehensive two dimensional gas chromatography–time-of-flight mass spectrometry
h_{ab}	Hue
HHP	High hydrostatic pressure
HPLC	High Performance Liquid Chromatography
HS-SPME	Headspace solid phase microextraction
IEC	Ion extraction chromatography
L^*	Lightness
LAB	Lactic acid bactéria
MA	Monomeric anthocyanins
mDP	Mean degree of polymerization
min	Minute/ minutes
MVC	Maillard derived volatile compounds
MS	Mass spectrometry
m/z	Mass-to-charge ratio
PCA	Principal component analysis

PPO	Polyphenoloxidase
Rd%	Contribution of red coloration to the overall colour of wine
RI	Retention index
RSD	Relative standard deviation
SIM	Selected ion monitoring
SPME	Solid-phase microextraction
TDN	1,1,6-trimethyl-1,2-dihydronaphthalene
TEAC	Trolox equivalent antioxidant capacity
TIC	Total ion chromatograms
TP	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Ye%	Contribution of yellow coloration to the overall colour of wine

CHAPTER I.

General introduction and Objectives

Index

I.1 High pressure as a food processing technology

Consumers demand high quality and convenient products with the natural flavour, taste and appearance of minimally processed food. Besides, they look for safe and natural products without additives, such as preservatives and water activity depressors. In order to harmonize or blend all these demands without compromising the safety of the products, it is necessary to implement novel preservation technologies in the food industry (Rastogi *et al.*, 2007).

Therefore, several alternatives or novel food processing technologies, without the use of heat, are being explored in order to provide safe, fresh-tasting, and nutritive foods. The most used non-thermal processing technologies are presented in Table I.1.

Table I.1. Non-thermal food processing methods, adapted from Welti-Chanes *et al.,* (2002).

Process	Description	Mechanism of inactivation
Ultrasound	Energy generated by sound waves of 20,000 Hz or more.	Intracellular cavitation (disruption of cellular structure and functional components up cell lysis).
UV-light/pulsed light	Intense and short-duration pulses of broad spectrum (ultraviolet to the near infrared region); UV radiant exposure, at least 400 J/m^2.	DNA mutations.
Pulsed electric field (PEF)	High voltage pulses between two electrodes (<1 s; 20–80 kV/cm; exponentially decaying, square wave, bipolar, or oscillatory pulses at ambient, sub-ambient, or above ambient temperature).	Electroporation of cell membranes and increase in permeability.
High pressure processing	Pressurization at 100–1000 MPa, below 0 ºC to >100 ºC, from a few seconds to over 20 min.	Breakdown of biological membranes; denaturation of enzymes and proteins; cellular mass transfer affected.

One emerging technology receiving a great deal of attention is high hydrostatic pressure (HHP), not only because of its food preservation capability, but also because of its potential to achieve interesting functional effects. Particularly for certain products, the application of HHP processing has shown considerable potential as an alternative

technology to heat treatments, in terms of assuring quality attributes in minimally processed food products (Welti-Chanes *et al.,* 2002).

Studies examining the effects of high pressure on foods date back to beginning of the twenty century. Hite *et al.,* (1914) were the first to report the effects of HHP on food microorganisms by subjecting milk to pressures of 650 MPa, obtaining a decrease in the viable number of microorganisms. However, the use of high pressure in food processing is an extension of a technology that is commonly employed in many other industrial processes, notably in the manufacturing of ceramics, diamonds, super-alloys, simulators, and sheet metal forming (Yaldagard *et al.,* 2008). The advances achieved in ceramics and metallurgical industries in the use of HHP techniques during the 1970s and 1980s has led to the possibility of treating food by this method at industrial level (Yaldagard *et al.*, 2008).

The preservation of food by high pressure only became a commercial reality in the last 20 years. The commercial interest of the food industry has occurred first in Japan, in April 1990, with the introduction on the Japanese retail market of a high-acid jam. In 1991, yogurts, fruit jellies, salad dressings, and fruit sauces were also introduced. Also, two Japanese fruit juice industries installed a semi-continuous high pressure equipment for citrus juice bulk processing. Only 5 years later, started appearing pressurized food products in the Europe and United States market (Enrique et al. 2007; Tewari 2003).

From its early beginnings, revenues from high pressure processed foods are evaluated as more than $2 billion annually. Today, the range of commercially available foods includes various fruit juices and meats in Europe, guacamole and oysters in the USA, and an extensive range of products in Japan, including rice cakes and fruit juices amongst a growing number of other products (Schaschke 2011).

Therefore, recent equipment advances, successful commercialization of high pressure products, and a consumer demand for minimally processed, high quality, and safe foods, lead to the research interest in high pressure technology has increased in the last years. This interest allowed a more detailed knowledge of the effect of high pressure on the food constituents and its influence on food quality parameters.This knowledge lead to the optimization of process parameters and, at the same time, the production of food products with better quality (Castro 2007).

I.1.1 High pressure principles and equipment

There are two principles that describe the effect of high pressure: one is based on the Le Chatelier's principle and the other on the isostatic principle.

The one based on the Le Chatelier's principle states that any phenomenon in equilibrium (chemical reaction, phase transition, and change in molecular configuration), accompanied by a decrease in volume, can be enhanced by pressure (Ramirez et al. 2009). The effect of pressure on a physicochemical process at equilibrium is governed by the volume change of the process (ΔV, $cm^3.mol^{-1}$). The volume change (ΔV) can be related to the reaction equilibrium constant K as follows:

$$\Delta V = -\mathrm{RT}\left(\frac{\partial \ln \mathrm{K}}{\partial \mathrm{P}}\right)_{\mathrm{T}} \qquad (1)$$

where P the pressure applied (MPa); T the absolute temperature (K); R the universal gas constant (8.314 $cm^3.MPa.mol^{-1}.K^{-1}$) and K, the equilibrium constant. The subscript T means that the process occurs at constant temperature. So, the application of high pressure favors reactions that generate a decrease in volume and generally retard reactions that involve volume increases. Most of the biochemical reactions result in changes in volume; consequently, biological processes are influenced by the application of pressure. The reactions strongly affected by pressure generally include reactants and products that differ in the number of groups that can be ionized. When the charge number does not change, the reaction is basically independent of pressure. In aqueous systems, a decrease in volume is generally a factor when the dissociation reactions yield an increase in the number of groups that can be ionized. This is generally caused by the electrostriction of water near the ions. Pressure tends to dissociate electrostatic interactions in such a way that more ions are exposed to water (Welti-Chanes *et al.*, 2005).

The HHP affects substantially non-covalent bonds (hydrogen, ionic, and hydrophobic bonds), which means that low molecular weight food components (responsible for nutritional and sensory characteristics) are normally less affected, whereas high molecular weight components (since tertiary structure is important for functionality) are sensitive to HHP (Tewari 2003).

The isostatic principle describes that the transmittance of pressure through the food is uniform and instantaneous, independently of the size and geometry of food (Tewari 2003). This is the major advantage of processing by HHP in relation to heat

treatment, in which the distribution of temperature is not uniform and it is necessary to increase the temperature treatment to achieve the desirable temperature inside the food, causing quality loss on the final product. The increase of pressure during processing leads to a uniform increase of food temperature due to adiabatic heating. The magnitude of this temperature increase depends on the initial temperature of the product and its composition. Water increases about 3 ºC for an increase of 100 MPa, whereas for greasy food the temperature can increase 6 ºC per 100 MPa (Balasubramanian and Balasubramaniam, 2003).

High pressure can be generated by direct or indirect compression. Direct compression is generated by pressurizing a medium with the small diameter end of a piston. The large diameter end of the piston is driven by a low-pressure pump. This method allows very fast compression (Enrique *et al.*, 2007).

Indirect compression uses a high-pressure intensifier to pump a pressure medium from a reservoir into a closed high-pressure vessel until the desired pressure is reached (Figure I.1) (Singh 2001). Generally, the level of pressure ranges from 100 to 1000 MPa and the pressure-transmitting medium is water, usually combined with mineral or vegetable oil for lubrication, with anticorrosive aims (Welti-Chanes *et al.*, 2005). Once the desired pressure is reached, it is maintained at that level and does not require more energy. After the required time has elapsed, the system is depressurized, the vessel opened and the product unloaded. The temperature inside the chamber can be controlled using cooling jackets or an internal heat exchanger in the pressure vessel or by recirculation of the cooling/heating medium (Welti-Chanes *et al.*, 2005).

As far as it concerns the industrial high pressure treatment, it can be applied as batch or as semi-continuous process. The selection of equipment depends on the kind of food product to be processed. Solid food products or food with large solid particles can only be treated in a batch mode. Liquids, slurries or other pumpable products have the additional option of semi-continuous production (Welti-Chanes *et al.*, 2005). In batch process, the risk of food contamination with the equipment is eliminated because the product is placed inside plastic containers, and once the treatment is finished, the containers are removed and the equipment does not require cleaning (Zimmerman and Bergman, 1993).

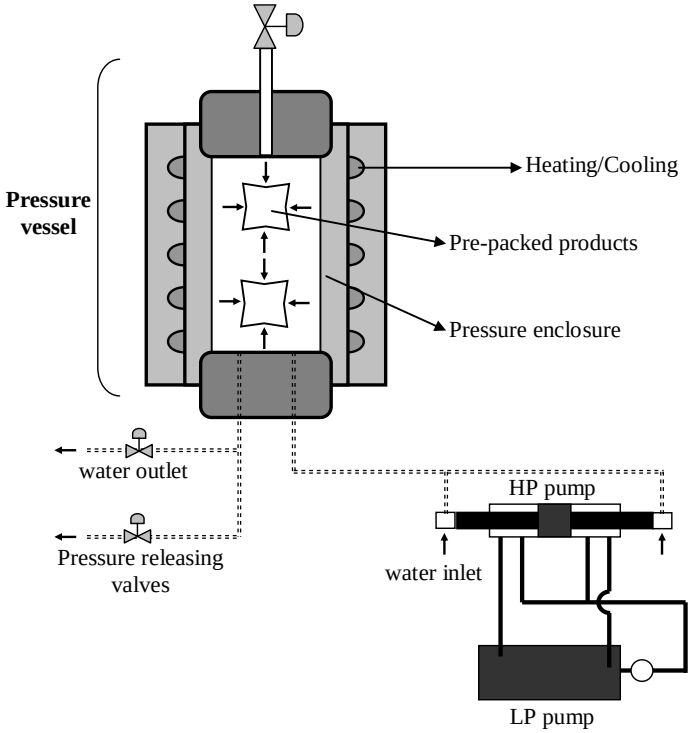


Figure I.1- Typical high-pressure processing system, with indirect compression for treating pre-packaged foods, adapted from Fernández de Simón *et al.*, (2014); Sun *et al.*, (2013).

HHP processing requires airtight packages that can withstand a change in volume corresponding to the compressibility of the product, as foods decrease in volume as a function of the pressure applied, while an equal expansion occurs on decompression (Hugas *et al.*, 2002). For this reason, the packaging used for HHP treated foods must be able to accommodate up to a 15 % reduction in volume and return to its original volume without loss of seal integrity or barrier properties (Norton and Sun, 2007).

I.1.2 Effect of high pressure on microorganisms

The effectiveness of any food preservation technique is primarily evaluated on the basis of its ability to eradicate pathogenic microorganisms present, enhancing the product safety. The secondary objective is inactivation of spoilage microorganisms to improve the shelf-life of the food (McClements *et al.*, 2001). The growth of microorganisms in foods can cause spoilage by producing unacceptable changes in taste, flavour, appearance, and texture. As a food preservation method, the effectiveness of HHP in destroying foodborne microorganisms depends on a number of intrinsic and extrinsic factors that must be taken into account when optimizing pressure treatments for particular foods (Patterson *et al.*, 2007).

The patterns of HHP inactivation kinetics observed for different microorganisms are quite variable. Some authors observed a change in the slope and a two phase inactivation phenomenon, the first fraction of the population being quickly inactivated, whereas the second fraction appears to be much more resistant (Enrique *et al.*, 2007; Erkmen 2009). In general, the pressure resistance increases from bacterial vegetative cells < yeasts < ascospores < bacterial spores (Patterson *et al.*, 2007). The nature of food is also important, as it may contain substances which might protect the microorganism from pressure damage. Microbial inactivation by high pressure has been extensively studied and has been concluded to be the result of a combination of factors.

In the inactivation of microorganisms by high pressure, the membrane is the most probable site of disruption. It was suggested that the structural impact of the HHP on yeast cells occurred directly in the membrane system, particularly in the nuclear membrane (Shimada *et al.*, 1993). High pressure seems to damage the molecular organization of the lipid-peptide complex by disrupting the phospholipidic acid bilayer membrane structure. Intracellular fluid compounds have been found in the cell suspending fluid after high pressure treatment demonstrating that leaks occur when cells are held under pressure (Perrier-Cornet *et al.*, 1999; Shimada *et al.*, 1993). Some functionalities of the membrane such as active transport or passive permeability can suffer alteration with the membrane damage and, therefore, perturb the physicochemical balance of the cell (Perrier-Cornet *et al.*, 1995). Besides membrane damage, a decrease in pH due to the enhancement of the ionic dissociation occur due to electrostriction during high pressure treatments (Yuste *et al.*, 2001). Other structures inside the cell have also been proposed as potential key targets for inactivation by high pressure, since many organelles such as the nucleous, mitochondria, endoplasmatic reticulum, Golgi apparatus, and lysosomes or vacuoles are all enveloped by membranes (Rendueles *et al.*, 2011). However, inactivation of key enzymes, including those involved in DNA replication and transcription, is also mentioned as a possible inactivation mechanism (Patterson 2005).

Treating food samples using HHP can destroy both pathogenic and spoilage microorganisms. However, there is a large variation in the pressure resistance of different bacterial strains and the nature of the medium can also affect the response of microorganisms to pressure (Rendueles *et al.*, 2011). The stage of growth of the bacteria is also important in determining pressure resistance, with cells in the stationary phase being more resistant than those in the exponential phase (McClements *et al.*, 2001). Also,

Gram-positive and Gram-negative bacteria differ significantly in terms of the chemical structure of their cell walls. The cell walls of Gram-negative bacteria are significantly weaker and consequently tend to be more pressure-sensitive than Gram-positive bacteria (Rendueles *et al.,* 2011). The elimination of bacterial endospores from food represents, probably, the greatest food processing and food-safety challenge to the industry. It is well established that spores are the most pressure-resistant life forms known, and so, only very high pressures (>800MPa) can eliminate bacterial spores at ambient temperatures (Eamonn *et al.,* 2005).

Yeasts are generally relatively sensitive to pressure, therefore treatment at pressures less than 400 MPa for a few minutes is sufficient to inactivate most yeasts. However, some strains within species have exhibited a lower rate at pressures of 500 MPa (Eamonn *et al.,* 2005; Patterson 2005). Smelt (1998) reported that, at about 100 MPa, the nuclear membrane of yeasts was affected and more than 400–600 MPa further alteration occurred in the mitochondria and the cytoplasm.

I.1.2.1 Factors influencing microbial sensitivity to high pressure

As stated, the pressure resistance of microorganisms varies considerably, depending on factors such as species, strain, stage of growth, and food composition. The factors that can affect the response of microorganisms to pressure must be considered,in order to optimize the treatments to assure microbiological safety (Eamonn *et al.,* 2005). Many food constituents appear to protect microorganisms from the effects of high pressure (Matser *et al.,* 2004; Rendueles *et al.,* 2011; Van Opstal *et al.,* 2004). Therefore, it is important to evaluate HHP treatments for each case.

pH

The pH of the food is one of the main factors affecting the growth and survival of microorganisms, therefore all microorganisms have a pH range in which they can grow and an optimum pH at which they grow quicker (Eamonn *et al.,* 2005). Bacterial spores are generally most resistant to the direct effects of pressure treatment at neutral pH (Smelt 1998). At acidic pH values, for most species, the extent of pressure-induced inactivation will generally be enhanced and the recovery of injured cells inhibited. Compression of foods during HHP treatment may shift the pH of the food as a function of the applied

pressure, and so the direction of pH shift and its magnitude must be taken into account for each food treatment process (Matser *et al.*, 2004; Norton and Sun, 2007).

Water activity (a_w)

Water in the liquid state is essential for the existence of all living organisms. Lowering the water activity (a_w) of food can significantly influence the growth of food spoilage or food-poisoning organisms that may be present in the raw materials or introduced during processing (Eamonn *et al.*, 2005).

The decrease of a_w appears to protect microorganisms against inactivation by HHP. The resistance to inhibition at low a_w values may be attributed to cell shrinkage, which probably causes a thickening in the cell membrane that reduces membrane permeability and fluidity (Linton *et al.*, 2000). The increased baroresistance of microorganisms at low a_w may also be attributed to partial cell dehydration due to the osmotic pressure gradients between the internal and external fluids, which may result in smaller cells and thicker membranes, and an increased pressure resistance (Palou et al. 1997). However, recovery of sub-lethally injured cells can be inhibited by low a_w (Smelt 1998). Consequently, the net effect of water activity on microbial inactivation by HHP treatment may be difficult to predict.

Temperature

Increasing temperature will generally increase the number of microorganisms inactivated. While many HHP processes are performed at ambient temperature, increasing or, to a lesser extent, decreasing temperature has been found to increase the inactivation rate of microorganisms during high pressure treatment (Knorr 1993). Temperatures above 45–50°C increase the rate of HHP inactivation of food pathogens and spoilage microbes (Welti-Chanes *et al.*, 2005). Hashizume *et al.*, (2014) reported that *S. cerevisiae* cells were more effectively inactivated by high-pressure treatments at elevated (40°C) or subzero (0°C and -20°C) temperatures. The decrease in resistance to pressure by vegetative cells at low temperatures (-5°C) may be due to changes in the membrane structure and fluidity, weakening of hydrophobic interactions, and crystallization of phospholipids (Rendueles *et al.*, 2011).

Pressure and holding time

Generally, an increase in pressure increases microbial inactivation. However, increasing the duration of the treatment does not necessarily increase the lethal effect. Above 200–300 MPa, the inactivation ratio of vegetative cells increases with process time (Rendueles *et al.*, 2011).

As mentioned before, the microbial response to high pressure treatments depends on the type of microorganism. Welti-Chanes *et al.*, (2005) reported several examples of microorganisms that demonstrated different resistances to pressure. For example, to achieve four decimal reductions with 10 min of treatment, *S. cerevisiae* and *Aspergillus awamori* required 250 MPa, whereas *Aspergillus niger* required 400 MPa in the same medium (mandarin juice). For each microorganism, there is a pressure-level threshold beyond which no effects are detected by increasing the exposure time.

There is a minimum critical pressure below which microbial inactivation by high pressure will not take place regardless of process time. Important processing parameters to be considered in HHP are the come-up times (period necessary to reach the treatment pressure) and pressure-release times (Eamonn *et al.*, 2005).

I.1.3 Effect of high pressure on proteins and enzymes

Proteins are usually denatured by high pressure. However the protein type, processing conditions and pressures applied are all important considerations. Generally, at low protein concentrations and low pressures (<300 MPa), reversible pressure-induced denaturation occurs, while higher pressures (>300 MPa) induce irreversible and extensive effects on proteins (Guerrero-Beltrán *et al.*, 2005). Denaturation may be due to the destruction of hydrophobic and ion pair bonds, and unfolding of molecules. At higher pressures, oligomeric proteins tend to dissociate into subunits that become vulnerable to proteolysis (Barba *et al.*, 2012). High pressure effects on proteins are related to the rupture of non-covalent interactions within protein molecules, and to the subsequent reformation of intra and inter molecular bonds within or between the molecules. Different types of interactions contribute to the secondary, tertiary, and quaternary structure of proteins, and the quaternary structure is mainly held by hydrophobic interactions that are very sensitive to pressure. However, significant changes in the tertiary structure are also observed beyond 200 MPa (Gamlath and Wakeling, 2011; Rastogi *et al.*, 2007)

Enzymes are a special class of proteins in which biological activity arises from active sites, brought together by the three-dimensional configuration of the molecule (Gamlath and Wakeling, 2011). Explanations for pressure-induced changes in the rate of enzyme-catalyzed reactions can be classified into: (1) direct changes in the structure of an enzyme, (2) changes in the reaction mechanisms (for example, a change in the rate-limiting step), and (3) changes in the substrate or solvent physical properties (*e.g.* pH, density, viscosity, phase) that affect enzyme structure or the rate-limiting step (Rastogi *et al.,* 2007; Welti-Chanes *et al.,* 2005).

HHP is most commonly used to inactivate food deleterious enzymes (such as polyphenoloxidase and lipoxygenase), thereby ensuring the maintenance of the high quality characteristics of the food (Eisenmenger and Reyes-De-Corcuera, 2009). Depending on the high pressure treatment, the same enzyme can be activated or deactivated. Cano *et al.,* (1997) studied the effect of HHP (50–400 MPa) in orange juice and strawberry puree for inactivation of some enzymes. Pectin methylesterase activation was observed in orange juice when treated between 200 and 400 MPa at room temperature. However, combinations of pressures lower than 200 MPa and mild temperatures inactivated the enzyme in juice. In strawberry puree it was observed that the peroxidase activity was reduced as pressure increased up to 300 MPa, but at pressures higher than 300 MPa an activation of peroxidase was observed.

Some enzymes are found to be pressure resistant, since the pressure level required to inactivate these enzymes surpasses the one needed to inactivate microbial vegetative cells. Besides, different sources show different pressure-temperature behaviours. For example, inactivation of polyphenoloxidase from apple, grape, avocado, and pear at room temperature (25 ºC) became noticeable at approximately 600, 700, 800, and 900 MPa, respectively, and followed first-order kinetics (Rastogi *et al.,* 2007).

The changes in enzymes activity tend to have low effect on the nutritional content of the food, but are important with respect to food quality especially in relation to colour (polyphenoloxidase) and texture of food (pectic enzymes), plus some influence in lipid oxidation (lipase and lipoxygenase) (Sulaiman and Silva, 2013; Welti-Chanes *et al*., 2005).

I.1.4 Effect of high pressure in quality related parameters

An important aspect of high pressure processing of foods concerns the physical and chemical nature of food structure and taste. The properties and quality of foods, which affect acceptability by consumers, are referred as organoleptic properties.

In order to select the most suitable processing conditions for a particular food product, sensory characteristics must be taken into account (Polydera *et al.,* 2004). Increasing treatment pressures will generally increase microbial inactivation in shorter times, but higher pressures may also cause detrimental changes in food quality that could affect negatively the appearance and some properties of the food, compared to the unprocessed product (Eamonn *et al.,* 2005).

Colour and flavour are important quality characteristics of liquid food and major factors affecting sensory perception and consumer acceptance of foods. HHP processing could preserve nutritional value and sensory properties of foods due to its limited effect on the covalent bonds of low molecular-mass compounds such the compounds responsible for colour and flavour. However, food is a complex system and the compounds responsible for sensory properties coexist with enzymes, metal ions, etc (Oey *et al.,* 2008a).

During high pressure processing (100-1000 MPa at 20 ºC – 60 ºC) can occur at the same time (i) cell wall and membrane disruption; (ii) enzyme catalyzed conversion processes; (iii) chemical reactions; and (iv) modification of biopolymers including enzyme inactivation, protein denaturation, and gel formation (Eisenmenger and Reyes-De-Corcuera, 2009; Enrique *et al.,* 2007; Oey *et al.,* 2008b). Therefore, different pressure and temperature combinations can be used to achieve desired effects on texture, colour and flavour of foods. The quality of high pressure processed foods can, however, change during storage due to coexisting chemical reactions, such as oxidation, and biochemical reactions when endogenous enzymes or microorganisms are incompletely inactivated (Oey *et al.,* 2008b).

I.1.4.1 Colour

HHP treatment (at low and moderate temperatures) has been reported to have a limited effect on the compounds responsible for the colour of fruits and vegetables (e.g. chlorophyll, carotenoids, anthocyanins, etc.). The colour compounds of HHP processed

fruits and vegetables can, however, change during storage due to incomplete inactivation of enzymes and microorganisms, which can result in undesired chemical reactions (both enzymatic and non-enzymatic) in the food matrix (Oey *et al.*, 2008a).

Chlorophyll is a green compound found in the leaves and green stems of plants. Chlorophylls *a* and *b* have different stabilities towards pressure and temperature. At room temperature, chlorophylls *a* and *b* exhibit extreme pressure stability but at temperatures higher than 50 ºC, high pressure treatment affects their stability (Butz *et al.*, 2002). For example, increasing pressure from 200 to 800 MPa at 70 ºC accelerates the degradation of chlorophyll *a* and *b* of broccoli by 19.4% and 68.4%, respectively (Van Loey *et al.*, 1998).

HHP treatment at ambient and moderate temperatures results in limited colour change of green vegetables. In many cases, the green colour of vegetables becomes even more intense (Matser *et al.*, 2004). This might be caused by cell disruption during HHP treatment resulting in the leakage of chlorophyll into the intercellular space yielding a more intense bright green colour on the vegetable surface (Krebbers *et al.*, 2002). During storage, the green colour of the vegetables HHP treated at room temperature turned into a pale yellow colour probably due to chemical reactions such as oxidation. By comparison, the vegetables pressurized at elevated temperatures, which results in inactivation of some enzymes, showed no further colour change during storage (Oey *et al.*, 2008a).

Carotenoids are important for the orange, yellow and red appearance of fruits and vegetables. HHP treatment has shown remarkable benefits in retaining or increasing the levels of total carotenoids in foods (Barba *et al.*, 2012). A significant increase in all types of carotenoids has been observed in tomato puree and orange juice (Sánchez-Moreno *et al.*, 2005) and melon (Wolbang *et al.*, 2008) around pressures of 400-600 MPa. Lycopene from tomato puree and lutein from broccoli and green beans also showed very high stability (100% retention) after HHP (Gamlath and Wakeling, 2011).

Anthocyanins are water-soluble vacuolar flavonoid pigments responsible for the red to blue colour of fruits and vegetables. Anthocyanins are normally stable during HHP treatment at moderate temperature (Barba *et al.*, 2012). However, anthocyanins in pressure-treated vegetables and fruits were not stable during storage (González-Cebrino *et al.*, 2013). There are various hypotheses on the degradation mechanism of anthocyanins in pressurized fruits during storage. The first hypothesis of anthocyanin degradation is a

reaction caused by incomplete enzyme inactivation. A link between enzyme inactivation (β-glucosidase, peroxidase and polyphenoloxidase) and anthocyanin stability has been found in several fruits (Altuner and Tokuşoğlu, 2013; Ferrari *et al.,* 2011). Suthanthangjai *et al.,* (2005) showed that cyanidin-3-glucoside and cyanidin-3-sophorosides (the major pigments in raspberry) had the highest stability during 9 days of storage at 4 ºC after pressurization at 200 or 800 MPa compared with pressure treatment at 400 or 600 MPa. A high loss of both pigments after HHP treatment at 400 and 600 MPa has been reported to be probably due to a lower degree of inactivation of β-glucosidase, peroxidase and polyphenoloxidase. Enzymatic degradation of anthocyanins by β-glucosidase is mainly due to the loss of glycosidic moiety leading to the formation of anthocyanidin and consequently affecting juice colour (Garcia-Palazon *et al.,* 2004). Specificity of β-glucosidase is another cause for selective degradation of anthocyanins present in fruit. For example a higher reduction in pelargonidin-3-glucoside, compared to pelargonidin-3-rutinoside in HHP processed strawberries during storage under similar processing conditions was reported by Zabetakis *et al.,* (2000), because β-glucosidase has a greater affinity towards glucose compared to rutinose. However, it must be noted that the effect of HHP processing parameters, such as pressure, temperature, and time in addition to physicochemical properties of fruit, such as total soluble solids and pH, have varying effects on the enzymes responsible for anthocyanins stability in HHP processed fruit products (Garcia-Palazon *et al.,* 2004; Suthanthangjai *et al.,* 2005; Zabetakis *et al.,* 2000). Some anthocyanin degradation in HHP processed juice combined with heat could be due to condensation reactions involving the covalent association of anthocyanins with other flavanols or organic acids present in fruit juices (Tiwari *et al.,* 2009). Corrales *et al.,* (2008) reported an insignificant decrease in cyanidin-3-glucoside in a model solution at processing conditions of 600 MPa at 20 ºC during 30 min. They also reported a 25% loss at 600 MPa, 70 ºC for 30 min compared to a 5% loss at 70 ºC for 30 min, indicating that HHP accelerates the decrease of anthocyanin content at elevated temperatures. This is due to condensation reactions involving covalent association of anthocyanins with pyruvic acid present in fruit juices leading to the formation of a new pyran ring by cycloaddition. Chemical compounds derived from these condensation reactions are responsible for changes in the colour of red wine towards brown or orange (Corrales *et al.,* 2008).

I.1.4.2 Flavour

Flavour is the sensory impression of a food that is determined mainly by the chemical senses of taste and smell. The human tongue can distinguish only among five distinct qualities of taste, of which sourness, sweetness and bitterness are the most important ones regarding the flavour of fruits and vegetables. The human nose, on the other hand, can distinguish among a vast number of volatile compounds, even in lower quantities. Any changes in the compounds responsible for the sourness, sweetness, bitterness or odour of fruits and vegetables may result in changes in their flavour (Oey, *et al.,* 2008a).

It is generally assumed that the flavour of fruits and vegetables is not altered by HP processing, since the structure of low molecular weight flavour compounds is not directly affected by high pressure. This has been observed by means of both chemical and sensory analysis, in a number of studies where fruit juices and purees have been treated at pressures of 200-600 MPa combined with ambient temperature (Bermúdez-Aguirre and Barbosa-Cánovas, 2010; Butz *et al.,* 2002; Lambert *et al.,* 1999). The sensory properties of many HHP treated fruit and vegetable products are still better than those of products preserved in the traditional way by heat treatment, but are not always better that unprocessed products. Based on sensory evaluation, the flavour of HHP treated (500–800 MPa during 5 min at ambient temperature) orange juice was not as fresh as the flavour of untreated orange juice (Fernández García *et al.,* 2001). As high pressure processing can enhance or inhibit enzymatic and chemical reactions, it could indirectly alter the content of some flavor compounds. However, it is difficult to evaluate how HHP induce changes in volatile compounds affecting the overall flavour of fruits and vegetables (Oey *et al.,* 2008a).

Hexanal is a volatile compound associated with the aroma of foliage and grass. Some changes have been reported in hexanal content in strawberries, onions, and tomato juice treated by HHP (Bermúdez-Aguirre and Barbosa-Cánovas, 2010). The increased concentration of hexanal (higher concentrations impart a rancid flavour) was considered to be a result of HHP induced oxidation of free fatty acids, such as linoleic and linolenic acid. Lipooxygenase and hydroperoxide lyase, which are naturally present in tomato, are partly responsible for the development of the rancid taste as they catalyse the oxidation of polyunsaturated fatty acids (Oey *et al.,* 2008a). Additionally, new compounds were found after HHP processing, enhancing the characteristic flavour of the fruit. Navarro *et*

al., (2002) reported that when HHP treated (400 MPa at ambient temperature during 20 min) strawberry puree was stored for 30 days at 4 ºC, increases in the contents of methyl butyrate, 2-methyl butyric acid, hexanoic acid, ethyl butyrate, ethyl hexanoate, 1-hexanol, and linalool were observed. Another study showed that the sweetness and acidity of the HHP treated (500 MPa at 2 ºC during 10 min) grape juice were maintained for 60 days during storage at 4 ºC but fresh fruit and grass aroma were slightly lower during storage (Daoudi *et al.,* 2002). Similar results were observed for HHP treated guava juices (Yen and Lin, 1999). The volatile flavour compounds in pressurized guava juice (600 MPa at 25 ºC during 15 min) remained stable during 30 days of storage at 4 ºC, but changes in the concentrations of volatiles were observed after 60 days of storage. The concentrations of methanol and ethanol increased and the concentrations of many ester and aldehyde compounds decreased, probably due to residual enzyme activity (Yen and Lin, 1999).

I.1.4.3 Other quality parameters

Food commodities or ingredients that provide health-related benefits beyond their basic nutrient supply have gained wide spread acceptance by consumers. There is a vast range of functional foods in terms of their chemical nature, target biomarker, and target health benefit. The beneficial effects of foods are associated with the bioactive components present, including phenolic compounds. Current trends in the functional food area are towards retaining the maximum level of bioactive components while maintaining fresh-like qualities of foods (Gamlath and Wakeling, 2011).

Phenolic compounds such as phenolic acids and flavonoids seem highly stable during HHP. A significant increase in the amount of total phenolics has been reported in strawberry (9%) and blackberry (5%) (Patras *et al.*, 2009b), and onion (12%) (Roldán-Marín *et al.,* 2009), while litchi had no significant changes (Prasad *et al.*, 2009). High pressure enhances mass transfer rates which increase cell permeability leading to an increased extraction of cellular components and increasing levels of pigments in vegetable and food products (Casquete *et al.,* 2014).

A study on the effect of HHP on isoflavones (complex glucosides and bioavailable forms of aglycone) in soybean seeds and soymilk reported insignificant changes to total isoflavone content and better retention of isoflavones compared with conventional thermal processing (Jung *et al.,* 2008). Pressurized (400 MPa at 40 °C during 1 min) orange juice, which is a very rich source of flavanone glycosides (degraded to aglycones

by human intestinal flora after ingestion), showed an increase in levels of naringenin (20%) and hesperetin (39%), but insignificant changes at 4 ºC for 10 days storage. It is suggested that at around 400 MPa some structural changes in the cell walls of the orange juice sacs may have led to an increase in the extraction of flavanones (Sánchez-Moreno *et al.*, 2005). As a consequence, changes in antioxidant capacity could also occur during HHP treatment. In general, the effect of HHP on the antioxidant activity depends on the vitamin and phenolic compounds stability, quality related enzymes, such as PPO levels, and pH conditions of the matrices (Gamlath and Wakeling, 2011). Orange juice showed also a good retention of ascorbic acid after HHP around 500-800 MPa at room temperature and for shorter periods (5 min). However, a slight reduction (10-15%) has been reported during three weeks storage at 4 °C. As the exposure time increased (90 min) the reduction of ascorbic acid accelerated at higher pressures (Indrawati *et al.*, 2004). The decrease of antioxidant capacity in orange juice during HHP processing is mainly caused by ascorbic acid degradation. Antioxidant content of HHP processed smoothies (450 MPa at ambient temperature during 1-5 min) decreased during storage and the level of reduction was greater than in thermally treated samples (70 ºC during 2 min) indicating that enzymatic degradation systems were not inactivated by the HHP conditions applied (Keenan *et al.*, 2010). However, some studies demonstrated that HHP processed fruit and vegetables purees had significantly higher antioxidant capacities when compared to thermally treated samples. Patras *et al.*, (2009a) showed that high pressure processed (400-600 MPa at 20 ºC during 15 min) tomato and carrot purees presented an antioxidant capacity 66% higher than thermally treated purees (70 ºC during 2 min). This was reflected in better retention of ascorbic acid (up to 90%) in high pressure treated samples.

The Maillard reaction is one of the most important and complex processes in food chemistry due to the large number of components able to participate through different pathways that give rise to a complex mixture of products, being a common reaction in foods which undergo thermal processing. Briefly, this reaction is initiated by a condensation between the carbonyl group of a reducing sugar and an amino compound (*e.g.* amino acids), giving rise to different compounds that include reductones, furfurals, and a variety of other substances (van Boekel 2006). Desired consequences like the formation of flavour and brown colour of some cooked foods but also the destruction of essential amino acids and the production of anti-nutritive compounds require the consideration of the Maillard reaction and relevant mechanisms for its control (Jaeger *et*

al., 2010). As chemical reactions are influenced by pressure according to the principe of Le Chatelier, the Maillard reaction must be taken into consideration while processing food (Tamaoka *et al.,* 1991). The influence of high hydrostatic pressure up to 600 MPa on the Maillard reaction was studied in model systems containing amino acids or ß-caein and sugars by Schwarzenbolz *et al.,* (2002). The formation of the amino acid derivate pentosidine was found to be increased by increasing the pressure whereas the formation of pyralline was reduced (Schwarzenbolz and Henle, 2010). Other studies found the acceleration of early Maillard reaction pathways with pressure, e.g. reaction products formed from tryptophan and glucose or xylose, and the slowdown of subsequent reaction steps (Hill *et al.,* 1999). High-pressure effects on the Maillard reaction between glucose and lysine were investigated by Moreno *et al.,* (2003) and the pressure-induced changes in pH were found to strongly influence the HP effects of different stages of the Maillard reaction. The formation and subsequent degradation of Amadori rearrangement products was accelerated by HHP (400 MPa, 60 ºC) and resulted in increased levels of intermediate and advanced reaction products, leading to higher brown colour. Similar results have been reported by Hill *et al.,* (1996) for the same glucose-lysine system. Therefore, the use of HHP at moderate temperatures (30- 60 °C) may promote the Maillard reaction altering the flavour, colour, and nutritional value of foods. However, since few studies have evaluated the effects of pressure on the Maillard reaction, the occurrence of this reaction through food storage is unknown.

I.2 Chemical constituents of Wine

Due to the mode of HHP technology application and operation, previously described in Chapter I.1.1, and its successful use in food industry to destroy microorganisms and inactivate enzymes, with minimal effects on sensorial and nutritional food quality, its seems to be a potential technology to be used in winemaking, *e.g* as an alternative to sulphur dioxide (SO_2). However, there are no references to the effect of high pressure on the chemical characteristics of a sulphur-free wine, in particular, those that may influence their antioxidant capacity and volatile characteristics. More than 500 compounds have been isolated and identified from various wines. Most of these compounds occur at concentrations between 10^{-4} to 10^{-9} grams per litre. At these levels, most are below the limit of human sensory perception. The vast majority of chemicals

found in wine are metabolic by-products of yeast activity during fermentation. By comparison, the number of aroma compounds derived from grapes are comparatively few. Nevertheless, these often constitute the compounds that make one wine distinct from another (Jackson 2000).

For an effective interpretation of the possible effect of high pressure technology in wine chemical constituents is required to know the chemical composition of the wine and the concentrations of the compounds normally found in this matrix.

I.2.1 Aliphatic acids

The most abundant aliphatic acid is the acetic acid, the essential component of volatile acidity. Its concentration, limited by legislation, indicates the extent of bacterial activity and the resulting spoilage of the wine. Other C_3 (propanoic acid) and C_4 acids (butanoic acid) are also associated with bacterial spoilage. The C_6, C_8 and C_{10} fatty acids can also be formed by yeasts. These acids are fermentation inhibitors at concentrations of only a few milligrams per liter, being responsible for stuck fermentations (Jackson 2000; Ribéreau-Gayon *et al.,* 2006).

Propanoic, 2-methyl-propanoic (isobutyric), 2-methyl-butanoic, 3-methyl-butanoic (isovaleric) and 2-phenylacetic acids are formed from α-ketoacids (products of transamination of amino acids) by decarboxylation. Isobutyric and isopentenoic acids come from valine and leucine, respectively. Other acids, such hexanoic (caproic), octanoic (caprilic) and, decanoic (capric) are formed by oxidation of fatty acids (Olivero and Trujillo, 2011).

I.2.2 Alcohols

Ethanol is indisputably the most important alcohol in wine. Although small quantities are produced in grape cells during carbonic maceration, the primary source of ethanol in wine is yeast fermentation. The prime factors controlling ethanol production are the sugar content, temperature, and yeast strain. The concentration of ethanol in wine is generally 100 g/L (12.6 % vol), although it may exceptionally be as high as 136 g/ L. (16% vol) (Jackson 2000). Ethanol acts as an important solvent in the extraction of pigments and tannins during red wine vinification. By affecting the metabolic activity of yeasts, ethanol also influences the type and amounts of aromatic compounds produced.

Furthermore, ethanol acts as an essential reactant in the formation of volatile compounds and adds its own distinctive odour (Ribéreau-Gayon *et al.,* 2006).

Higher alcohols, defined as compounds with more than two carbon atoms with just one alcohol function, occur in wines in concentrations between 150 and 550 mg/L. In spite of being present in lower amounts than ethanol, the higher alcohols can play an important role in wine aroma. They are formed during fermentation, resulting mainly from the metabolic activity of yeasts. The main higher alcohols from fermentative origin are 2-methyl-propanol (isobutyl alcohol) (9-174 mg/L), 2-methyl-butanol (amyl alcohol) (87-564 mg/L) and 3-methylbutanol (isoamyl alcohol) (87-564 mg/L) (Ribéreau-Gayon *et al.,* 2006). In general, factors that increase the fermentation rate (yeast biomass, oxygenation, high temperature, and the presence of matter) also increase the formation of higher alcohols (Ribéreau-Gayon *et al.,* 2006). Higher alcohols, during fermentation, can be formed by amino acids degradation (catabolic pathway), following mechanisms like deamination, decarboxylation and reduction or from sugars metabolism by pyruvate pathway, having α-ketoacids as intermediates (anabolic pathway) (Longo *et al.,* 1992).

Glycerol (a polyalcohol) is probably the chemical compound with the highest concentration in wine after water and ethanol. The minimum glycerol concentration in wine is 5 g/L, but it may reach values as high as 15–20 g/L, depending on the fermentation conditions (Ribéreau-Gayon *et al.,* 2006). The 2-phenylethanol and benzyl alcohols are the main aromatic alcohols found in wines. These compounds are responsible for important sensory marks (flower and sweet aroma). During fermentation, the 2-phenylethanol is formed in considerable amounts (up to 80 mg/L), being one of the main flavour compounds involved in wine aroma (Jackson 2000).

I.2.3 Acetals

An acetal is formed when an aldehyde comes into contact and reacts with two alcohol molecules. Acetals are formed during fermentation, but their content increase significantly during the oxidative conditions of aging process (Perestrelo *et al.,* 2011). The high acetaldehyde content in wine contributes to the acetalization reaction with glycerol, which is favoured at higher pH values, leading to four heterocyclic acetal alcohol formation: cis- and trans-5-hydroxy-2-methyl-1,3-dioxane, and cis- and trans-4-hydroxymethyl-2-methyl-1,3-dioxalane. Heterocyclic acetal alcohols were identified and reported as potential age markers of Porto (da Silva Ferreira *et al.,* 2002) and Madeira

wines (Câmara *et al.,* 2006). Other acetals, such as 1,1-diethoxyethane, that result from a reaction between acetaldehyde and ethanol (Figure I.2), and 2,4,5-trimethyldioxolane, were also detected in table wines (Weldegergis *et al.,* 2011b).

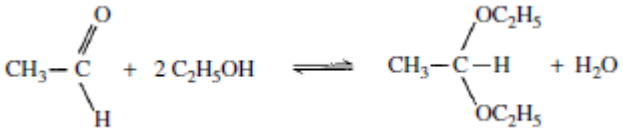


Figure I.2. Acetalization of ethanal and formation of 1,1-diethoxyethane.

I.2.4 Esters

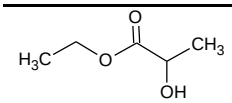
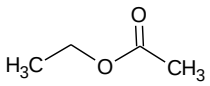
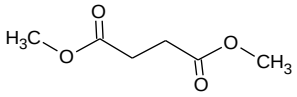
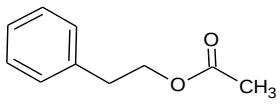
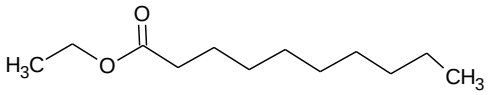
Esters are considered the main chemical group of the volatile fraction of wine. There are a large number of different alcohols and acids in wine, so the number of possible esters is also very large. Ethyl acetate is the most common for kinetic reasons, i.e. the large quantities of ethanol present and the fact that primary alcohols are the most reactive. Esters in wine have two distinct origins: enzymatic esterification during the fermentation process and chemical esterification during long-term aging. The same esters may be synthesized by both ways (Ribéreau-Gayon *et al.,* 2006). Several factors affect the esters formation (formed by both chemical and enzymatic pathways): wine composition, must acidity, defecation, clarification, airing, fermentation temperature, and wine age. The absence of oxygen, low temperatures and must clarification lead to a decreased/difficulty in the esters formation (Ancín-Azpilicueta *et al.,* 2009).

The esters play an important role on the aroma composition of wine because usually the larger the amount of esters present more accentuated the floral and fruity aroma will be (Jackson 2000). During wine aging, the ester composition changes due to shifts toward chemical equilibrium. These changes are influenced by wine pH, storage type (in bottle or barrel) and temperature (Garde-Cerdán and Ancín-Azpilicueta 2006). The concentrations of acetate esters of higher alcohols, such as 2-phenylethyl acetate, decrease during wine ageing. These compounds are produced by enzymatic reactions in excess of their equilibrium concentrations, consequently, they gradually hydrolyse during storage until the equilibrium with their corresponding acids and alcohols is reached (Ancín-Azpilicueta et al. 2009; Câmara et al. 2006). Also, during wine aging, fatty acid ethyl esters can interact with the lees present in barrels, and so the concentrations of these

compounds can decrease during wine aging. Contrarily, the ethyl esters of organic acids, such as dimethyl succinate, increase significantly with time of storage, (in barrel as well as bottle) due the chemical esterification during the course of aging (Câmara et al. 2006).

In Table I.2 are represented the most abuntant esters and the concentrations normally found in wine.

Table I.2. Some esters identified in wine (adapted from Ribéreau-Gayon *et al.*, 2006).

Structure formula	Ester name	Concentration in wine (mg/L)
	Ethyl lactate	0.5-400
	Ethyl acetate	0.15-300
	Dimethyl succinate	0.5-30
	2-phenylethyl acetate	0.05-18.5
	Ethyl decanoate	0.05-5

I.2.5 Lactones

Lactones are formed by an esterification reaction between an acid function and an alcohol function in the same molecule. This reaction produces an oxygen heterocycle. As other chemical groups, lactones can appear in grapes or may be formed during winemaking or during the evolution and wine aging (Pérez-Olivero *et al.,* 2014). Volatile lactones, produced during fermentation, are likely to contribute to wine aroma, since they are responsible for fruity and caramel aromas (Câmara *et al.*, 2006; Oliveira e Silva *et al.*, 2008; Perestrelo *et al.,* 2011).

The γ- and δ-lactones result from the cyclization of the hydroxy acids in the 4 or 5 position, respectively (Figure I.3). In wines, the γ-lactones are more frequent and are formed from γ-ketoacids that come from the carbonated chain along of the fatty acids synthesis or from the deamination of amino acids during the fermentation process.

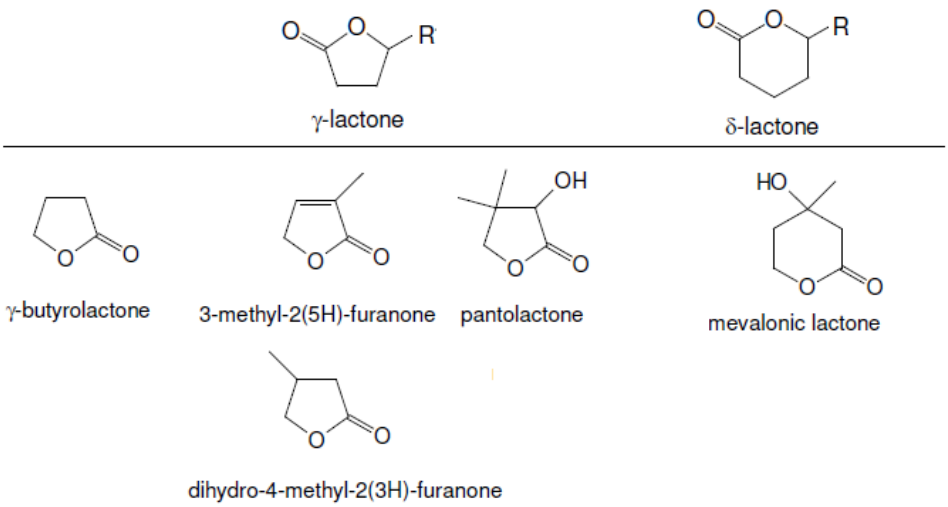


Figure I.3. General structure of γ- and δ-lactones (R-lateral chain) and some lactones identified in wines.

The most abundant lactone is the γ-butyrolactone, that is present in wine at concentrations of few mg/L, resulting from the lactonization of the γ-hydroxybutyric acid, an unstable molecule produced by deamination and decarboxylation of glutamic acid, according to the Ehrlich reaction (Giaccio *et al.*, 2010) (Figure I.4). The 4 ethoxycarbonyl-γ-butyrolactone and 4-ethoxy-γ-butyrolactone are found in concentrations from a few to some tens of µg/L (Bayonove *et al.*, 1998b). Also, it is possible to found some δ-lactones in wines such δ-octalactone, δ-nonalactone, and δ–decalactone.

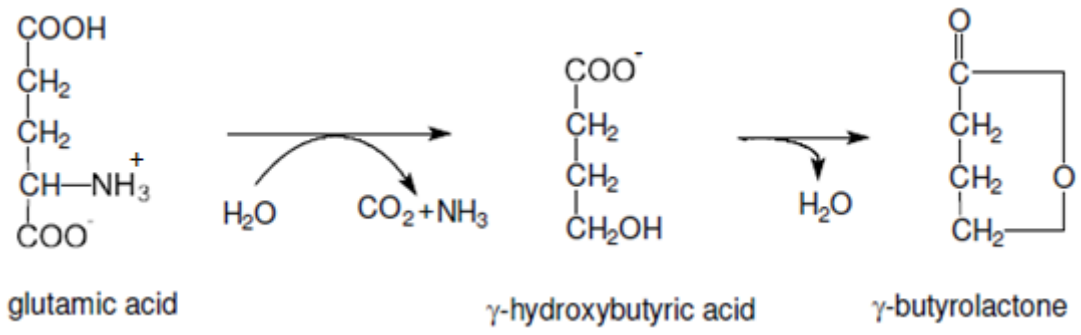


Figure I.4. Formation of γ-butyrolactone (adapted from Ribéreau-Gayon *et al.*, 2006).

I.2.6 Terpenes and Oxygenated Derivatives

A large proportion of terpenes (around 90%) are present as non-volatile glycosides in the grape. This compounds can be hydrolysed (enzymatically or chemically) to the corresponding free forms during fermentation and ageing (Weldegergis, Villiers, et al. 2011). Terpenoids can be found in grapes, musts and wines and, according to the number of combined isoprene units, they can be classified into mono- (C_{10}) and sesquiterpenoids (C_{15}) (Ebeler 2001). These compounds are important varietal aroma compounds and can exist in concentrations in the order of 100 to 1000 mg/L in wines (Ribéreau-Gayon *et al.*, 2006).

The monoterpenoids, constituted by two isoprenic base units, can occur in both free and glycosidically-linked forms. The most dominant monoterpenoids are linalool, geraniol, nerol, α-terpeniol, hotrienol, and citronellol (Luan *et al.*, 2004). Oxidative pathways are active in *Vitis vinifera* L., converting terpenoid constituents of grapes into oxygenated derivatives that accumulate in glycosidically-linked forms. Although the glycoconjugates themselves are odourless, they are easily transformed under pH conditions of wine into volatile constituents, some of which have significant sensory properties (Coutinho 2007). Linalool, for example, in an aqueous acid medium, can originate hydroxylinalool through hydration in the C7 position, α-terpineol by cyclization and also, geraniol and nerol by isomerization during the wine conservation and aging (Ribéreau-Gayon *et al.*, 2006) (Figure I.5). Besides linalool, geraniol and nerol, several

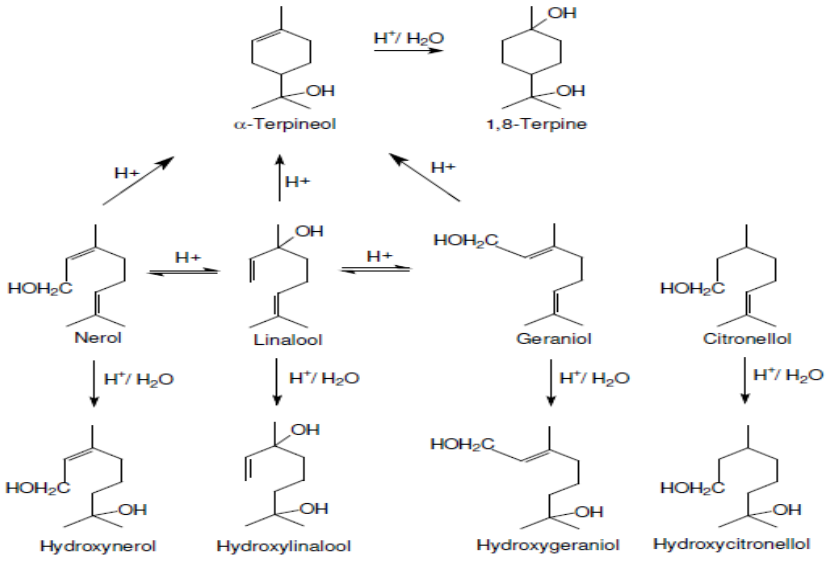


Figure I.5. Possible reactions of monoterpenols in acid medium during conservation and evolution of wine (adapted from Coutinho, 2007).

highly odouriferous cyclic ethers and lactones have been identified as key compounds that are generated by cyclization of oxygenated products from monoterpene alcohols in musts (Figure I.6) (Ebeler 2001; Luan *et al.*, 2004).

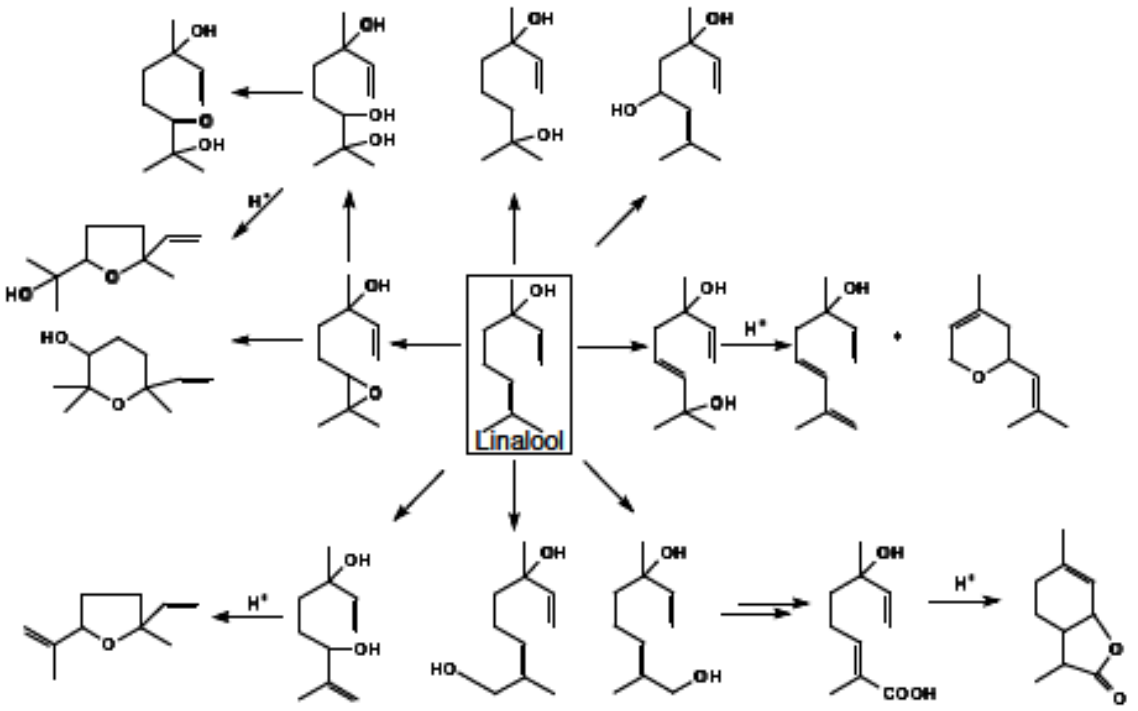


Figure I.6. Oxygenation reactions products of linalool (adapted from Luan et al. 2004).

The sesquiterpenic compounds present in wines may arise directly from grape and/or may have their origin on the rearrangement processes during winemaking process and/or aging (Petronilho *et al.*, 2014). These secondary metabolites are predominantly formed from farnesyl pyrophosphate or nerolidyl pyrophosphate (Rocha *et al.*, 2006). After losing the pyrophosphate residue, different ways of cyclisations are followed (Petronilho *et al.*, 2014). Skeletal rearrangement via carbocation intermediates with hydride or methyl group migration at low pH or temperature conditions can give rise to an enormous type of structures (Bülow and König, 2000) (Figure I.7).

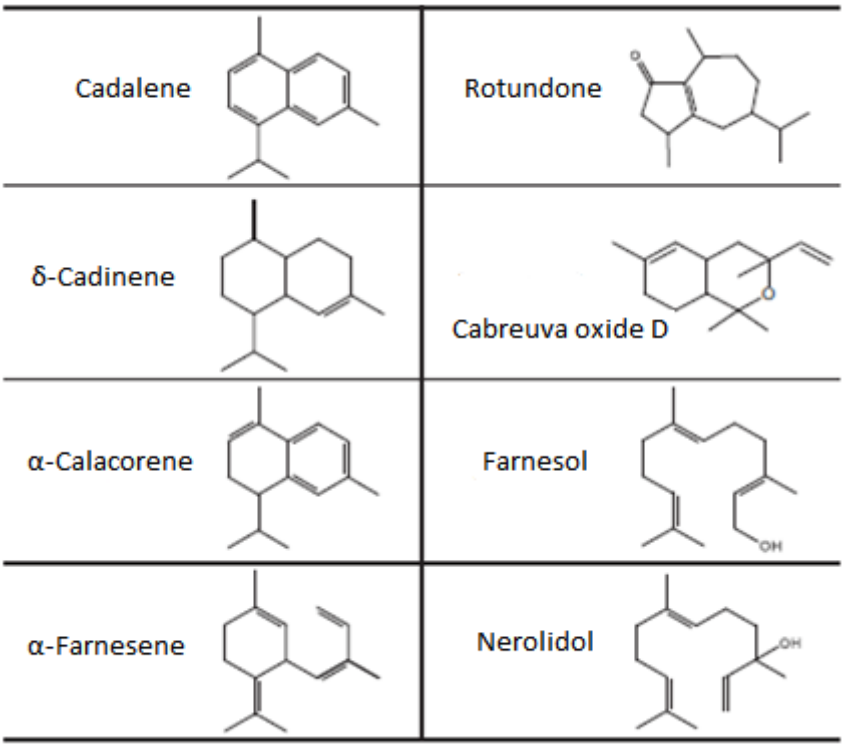


Figure I.7. Sesquiterpenic compounds identified in grapevine related matrices, representing the four detected chemical families: hydrocarbons, ketones, oxides, and alcohols. (adapted from Petronilho *et al.*, 2014).

I.2.7 C_{13} norisoprenoids

C_{13} norisoprenoids have been associated with high quality wine characteristics due to their low odour threshold (µg L^{-1}) and pleasant odour descriptors related to tea, violet, exotic flowers, stewed apple, eucalyptus, and camphor (Genovese *et al.*, 2007; Vinholes *et al.*, 2009). These compounds can represent 0.07 to 1.50 % of the total volatile composition of wines in concentrations ranging ng L^{-1} and mg L^{-1} (Baumes *et al.*, 2002; Genovese *et al.*, 2007). The C_{13} norisoprenoid compounds can be classified in two main groups, megastigmanes and non-megastigmanes. The megastigmane group has a sub-classification that differs according to the position of the oxygen functional group: i) Damascones -oxygen at carbon 7, like β-damascone and, ii) Ionones -oxygen at carbon 9 as observed for β-ionone (Figure I.8). The non-megastigmane forms are all the other C_{13} norisoprenoids derivatives (Ribéreau-Gayon *et al.*, 2006). Some C_{13} norisoprenoids, such as β-damascenone, β-ionone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and vitispirane isomers have been frequently found in wines (Vinholes *et al.*, 2009; Weldegergis et al., 2011a; Weldegergis et al., 2011b). These compounds are products of

direct degradation of carotenoid molecules such as β-carotene, lutein, neoxanthin, and violaxanthin or they can also be released after hydrolysis of glycoside molecules during the wine making or ageing processes (Lloyd *et al.,* 2011; Rodríguez-Bustamante and Sánchez, 2007). TDN, for instance, has been described as coming from direct degradation of β-carotene and other compounds, namely the aglycones megastigme-4,7-dien-3,6,9-triol and 2,6,10,10-tetramethyl-1-oxaspiro[4,5]dec-6-ene-2,8-diol have also been reported as its precursor (Vinholes *et al.,* 2009).

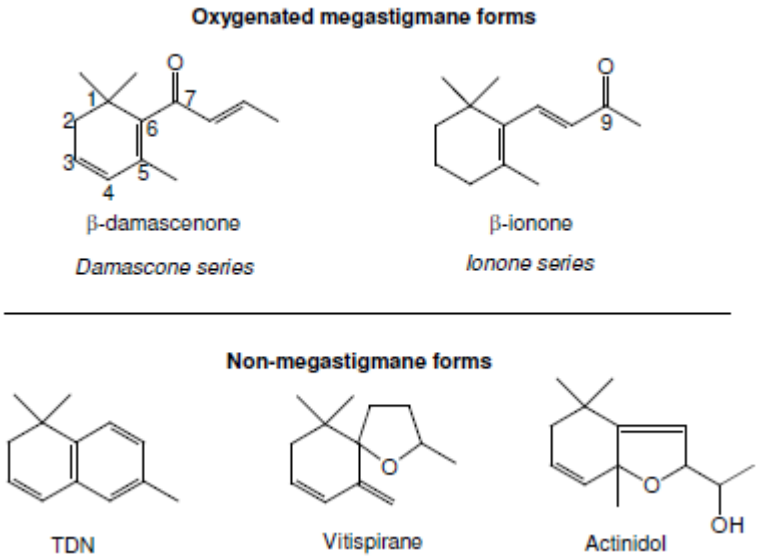


Figure I.8. Main families of C_{13} norisoprenoid derivatives in grapes and wine (adapted from Ribéreau-Gayon *et al.,* 2006).

I.2.8. Organic acids

Organic acids make major contributions to the composition, stability and organoleptic qualities of wines, especially white wines. Their preservative properties also enhance wines microbiological and physicochemical stability. Quantitatively, the organic acids control the pH of wine (Ribéreau-Gayon *et al.,* 2006). The tartaric and malic acids are the most abundant acids in wine. However, if the wine undergoes malolactic fermentation, as occurs in red wines, malic acid is replaced by lactic acid (Jackson 2000). The role of organic acids in maintaining a low pH (3.1 to 3.6) is crucial to the colour stability of red wines. As the pH increases, anthocyanins decolorize and may eventually

turn blue (de Freitas and Mateus, 2006). Acidity also affects phenolic compounds ionization. The ionized form of phenols is more readily oxidized than is the non-ionized form. Accordingly, wines with high pH (>3.9) are very susceptible to oxidation and loss of their fresh aroma and young colour (Karbowiak et al. 2010). Ascorbic acid appears naturally in grapes at a concentration range from 10 to 100 mg/L. However, it rapidly disappears during the winemaking, due to oxidation. In some white wines addition of few milligrams per liter of ascorbic acid may be used as adjuvant to SO_2 (Ribéreau-Gayon *et al.*, 2006). Independently of their origins, most of the main organic acids in must and wine consist on polyfunctional molecules, and many are hydroxyl acids. These functional groups confer polar and hydrophilic characteristics to these compounds. As a result, they are soluble in water, and even in dilute alcohol solutions, such as wine. Their polyfunctional character is also responsible for the chemical reactivity that enables them to develop over time as wine ages (Jackson 2000). In Table I.3 are represented the most important organic acids present in wine.

Table I.3. Most important organic acids present in wine (adapted from Ribéreau-Gayon *et al.*, 2006).

Acids	**Concentration in wine (g /L)**
Tartaric	2-5
Malic	1-6.5
Lactic	0.3-3
Citric	0.5-1
Succinic	0.5-1

I.2.9 Carbonylated Compounds

Aldehydes and ketones are compounds also named carbonylic compounds due to the presence, in both groups, of a carbonyl group (C=O). In these compounds, acetaldehyde is the most abundant in wine. The many ways it can be produced and its high reactivity, as well as its rapid combination with sulphur dioxide at low temperature

and its organoleptic properties, make acetaldehyde a very important component of wine (Ribéreau-Gayon *et al.,* 2006). The presence of acetaldehyde, produced by the oxidation of ethanol, is closely linked to oxidation–reduction phenomena. It can also be produced by the decarboxylation of pyruvate during alcoholic fermentation. Furthermore, acetaldehyde plays a role in the colour changes occurring in red wines during aging by facilitating the copolymerization of phenols (anthocyanins and catechins) (de Freitas and Mateus, 2006; Oliveira *et al.,* 2011).

A few other aldehydes are present in wine in trace amounts and contribute to the wine aroma. The neutralizing effect of sulfur dioxide on the fruitiness of certain white wines is due to the fact that it combines with the aldehyde fraction in the bouquet (Villamor and Ross, 2013). The molecules with ketone function, such as propanone, butanone and pentanone, have been identified in wines. The most important are 3-hydroxy-2-butanone (acetoin), and 2,3-butanedione (diacethyl). Diacethyl can have the pyruvic acid as its precursor, but it can also result from the oxidation of the acetoin and can be formed during excessive maturation of the grape by the decarboxylation and oxidation of α-acetolactate (Moreno-Arribas 2000). With the exception of acetaldehyde and acetoin (Table I.4), most of this type of compounds is present in trace amounts.

Table I.4. Some aldehydes and ketones identified in wine (adapted from Ribéreau-Gayon *et al.,* 2006).

Compound name	**Concentration in wine (mg/L)**
Acetaldehyde	7- 252
3-Hydroxy-2-butanone	0-140
Ethanedial	2-4.5
2,3-Butanedione	0.2-4.1
Phenylethanal	0.12
5-hydroxymethyl-2–furfural	0-87
2-Furfural	0-10.3

I.2.10 Carbohydrates

In dry wines, the residual sugar content consists primarily of pentoses, such xylose and mostly arabinose (0.3–2 g/L), and some hexoses such as glucose and fructose (in the order of 1 g/L). The hexoses are mainly fructose, because glucose is preferentially fermented by the great majority of yeasts. For this reason, the glucose/fructose ratio, which is around 1 in grape must, decreases regularly during fermentation. Pentoses are not fermentable by yeast and are more common in red than white wines (Jackson 2000; Ribéreau-Gayon *et al.*, 2006).

The polysaccharide level in finished wine can reach 740 mg/L (Moreno-Arribas 2000) but, depending on their composition, structure and concentration, is relevant for explaining and controlling wine stability and retention of aroma compounds (Coimbra *et al.*, 2005). They are originated both from grape and microorganisms. Arabinans, type II arabinogalactans, rhamnogalacturonans and galacturonans arise from native cell-wall pectic polysaccharides of grape berry after degradation by pectic enzymes during grape maturation and during the first steps of wine making. Yeasts produce mannans and mannoproteins during and after fermentation, whereas glucans are produced by *Botrytis cinerea*, which may infect grape berries (Coimbra *et al.*, 2005).

I.2.11 Minerals

The wine contains about 2 to 4 g/L of salts of mineral acids and some organic acids. The major mineral anions existing in the wine are phosphates (white wine: 70-500 mg/L; red wine: 150 mg/L to 1 g/L) and sulfates (100 to 400 mg/L), although the concentration of sulfates tends to increase with aging due to oxidation of SO_2, may achieving concentrations of 2 g/L. Chlorides are also present in concentrations below 50 mg/L and nitrates in trace amounts (Burin *et al.*, 2010a; Ribéreau-Gayon *et al.*, 2006).

Potassium is the dominant cation, with concentrations ranging from 0.5 to 2 g/L. The calcium concentration ranges from 80 to 140 mg/L, while the sodium has values between 10 and 40 mg/L.Therefore, wine has more magnesium (60-150 mg/L) than sodium. The average concentration of iron and copper ions in wine is 2.8 to 16 mg/L and 0.11 to 3.6 mg/L, respectively (Burin *et al.*, 2010a; Jackson 2000; Ribéreau-Gayon *et al.*, 2006).

I.2.12 Nitrogen compounds

There are two forms of nitrogen in musts and wines, mineral nitrogen and organic nitrogen. The mineral nitrogen exists in form of ammonium salts (NH_4^+). There may be a few tens of mg/L of inorganic nitrogen in wine after aging on the lees, or even after malolactic fermentation (Martínez-Rodríguez and Polo, 2000; Mauricio *et al.*, 2001). Indeed, lactic bacteria do not assimilate ammonia nitrogen and may even excrete it (Ribéreau-Gayon *et al.*, 2006). Organic nitrogen is divided in eight families: amino acids, oligopeptides and polypeptides (under 10,000 Dalton), protein (above 10,000 Dalton), amides, biogenic amines, nucleic nitrogen, amino sugar nitrogen, and pyrazines (Jackson 2000).

Amino acids are an important source of nitrogen in musts and wines. The total concentration of free amino acids can vary from 1 to 4 g/L (Bouloumpasi *et al.*, 2002; Košir and Kidrič, 2001; Lehtonen 1996). The predominant amino acids in wine are alanine, serine, arginine, proline, and glutamic acid and glutamine that can reach concentrations of the order of hundreds of milligrams per liter (Bouloumpasi *et al.*, 2002). These substances are very useful, due to their antimicrobial, surfactant, and emulsifying properties (Dartiguenave *et al.*, 2000).

Glutathione is an important tripeptide that contains a cysteine residue that reacts partially with quinones resulting from oxidation of phenols. Grapes and wine contain many proteins with a wide range of molecular weights (30 kDa–150 kDa). Some unstable proteins are responsible for protein casse in white wines. Other proteins are associated with a carbohydrate fraction (Jackson 2000). The amides family is represented by small quantities of urea (Ribéreau-Gayon *et al.*, 2006), as well as ethyl carbamate. This compound is very strictly controlled for health reasons, such is potentially toxic, and was re-classified in 2007 as probable human carcinogen compound (Group 2A) by the International Agency for Research on Cancer (Perestrelo *et al.*, 2010).

Nucleic nitrogen is present in purine and pyrimidine bases, nucleosides and nucleotides, as well as nucleic acids. Amino sugar nitrogen consists of hexoses in which an –OH is replaced by $–NH_2$. Small quantities of glucosamine and galactosamine have been found in protein nitrogen in wine (Ribéreau-Gayon *et al.*, 2006).

Biogenic amines in wine can be formed mainly as a consequence of the decarboxylation of amino acids by various microorganisms associated with the different stages of wine production and storage, and wines usually contain a few milligrams per

liter (Beneduce *et al.*, 2010). The main biogenic amines associated with wine are putrescine, histamine, tyramine and cadaverine, followed by phenylethylamine, spermidine, spermine, agmatine and tryptamine (Figure I.9) (Smit *et al.*, 2008). The presence of these compounds is considered by some authors a fundamental parameter for the detriment of wine, and high concentrations of biogenic amines can cause undesirable physiological effects in sensitive humans (Landete *et al.*, 2005).

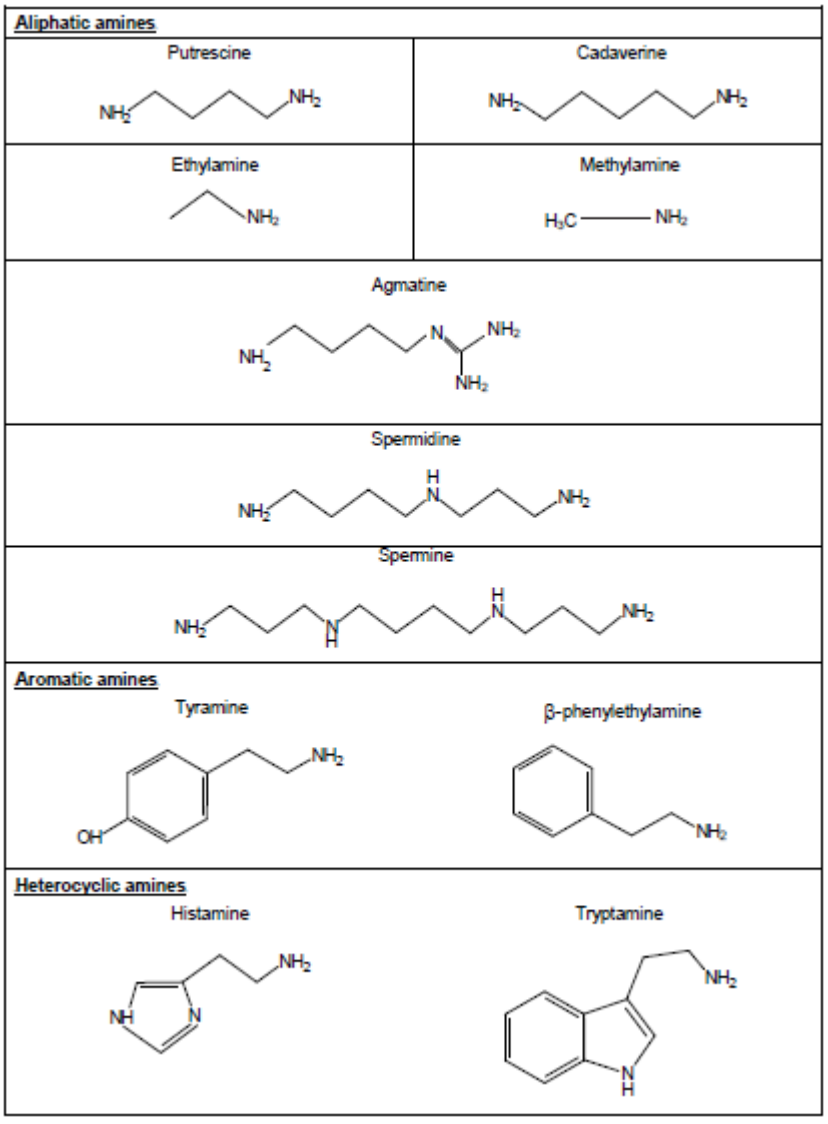


Figure I.9. The main biogenic amines presented in wine (adapted from *Smit et al.*, 2008).

Pyrazines are heterocycles with six links containing two nitrogen atoms and four carbon atoms bearing radicals and can appear in wines. The methoxypyrazines have been reported to be produced by the metabolism of amino acids (Ribéreau-Gayon *et al.*, 2006). Some studies suggest that some methoxypyrazines have a microbiologic origin in wine (Allen *et al.*, 1995). Pyrazines can also be formed by Maillard reactions (Van Lancker *et*

al., 2012). The 2-methoxy-3-isopropylpyrazine, 2-methoxy-3-sec-butylpyrazine and 2-methoxy-3- isobutylpyrazine have a vegetal odour resembling green pepper with some earthy, potato or herbaceous nuances (Fan and Qian, 2006; Pripis-Nicolau *et al.*, 2000).

I.2.13 Sulphur compounds

Sulphur compounds have in their structure, at least, one sulphur atom. In wine, a considerable number of sulfur volatile compounds from several origins have been identified. Thiols come from grapes, having an important contribution to the varietal aroma of certain wines (Coutinho 2007).

The intensity of the odour perception of sulfur compounds seems to be related with the molecular weight. The sulphur compounds can be classified in two main groups: those with lower molecular weight (boiling points inferior to 90 ºC) and those with higher molecular weight (boiling points superior to 90 ºC) (Ribéreau-Gayon et al. 2006) (Table I.5). The first group is usually associated to defects in wines and the second group is more

Table 1.5. Some sulphur compounds identified in wines (Adapted from Ribéreau-Gayon *et al.*, 2006).

Formula	Compound name	Concentration in wine (µg/L)
Sulphur compounds with low molecular weight		
H_2S	Hydrogen sulphide	0.3
CH_3-S-CH_3	Dimethyl sulphide	1.4
CH_3-SH	Methanethiol	0.7
CS_2	Carbon disulphide	1.7
Sulphur compounds with high molecular weight		
CH_3-S-CH_2-CH_2-CH_2-OH	3-Methylthiopropan-1-ol (methionol)	838
CH_3-S-CH_2-CH_2-OH	2- Methylthioethanol	56
CH_3-S-CH_2-CH_2-CH_2-COO-CH_3	3-Methylthiopropyl acetate	50-115
HS-CH_2-CH_2-OH	2-Mercaptoethanol	72

abundant and may contribute to the global white wine aroma. These compounds are produced by yeasts during fermentation, as well as during the vinification process, aging in oak barrels, or during storage in bottles (Fedrizzi et al. 2007; Siebert et al. 2010).

I.2.14 Phenolic compounds

Phenolic compounds are very important in wine since they are responsible for several organoleptic properties, namely colour, astringency, and bitterness (Ribéreau-Gayon *et al.*, 2006). Wine phenolic compounds consist on both grape phenolic compounds and new phenolic compounds derived from them during winemaking and ageing process (Soto Vázquez *et al.*, 2010; Sun *et al.*, 2011). These compounds are also associated with the beneficial effects related with moderate wine consumption, especially in relation to cardiovascular and degenerative diseases, due to the antioxidant capacity of polyphenols (Garrido and Borges, 2011; Radovanović and Radovanović, 2010; Touriño *et al.*, 2008). Their structures enable them to scavenge and neutralize free radicals (Rivero-Pérez *et al.*, 2008; Tabart *et al.*, 2009). These compounds are oxidized sequentially to semiquinones and quinones, while oxygen is reduced to hydroperoxyl radicals and hydrogen peroxide. This process is catalyzed by the redox cycle Fe^{3+}/Fe^{2+}. Hydrogen peroxide is then reduced by Fe^{2+}, in the Fenton reaction, to hydroxyl radicals, which oxidize hydroxyl groups of saturated compounds (Figure I.10). Radical intermediates can also react with oxygen to form an additional pathway to their reduction (Karbowiak *et al.*, 2010; Oliveira *et al.*, 2011).

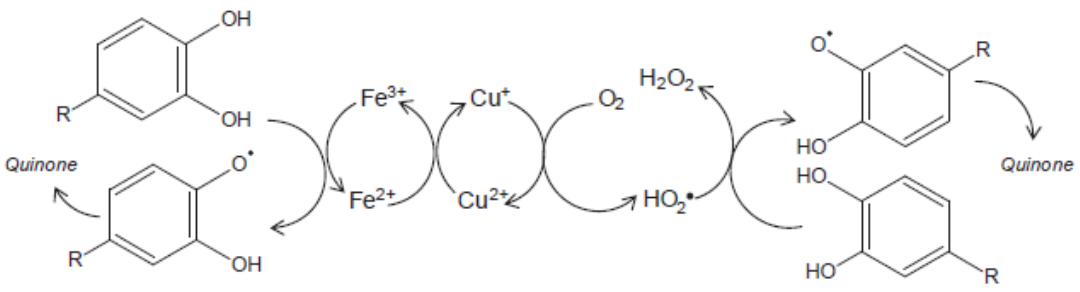


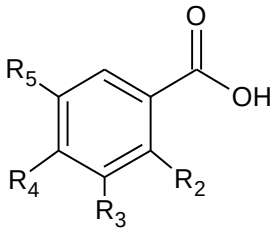
Figure I.10. Proposed catalytic action of iron and copper ions in the oxidation of catechols to produce quinones and hydrogen peroxide (adapted from Oliveira *et al.*, 2011).

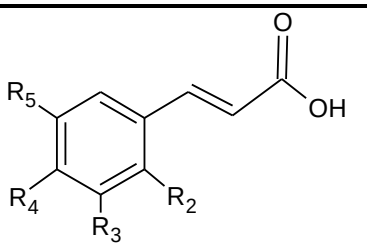
The phenolic compounds represent a family of many compounds, since they are grouped under that name all the molecules that have at least one benzene ring substituted by a variable number of hydroxyl groups. However, the family is divided into two main groups: non-flavonoids, present mainly in the pulp of grapes, and flavonoids, present in the solid parts of the grape (seed and skin) (Busse-Valverde *et al.*, 2012; Kelebek *et al.*, 2010; Perestrelo *et al.*, 2012).

I.2.14.1 Non-flavonoids

The grapes and wines present phenolic acids in concentrations range of 100 to 200 mg/L in red wine and 10 to 20 mg/L in white wine (Jackson 2000; Ribéreau-Gayon *et al.*, 2006). The phenolic acids are divided into two classes: the derivatives of benzoic acid and those of hydroxycinnamic acid (Table I.6). In grapes, benzoic acids are mainly present as glycoside combinations, from which they are released by acid hydrolysis, and

Table I.6. Some phenolic acids identified in wines.



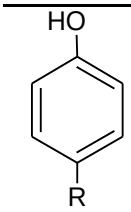
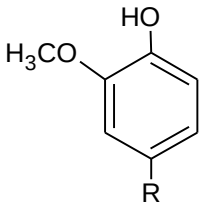
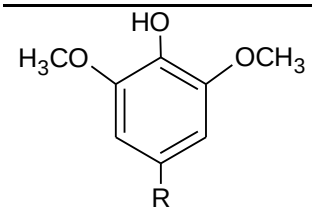


Benzoic acids	**R_2**	**R_3**	**R_4**	**R_5**	**Cinnamic acids**
p-Hydroxybenzoic acid	H	H	OH	H	*p*-Coumaric acid
Protocatechuic acid	H	OH	OH	H	Caffeic acid
Vanillic acid	H	OCH_3	OH	H	Ferulic acid
Gallic acid	H	OH	OH	OH	
Syringic acid	H	OCH_3	OH	OCH_3	Sinapinic acid
Salicyclic acid	OH	H	H	H	
Gentianic acid	OH	H	H	OH	

esters (gallic and ellagic tannins). Free forms are more prevalent, mainly in red wine, due to the hydrolysis of these combinations and heat breakdown reactions of more complex molecules, especially anthocyanins (Carvalho *et al.,* 2010; Mateus and de Freitas, 2001; Vidal *et al.,* 2002). Several cinnamic acids are present in wines mainly esterified, in particular with tartaric acid, therefore they have been identified in small quantities in the free form (Schwarz *et al.,* 2003).

Phenolic acids are colorless in a dilute alcohol solution, but they may become yellow due to oxidation. From an organoleptic standpoint, these compounds have no particular flavour or odour (Ribéreau-Gayon *et al.,* 2006). However, they are precursors of the volatile phenols (Table I.7) produced by the action of certain microorganisms. Ethyl

Table 1.7. Some volatile phenolic compounds identified in wines.

	R	**Name**
	CH_2-CH_3	Ethyl phenol
	$CH=CH_2$	Vinyl phenol
	CH_2-CH_2OH	Tyrosol
	H	Guaiacol
	CH_3	Methyl guaiacol
	CH_2-CH_3	Ethyl guaiacol
	$CH=CH_2$	Vinyl guaiacol
	CH_2-CH_2-CH_3	Propyl guaiacol
	$CH=CH$-CH_3	Allyl guaiacol
	H	Syringol
	CH_3	Methyl syringol

phenol and ethyl guaiacol are found in red wines whereas vinyl phenol and vinyl guaiacols are found in white wines (Arapitsas *et al.,* 2004; Jeleń *et al.,* 2011; Larcher *et al.,* 2012). It has been established that these compounds result from the breakdown of *p*-coumaric acid and ferulic acid (Larcher *et al.,* 2012). Tyrosol is present in both red and

white wine (20–30 mg/L) and is formed during alcoholic fermentation (Jackson 2000). When wines are aged in new oak barrels, the toasting of the wood involved in barrel manufacture causes the breakdown of lignins and the formation of various components from the same family, with a variety of smoky, toasty and burnt smells (Table I.7), such as guaiacol, methyl guaiacol, propyl guaiacol, allyl guaiacol (isoeugenol), syringol and methyl syringol (Arapitsas *et al.*, 2004; Chira and Teissedre, 2013a; Escudero *et al.*, 2007; Ribéreau-Gayon *et al.*, 2006; Ristic *et al.*, 2011).

The stilbenes are another family of phenolic compounds present in grapes and wines. These compounds have two benzene rings bonded by an ethane or ethylene chain (C_6-C_2-C_6). In the stilbenes family, resveratrol (Figure I.11) is recognized as compound capable of preventing or reducing a wide range of diseases (Burns *et al.*, 2000; Galmarini *et al.*, 2013), and is present in red wine at a concentration range of 1 to 3 mg /L (Ribéreau-Gayon *et al.*, 2006).

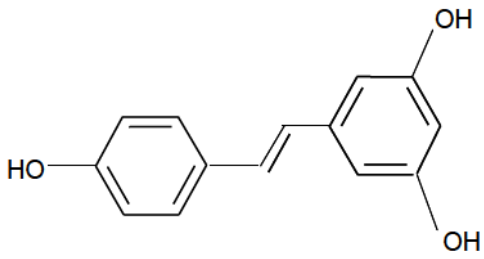


Figure 1.11. Structure of *trans*- resveratrol.

A different class of non-flavonoids is the hydrolysable tannins, which can be hydrolyzed by the action of hot water or enzymes. They can be formed from gallic acid and ellagic acid and in the simplest element of this type of tannin the gallic acid is esterified with a molecule of glucose (Figure I.12) (Jackson 2000; Ribéreau-Gayon *et al.*, 2006).

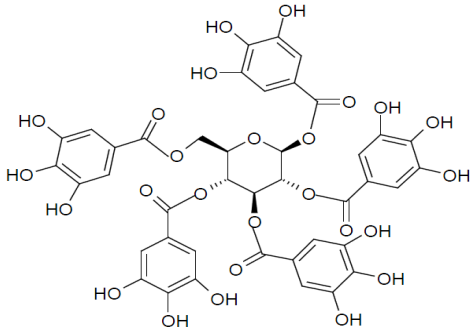


Figure I.12. Structure of a hydrolysable tannin.

I.2.14.2 Flavonoids

Flavonoids constitute the largest group of naturally occurring phenolic compounds (Ribéreau-Gayon *et al.*, 2006). Their basic structures (Figure I.13) are built upon a C6-C3-C6 skeleton, consisting of two aromatic rings (A- and B-ring) linked by an oxygen containing a pyran-derived ring (C-ring) (Valls *et al.*, 2009).

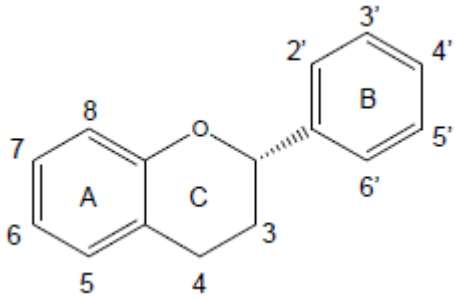
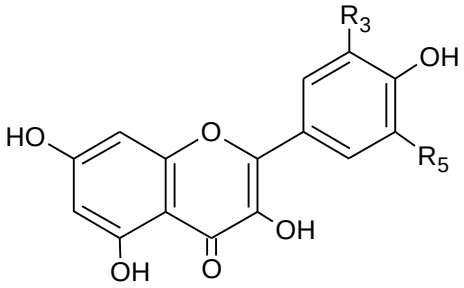


Figure I.13. Basic structure of flavonoids showing A, B, and C rings and the numbers for the various positions in the flavonoids structure.

In the flavonoid group, the flavonols are compounds characterized by the presence of an insaturation in heterocyclic ring and a hydroxyl group in the position 3. The flavonols are differentiated by substitution of the lateral nucleus, producing kaempferol, quercetin and myricetin (Figure I.14). These molecules are present in red wine in concentrationsin the order of 100 mg/L, while in white wine the concentration is between 1 and 3 mg/L (Ribéreau-Gayon *et al.*, 2006).



R_3	**R_5**	**Name**
H	H	Kaempferol
OH	H	Quercetin
OH	OH	Myricetin

Figure I.14. Some flavonols identified in wines.

Flavanols

One important group of flavonoids are the flavanols that 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, gallocatechin, and epigallocatechin are the most common flavanol monomers (Ribéreau-Gayon *et al.,* 2006) (Figure I.15).

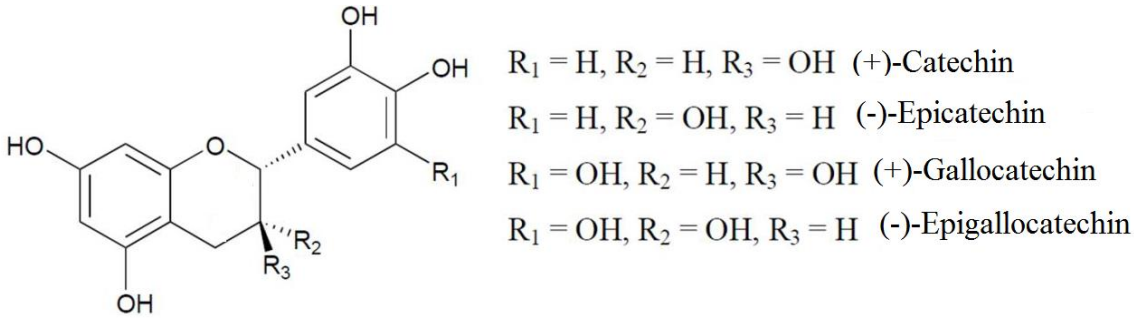


Figure I.15. Most important flavanol monomers identified in wines in wine.

The proanthocyanidins are extracted during alcoholic fermentation and post-fermentation maceration. Their content in red wines are between 1 and 4 g/L depending on the grape variety and the winemaking method. In the case of white wines, this content are between 100 and 300 mg/L (Ribéreau-Gayon *et al.,* 2006). Besides the winemaking method, these levels also depend on many factors such as the nature of the grape (Chira *et al.,* 2009; Fang *et al.,* 2008), and the soil or the climate (Andrés-de Prado *et al.,* 2007; Downey *et al.,* 2003). Different classes of tannins are characterized by the degree of hydroxylation of the B ring of the constituent units. These classes can be distinguished in procyanidins and prodelphinidins and the names of these two groups comes from their properties to turn into red anthocyanidins, cyanidin and delphinidin respectively, when depolymerized under oxidative conditions (Ribéreau-Gayon *et al.,* 2006). Procyanidins are polymers of (+)-catechin and (-)-epicatechin, and the prodelphinidins polymers of (+) -gallocatechin and (-)-epigallocatechin. Condensation of flavanols are between a flavan-3-ol electrophilic unit (position 4) and a flavan-3-ol nucleophilic unit (postions 6 and / or 8), which leads to the formation of interflavanic bonds between C4-C8 and/or C4-C6 (Figure I.16).

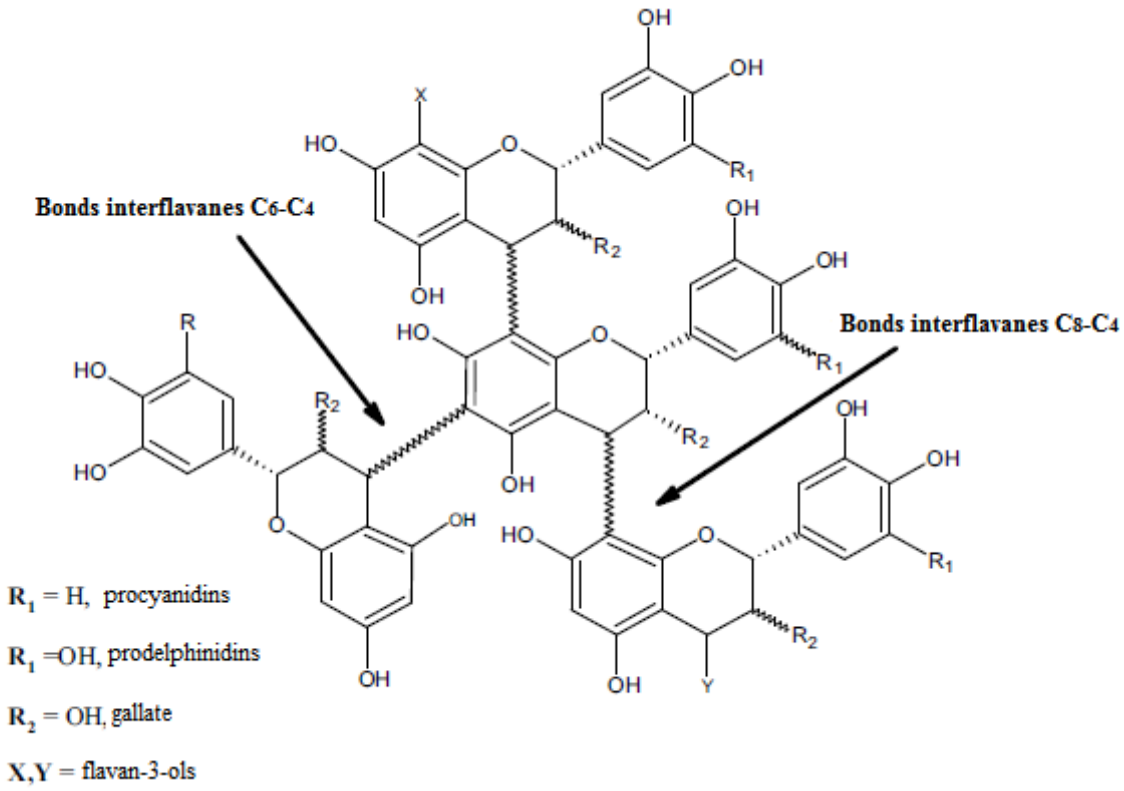


Figure I.16. Structure of flavanols condensation.

The proanthocyanidins play an important role in the taste quality of the wines, since they have the ability to interact with salivary proteins, forming stable complexes that cause a sensation of dryness and constriction, denominated as astringency (Carvalho *et al.,* 2006). The molecular weight of proanthocyanidins, expressed as mean degree of polymerization (mDP) is one of their most important properties, since it influences wine astringency and bitterness (Peleg *et al.,* 1999). Monomers are more bitter than astringent, whereas the reverse is true for large molecular weight derivatives (Chira *et al.,* 2009).

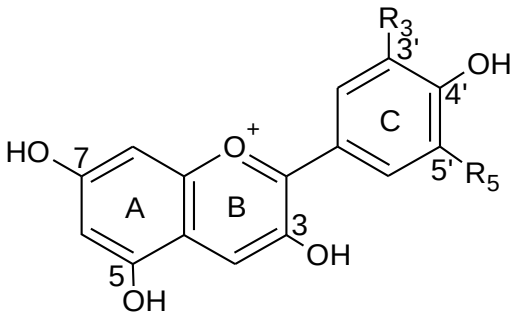
During wine aging, different reactions can occur, giving rise to proanthocyanidins with different degree of polymerization. In the first months of wine storage, the mDP tends to increase due to condensation reactions of flavan-3-ols in the wine (Ribéreau-Gayon *et al.,* 2006). However, along aging, a decrease in mDP and prodelphinidins could occur due to the easier degradation of higher molecular weight proanthocyanidins and also due to their precipitation after condensation with other compounds, namely polysaccharides and proteins (Cheynier *et al.,* 1997; Chira *et al.,* 2011; Cosme *et al.,* 2009). Vidal *et al.,* (2002) also attributed the decrease in mDP to a cleavage reaction that

occurs in acidic media like wine. In this case, these reactions dominate in relation to the polymerisation reaction of proanthocyanidins that also occur.

Anthocyanins

The anthocyanins correspond to the red pigments of grapes and are present in red wines at concentrations on the order of 200 to 500 mg/L (Ribéreau-Gayon *et al.,* 2006). Anthocyanin classification is based primarily on the position of the hydroxyl and methyl groups on the B ring of the anthocyanidin molecule (Figure I.17). On this basis, grape anthocyanins are divided into five classes, namely cyanidin, peonidin, delphinidin, petunidin and malvidin. These compounds are much more stable in the glycosylated form than in the glycone form (anthocyanidins) (He *et al.*, 2012). For the genus *Vitis*, the glycosylation occurs at position *O*-3 giving a higher solubility and stability to these compounds. The glycosidic fragments could be esterified at position 6 with some acids, including acetic acid, *p*-coumaric acid, and caffeic acid (von Baer *et al.,* 2008).

The proportion and amount of each class of compounds vary widely among cultivars and with growing conditions. The proportion of anthocyanins markedly influences both hue value and colour stability. Both properties are directly affected by the hydroxylation patern of the anthocyanidin B ring (Alcalde-Eon *et al.,* 2006; Boulton 2001; González-Manzano *et al.,* 2007).



R_3	R_5	Name
OH	OH	Delphinidin
OH	H	Cyanidin
OH	OCH_3	Petunidin
OCH_3	H	Peonidin
OCH_3	OCH_3	Malvidin

Figure I.17. Anthocyanidins identified in wines from *Vitis vinifera*.

The anthocyanins have been investigated in aqueous solution and have been shown to exist in different forms in equilibrium depending on the pH. Under very weak acidic conditions, four anthocyanin structural types exist in equilibrium (Figure I.18) (Castañeda-Ovando *et al.,* 2009). In very acidic aqueous solutions, anthocyanins occur as

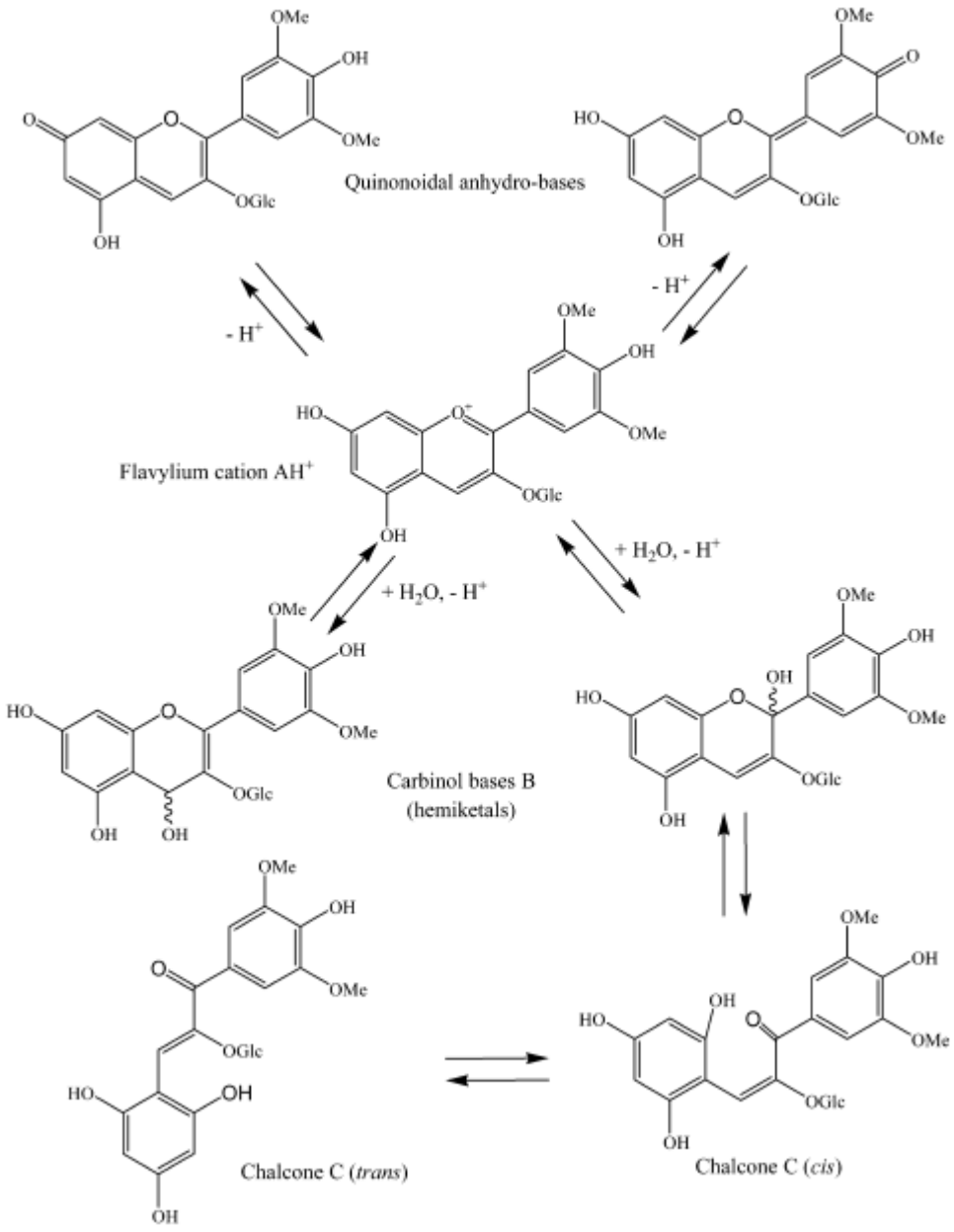


Figure 1.18. Malvidin 3-*O*-glucoside equilibria in aqueous media (adapted from Es-Safi *et al.,* 2008).

red flavylium cations AH^+. In aqueous media, increasing the pH leads to a reduction of color intensity because of a decrease in the concentration of the flavylium cation AH^+ that is converted into its colorless hemiketal form B through nucleophilic attack of water. This

pseudo-base form is in equilibrium with the colorless hydroxy chalcone form C. At low acidic, neutral, and basic pH values, deprotonation of the flavylium cation also occurs, giving rise to the violet/blue quinonoidal forms (Es-Safi *et al.*, 2008; He *et al.*, 2012; Monagas *et al.*, 2003).

In the presence of sulphur dioxide, free anthocyanins are strongly discolored. At pH 3.2, 96% of sulphurous acid are in form of HSO_3^- (Ribéreau-Gayon *et al.*, 2006). HSO_3^- reacts with the flavylium cation (A^+) on carbon 2 or carbon 4 leading to the formation of colorless compounds of type $AHSO_3$ (Mazza and Francis, 1995). The formation of such colorless compounds is a function of the concentration of free anthocyanins. The effect of SO_2 on the combined and polymerized anthocyanins is less. In addition, this substitution prevents the condensation of anthocyanins with other molecules (Mirabel *et al.*, 1999).

During wine maturation and aging, phenolic compounds, including anthocyanins and flavanols, are subject to various chemical transformations due to oxidation-reduction reactions, condensation, polymerization and complexation with other compounds such as proteins, polysaccharides or metals (Ricardo-da-Silva *et al.*, 1991). Consequently, anthocyanins and tannins are gradually being transformed into oligomeric and polymeric pigments more stable and with physical and chemical characteristics distinct from their precursors, contributing to the change in the organoleptic properties such as colour and taste of wine (Monagas *et al.*, 2005).

The colour evolution of red wines is a complex process that is in part attributed to copigmentation phenomena and to the progressive displacement of the original anthocyanins by newly formed pigments (Marquez *et al.*, 2013; Mateus *et al.*, 2002; Pissarra *et al.*, 2004) (Figure I.19). These pigments usually arise from the interaction between anthocyanins and other phenolic compounds, especially flavanols such as catechins (He et al. 2012). Different mechanisms have been suggested to explain the formation of these new pigments (Blanco-Vega *et al.*, 2011; Gómez-Míguez *et al.*, 2006; Mateus *et al.*, 2002, 2004; Oliveira *et al.*, 2013; Sánchez-Ilárduya *et al.*, 2014). Processes such as direct reaction between anthocyanins and flavanols (Remy *et al.*, 2000), reaction between anthocyanins and flavanols through ethyl bridges (Es-Safi *et al.*, 1999; Francia-Aricha *et al.*, 1997), or the reaction between anthocyanins and other small compounds such as glyoxylic acid, vinylphenol and pyruvic acid (Benito *et al.*, 2011) have already been demonstrated in model solutions. Among these pigments, the pyranoanthocyanins

are currently acknowledged as one of the most important classes of anthocyanin derivatives. Correct assignment of their structures and formation in red wine has been achieved relatively recently over the last two decades. Their general structure includes an additional ring D formed between the OH group at C-5 and the C-4 of the anthocyanin pyranic ring. This new pyranic ring D may have different kinds of substituents linked directly at C-10 (de Freitas and Mateus, 2011).

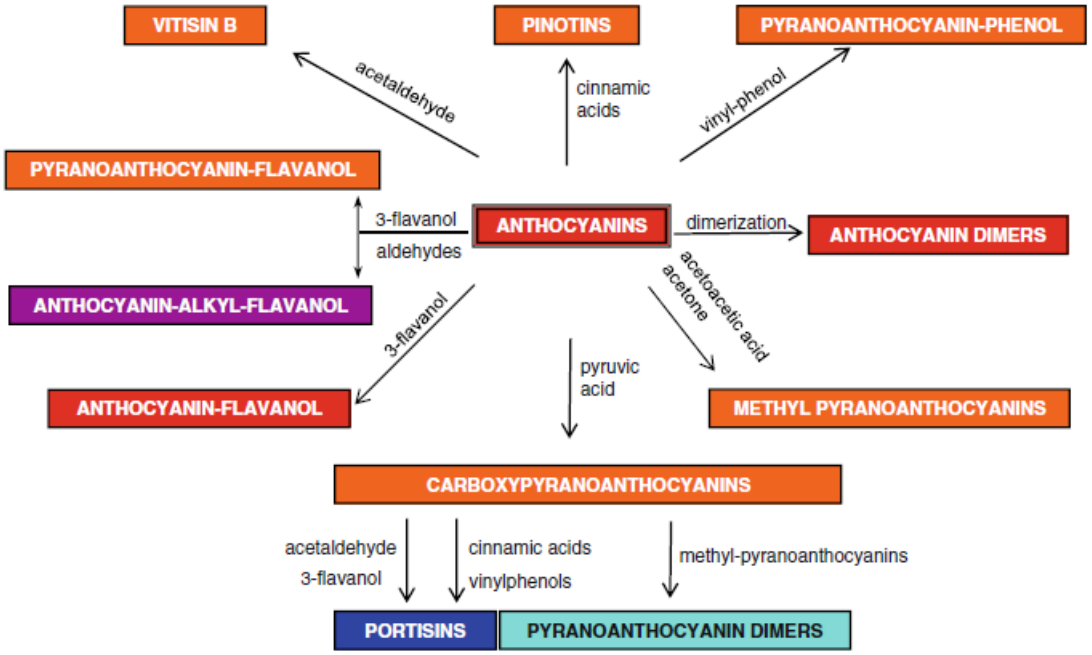


Figure I.19. Anthocyanin-derived pigments formation during red wine aging (adapted from de Freitas and Mateus, 2011).

Figure I.20 shows the formation of pyranoanthocyanins during wine aging from condensation reaction of malvidin-3-*O*-glucoside and acetaldehyde, pyruvic acid and vinylphenol. However, pyranoanthocyanins with analogous structures are also formed from other anthocyanin-3-*O*-glucosides and their acetylic, *p*-coumarilic and caffeoilic derivatives (Blanco-Vega *et al.*, 2011; Mateus *et al.*, 2004; Sánchez-Ilárduya *et al.*, 2014). All these events result in the formation of more stable pigments that stabilize wine colour (Carvalho *et al.*, 2010; Marquez *et al.*, 2013; Oliveira *et al.*, 2010; Pechamat *et al.*, 2014; Talcott *et al.*, 2003). 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).

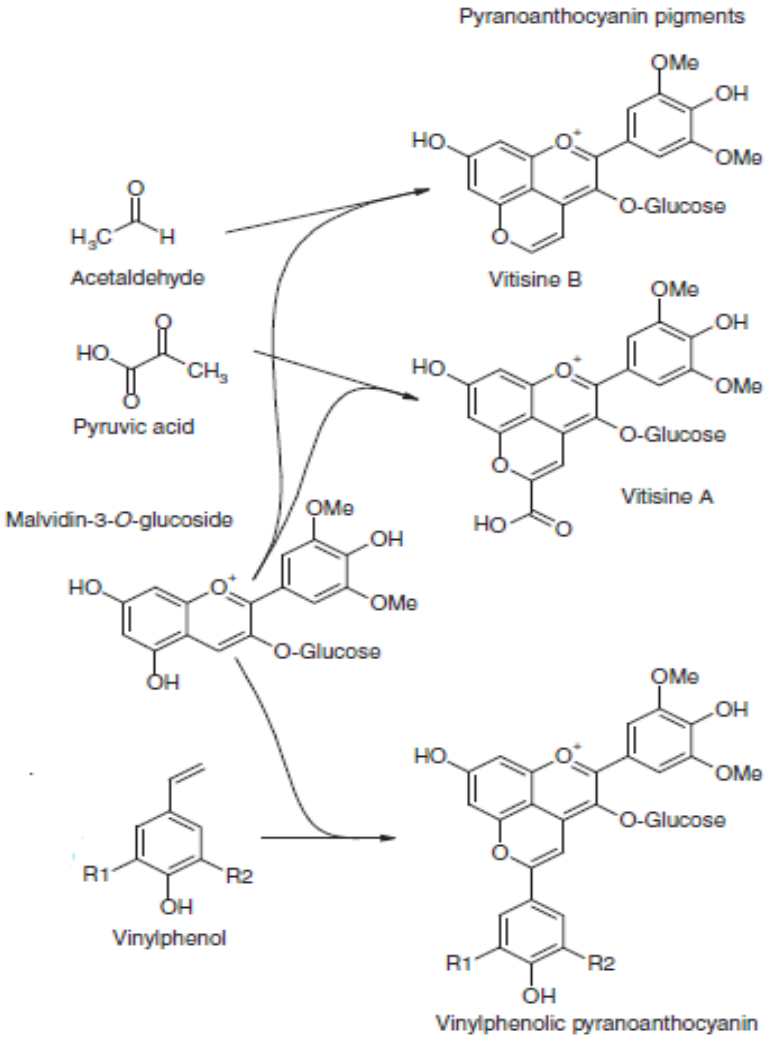


Figure I.20. Formation of pyranoanthocyanins during wine aging (adapted from Benito *et al.*, 2011).

I.3 High pressure in winemaking

In the oenological sector, the use of HHP treatments has already been tested to preserve the quality and sustainability of grape juice and must (Daoudi *et al.*, 2002; Talcott *et al.*, 2003), and also to preserve the wine (Buzrul 2012; Delfini *et al.*, 1995; Mok *et al.*, 2006; Morata *et al.*, 2012; Puig *et al.*, 2003). In 1995, Delfini *et al.*, (1995) demonstrated that microorganisms added to the wine, such as *Leuconostoc oenos*, *Lactobacillus* spp, *Acetobacter*, and *Botrytis cinerea* were killed with pressure treatments of 400 MPa during 2 min at 20 ºC. Puig *et al.*, (2003) investigated the microbiological and biochemical stabilization of wines by use of HHP. Two yeasts (*S. cerevisiae* and *Brettanomyces bruxellensis*), two lactic acid bacteria (LAB), and two acetic acid bacteria

(*A. aceti* and *A. pasteurianus*) (*L. plantarum* and *Oenococcus onei*) were inoculated into a white wine and a red wine with SO_2. HHP treatments (400 – 500 MPa for 5 - 15 min at 4 °C - 20 °C) resulted in almost complete inactivation (6 $\log_{10}$ reduction for yeasts and 8 $\log_{10}$ reduction for bacteria) of the microorganisms. It was also observed that HHP treatments did not affect PPO activity, alcohol level, total and volatile acidity, free and total SO_2, protein stability, malic acid, lactic acid, reducing sugars and pH compared to untreated wines. Sensory evaluation was done with 12 series of tasting using 8 tasters. Tasters detected plastic taste in samples of three series which was due to the plastic container used in HHP treatment, but no other organoleptic differences was found between untreated and HHP treated wines.

Mok *et al.,* (2006) reported the effect of pressure treatments ranging from 100 to 350 MPa until 30 min on microorganism (aerobic bacteria, yeast, and LAB) of wine. They showed that the microbial inactivation increased with the pressure treatment and with time. It was also reported that aerobic bacteria were more susceptible to the HHP treatments than yeast and LAB (Mok *et al.,* 2006). Sensory evaluation done using 10 tasters revealed that there were no differences in the aroma, taste, and overall sensory quality between the HHP treated (350 MPa for 10 min) and untreated samples. However, some studies conducted in muscadine grape juice demonstrated that HHP treatments, depending on pressure and time, may activate some enzymes, such as polyphenoloxidase (PPO), leading to a decrease in antioxidant capacity and anthocyanins content (Del Pozo-Insfran *et al.,* 2007; Talcott *et al.,* 2003). Morata *et al.,* (2012) showed that pressure treatments (100 MPa for 24 h at 25 ºC) are efficient in the control of *Dekkera/Brettanomyces* growth in red wines, although the HHP-treated wines showed a smaller total anthocyanin content (by about 15 %) than the non-HHP-treated controls. Furthermore, more severe high pressure treatments (650 MPa for 1 and 2 h) revealed to change the physicochemical characteristics of red wine, namely the decrease of colour intensity and the content of phenolic compounds. In terms of sensorial properties, the sour and fruity aroma of the wine became weaker after 2 h of pressurization whereas the intensities of several gustatory attributes, including astringency and alcoholic and bitter taste, were enhanced slightly (Tao *et al.,* 2012). This result indicate that HHP can also be potentially used to change the equilibrium of chemical reactions in wine and modify the organoleptic properties of wine rapidly, accelerating the wine aging process (Tao *et al.,* 2012, 2014).

Recently, besides the use for wine conservation, HHP has been also tested to delay haze formation in wine during storage (Tabilo-Munizaga *et al.*, 2014). Pressure treatments of 450 MPa for 3 and 5 min showed to decrease α-helical structure in wine proteins, due to the increase of intermolecular interactions between proteins and other non-protein compounds in the wine matrix. These structural changes improved thermal stability of wine proteins and thus delay haze formation in wine during storage (Tabilo-Munizaga *et al.*, 2014).

The principal limitation of the HHP treatment is the current impossibility to be used in a continuous process. Therefore, the use of HHP for wine conservation is only viable in the final stage of winemaking, replacing the addition of SO_2 before bottling, for a pressure treatment after bottling. The requirement of packaging the wine in a resistant and flexible package before the treatment is expected to be a challenge for product presentation. It should be noted that installation of an HHP equipment in a winery would definitely bring an extra cost. However, the HHP treated wine would be healthier and would most probably attract the consumer attention. Moreover, the application of HHP process in winemaking is still at an early stage of development and the effect on the physical-chemical characteristics of wine is still largely unknown, namely in respect to antioxidant activity, phenolic and volatile compounds composition.

It is important to refer that in the studies about HHP effect in wine, both physico-chemical and sensorial properties of the HHP treated wine have been only analysed after the pressure treatment, without ever having references on these properties during storage. As mentioned above, chemical reactions occur during wine maturation, mainly between phenolic compounds, and it is important to understand what the effect of HHP treatments, together with the absence of SO_2, in the course of these reactions during wine storage.

Furthermore, the beneficial qualities that a moderate consumption of wine have, due essentially of the bioactive properties of the wine phenolic compounds (namely, antioxidant, anticancer, anti-inflammatory, blood sugar-lowering, and cardiovascular beneficial effects) must be ensured in the HHP treated wine for the benefit of consumers.

I.4 Aim of the work

Since, it seems that HHP treatments modify the chemical and sensorial properties of wine, and in order to increase the fundamental knowledge about the effect of HHP on wine, the aim of this PhD thesis is to study the influence of HHP treatments on wine chemical and sensorial characteristics. These studies are still required to further evaluate the feasibility of this technology to preserve or /and aging wine.

To achieve the main objective it will be fulfilled the following specific aims:

-Study the effect of HHP in the physicochemical and sensorial properties of sulphur dioxide-free wines during bottle storage.

- Study the influence of HHP treatments in the sulphur dioxide-free wine volatile composition.

-Study the effect of HHP treatments on the structure and interaction of wine compounds, using wine model solutions.

- Study the impact of HHP treatments on red wine phenolic composition.

The application of high hydrostatic pressure treatments in winemaking for wine preservation, as an alternative to sulphur dioxide, was evaluated in the **Chapter 2**, being Chapters 2.1 and 2.2 related to the effect of HHP in the physicochemical and sensorial properties of sulphur dioxide-free red and white wines, respectively, during bottle storage. In addition, the impact of the pressure treatments in wine volatile composition is presented in the Chapter 2.3.

In **Chapter 3** is reported the effect of HHP treatments on the structure and interaction of wine compounds, such the formation of Maillard reaction-derived volatile compounds (Chapter 3.2) and new phenolic compounds (Chapter 3.1), using wine model solutions.

The application of high hydrostatic pressure treatments in winemaking to treat young wines was evaluated in the **Chapter 4**. For this propose, the effect of a high hydrostatic pressure treatment in the phenolic composition of a red wine was studied (Chapter 4.1), and compared with the effect of different enological practices, such oak barrels, oak chips and micro-oxygenation with oak chips (Chapter 4.2).

Chapter 5 presents a general discussion of all the results obtained, representing not only a summary of the main results obtained as also a global analysis of the different results. Final remarks and recommendations for future research activities are also proposed.

EXPERIMENTAL WORK AND RESULTS

CHAPTER II

Application of high hydrostatic pressure treatments in winemaking as an alternative to sulphur dioxide

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CHAPTER II.1. Effect of high pressure treatments on the physicochemical properties of a sulphur dioxide-free red wine

CHAPTER II.2. Impact of high pressure treatments on the physicochemical properties of a sulphur dioxide-free white wine during bottle storage

CHAPTER II.3. High pressure treatments accelerate changes in the volatile composition of sulphur dioxide-free wine during bottle storage

CHAPTER II.1

Effect of high pressure treatments on the physicochemical properties of a sulphur dioxide-free red wine

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Effect of high pressure treatments on the physicochemical properties of a sulphur dioxide-free red wine

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Keywords: Colour; phenolic compounds; Anthocyanins; sensorial analyses; high pressure

II.1.1 Overview

Sulphur dioxide (SO_2) is probably one of the most versatile and efficient additives used in winemaking due to its antiseptic and antioxidant properties. This compound is also important for minimizing phenolic polymerisation rate and colour loss during wine aging (Karbowiak *et al.*, 2010; Ribéreau-Gayon *et al.*, 2006). However, allergies caused by SO_2 derived compounds, namely the sulphites, are becoming more frequent, causing symptoms such as headaches, nausea, gastric irritation, and breathing difficulties in asthma patients (Vally and Misso, 2012). Consequently, the legislated maximum concentration of SO_2 allowed in wines has been gradually reduced, which is nowadays 150 mg/L for red wines and 200 mg/L for white wines (Regulation (EC) No 607/2009). For this reason, the search of new healthy safe strategies able to replace totally or partially the action of SO_2 in wines has increased in the last years.

The application of HHP in winemaking for substitution of the use of sulphur dioxide is still at a very early stage of development, since knowledge about the effect on physicochemical and sensorial characteristics of the wine during storage is very scarce. The aim of this work was to increase the fundamental knowledge about the effect of HHP on wine, contributing for the evaluation of the feasibility of using HHP for wine long term preservation in the absence of SO_2. This was pursued by studying the physicochemical and sensorial properties of a red wine treated with HHP along its storage in bottles during 12 months. For this purpose, a red wine was produced without the addition of SO2 and was

pressurized at two pressure conditions, 500 and 425 MPa, for 5 min at 20 ºC. A wine with 40 ppm of SO2 and a wine with no preservation treatment were used as controls. Colour, antioxidant activity, total phenolic compounds, and monomeric anthocyanins composition of the wines were evaluated.

II.1.2 Materials and methods

II.1.2.1 Chemicals

Absolute ethanol p.a, tartaric (99%) acid and gallic acid (99%) were purchased from Panreac (Barcelona, Spain), while sodium carbonate anhydrous (99%) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic) (ABTS, 99%) were purchased from Fluka (St. Louis, MO). Potassium persulfate (99%), Folin-Ciocalteu reagente, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 99%), and formic acid (HPLC grade) were purchased from Sigma–Aldrich (Seelze, Germany), while acetonitrile (HPLC grade) was purchased from from LAB-SCAN (Gliwice, Poland).

II.1.2.2 Wine samples and high pressure treatments

Red wine samples without the addition of SO_2 were produced by Dão Sul SA (Carregal do Sal, Portugal) using Touriga Nacional grapes from Dão Appelation, at 2010 and 2011 vintages. After alcoholic fermentation, the wines were transferred to polyethylene bottles of 250 mL, stoppered, and were pressurized during 5 min at 20 ºC at 425 MPa and 500 MPa in a hydrostatic press (Avure Technologies, Model 215L-600, USA), giving origin to the samples named *425 MPa* and *500 MPa*, respectively. Two lots of the same wine were also bottled in the polyethylene bottles, one with the addition of 40 ppm of SO_2, the typical amount used in the wine industry (sample named as *SO_2*), and another with neither addition of SO_2 nor submitted to any high pressure treatment (sample named as *Untreated*). All wine samples were stored at 80% relative humidity, in the absence of light at a temperature ranging between 10 ºC and 15 ºC. The wines were analysed in triplicate along storage in the bottles.

II.1.2.3 Spectrophotometric determinations

Colour

The measurement of the wine colour was carried out using the CIELab space. The absorption spectra were recorded using a PerkinElmer Instruments Lambda 35 spectrophotometer and a 1 mm optical path glass cell. The whole visible spectrum (380-780 nm) was recorded and Illuminant D65 and 10° Observer were used in the calculations. The CIELab parameters were determined using the original PerkinElmer UV WinLab® Software according to regulations by the International Commission on Illumination: red/green colour (a^*) and yellow/blue colour (b^*) components, and luminosity (L^*). The parameters correlated with the colour perception, namely the polar coordinates chroma (C^*_{ab}) and hue angle (h_{ab}) were determined according to the equations $C^*_{ab} = (a^{*2} + b^{*2})^{0.5}$ and $h_{ab} = \tan^{-1}(b^* / a^*)$. Colour differences ($\Delta E^*$) between wines were calculated from the equation $\Delta E^* = (\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2})^{0.5}$, where $\Delta L^* = L^*_{(sample)} - L^*_{(ref)}$, $\Delta C^* = C^*_{ab(sample)} - C^*_{ab(ref)}$ and ΔH^* (hue difference) = $2 \sin(\Delta h_{ab}/2)(C^*_{ab(ref)} \times C^*_{ab(sample)})$, with $\Delta h_{ab} = h_{ab(sample)} - h_{ab(ref)}$ (hue angle difference) (Berké and de Freitas, 2007).

Total phenolic content and antioxidant activity

The total phenolic (TP) content of the samples was determined by the Folin–Ciocalteu method, as described by Singleton (1985). The samples were appropriately diluted in a solution of 10% ethanol. The calibration curve was performed using gallic acid as standard in a concentration range between 50 and 500 mg/L. The results were expressed as gallic acid equivalents.

The antioxidant activity (AOA) was determined by the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid (ABTS) method (Pellegrini *et al.,* 2000). The samples were appropriately diluted in a solution of 10% ethanol. The calibration curve was performed using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard in a concentration range between 50 and 400 µM. The results were expressed as Trolox equivalent antioxidant capacity (TEAC).

Total proanthocyanidins content

Total proanthocyanidins were estimated according to Chira *et al.,* (2011). This method is based on the Bate-Smith reaction, in which the proanthocyanidins in acid medium release

anthocyanidins by heating. The wines were diluted to 1/50 in a *10% ethanol solution*. One mL of the samples was added to 0.5 mL of water and 1.5 mL of 12 M HCl and the mixture was homogeneized. Two tubes for each sample were prepared: one was heated for 30 min in boiled water (sample A), while the other was maintained at room temperature (sample B). To each tube, 0.25 mL of 95% ethanol were added. The absorbance at 550 nm was read under 10 mm optical path. The content in proanthocyanidins was determined using a calibration curve obtained with a mixture of procyanidin oligomers as standard, obtained according to de Freitas *et al.*, (1998).

Anthocyanins content

The anthocyanin content of samples was estimated using the pH shift method described by Burns *et al.*, (2000). Two test tubes were set up, each containing 1 mL of wine and 1 mL of 0.1% HCl prepared in 95% ethanol. Ten mL of 2% HCl (pH 0.6) were added to one tube and 10 mL of phosphate/citrate buffer pH 3.5 were added to the other tube. Absorbance was read at 700 nm to allow for correction of the haze and then at 520 nm for anthocyanin determination. Anthocyanins were quantified as malvidin-3-glucoside equivalents, the major anthocyanin in red wine, using the extinction coefficient of ε = 28000 $Lm^{-1}mol^{-1}$. At pH <1 anthocyanins are in their red flavylium form, allowing the determination of the total anthocyanins. At pH 3.5 the flavylium form of the anthocyanin is primarily in equilibrium with the colourless carbinol. Therefore, at this pH the absorbance observed is due to polymeric anthocyanins. The free anthocyanin content is estimated by the difference in absorbance between pH <1 and pH 3.5.

All samples were analysed in triplicate for the estimation of TP, AOA, proanthocyanidins, and anthocyanins content.

II.1.2.4 Analysis of monomeric anthocyanins by HPLC

The monomeric anthocyanins (MA) present in the wine samples were analysed by HPLC using the method described by (Gonçalves *et al.*, 2012). 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 was used. 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. The eluents were: (A) 40% formic acid, (B) 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 at 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.

Anthocyanins were identified according to their UV-Vis spectrum (Dallas and Laureano, 1994). The chromatographic peaks of all anthocyanins were identified by comparing their retention times with the retention time of the respective standard. The quantification of the monomeric anthocyanins was done based on 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 the standard used. All analyses were done in triplicate.

II.1.2.5 Phenolic extraction of the deposits present in wine bottles

In order to recover the phenolic compounds present in the deposits and covering the walls of each bottle, an acidic methanol extraction was done on the 2011 vintage wine samples after 6 months of storage. Firstly, the wine samples were decanted, and to the remaining insoluble material 20 mL of methanol/formic acid (99:1, v/v) solution were added and the mixture was left under constant stirring during 24h. The methanolic solution was then centrifuged (3500 *g* for 5 min) to remove the insoluble material. The phenolic extracts were analysed by spectrophotometry to determine the total phenolic, proanthocyanidin, and anthocyanidin contents.

II.1.2.6 Sensorial analysis

A blind tasting test was done to the wine after 9 months of bottling by 7 expert panellists of the wine producer (5 men and 2 women). Wines (30 mL) were presented in transparent glasses coded with a three-digit random code and distributed in a completely randomized order. In each session a descriptive analysis of each wine was conducted.

All tasters were informed that the wines had different treatments, but the panellists did not have any details of the experimental design. Each panellist was presented with the four samples: *untreated*, *SO_2*, *425 MPa* and, *500 MPa*. Wines were evaluated on a predefined

score sheet (scale from 0 to 5) that included 17 descriptors in three categories: colour (limpidity, red colour, violet colour, and brown colour), aroma attributes (balance, fruity, floral, cooked fruit, spices, leather, and metallic) and taste attributes (body, astringency, bitterness, acidity, balance, and persistence). The score sheet was drawn up by the project team in accordance with the objectives of the trials. Also a global evaluation was done on a scale from 0 to 20 in 4 categories: colour, aroma, taste and global attributes. Averages of the scores for each descriptor were calculated.

II.1.2.7 Statistical analysis

Statistical data analysis was performed by Analysis of Variance (ANOVA). Tukey's HSD Test was used as comparison test when significant differences were observed by ANOVA ($p<0.05$).

Principal components analysis (PCA) was conducted in order to extract the main sources of variability and thus grouping the wine samples. PCA was performed on the normalized values of the following parameters: a^*, b^* and L^*, antioxidant activity, and total phenolic content for the pressurized and unpressurized wines at the beginning and after 12 months of storage.

II.1.3. Results and discussion

II.1.3.1 Physicochemical analysis of the 2010 vintage wine

Colour

The CIELab (L^*, a^*, and b^*) parameters calculated for the four wine samples of 2010 vintage (untreated, with 40 ppm of SO_2, and pressurized at 425MPa and 500 MPa) during 12 months are shown in Figure. II.1.1. An increase in a^*, b^*, and L^* parameters was observed for the wines along storage, indicating a change in the wine colour, shifting to more red, yellow, and transparent, respectively. This change was also verified through the increment in the Chroma (C^*_{ab}) and hue (h_{ab}) values. These results are in agreement with other reports that observed that during aging, red wine acquires a more orange and clear colour, corresponding to increments in b^*, L^* and h_{ab} (Boido *et al.,* 2006; Garcia-falcon *et al.,* 2007; Gutierrez *et al.,* 2005). The colour change during wine ageing is mainly due to the reaction of the anthocyanins, with formation of polymeric compounds. These compounds

had different physicochemical features that could stabilize wine colour changing it from the red-purple of young wines to the more orange-red hue of aged wines (He *et al.*, 2006; Mateus and de Freitas, 2001).

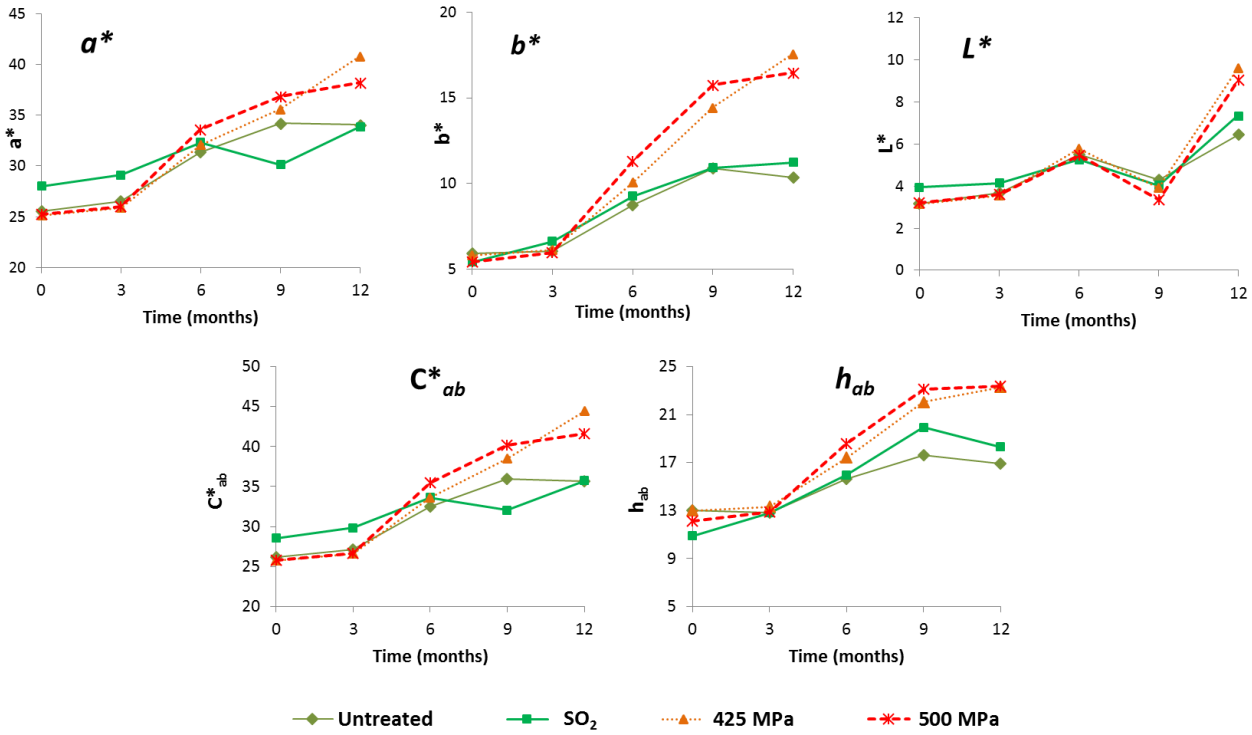

Figure. II.1.1. Evolution of CIELab parameters (*a**, *b**, *L**, *C**$_{ab}$ and h_{ab}) of wine simples, 2010 vintage, during 12 months of aging in bottle.

The pressurized samples, at 12 months of storage, presented higher values of CIELab parameters, especially *b**, when compared with the unpressurized wines (p<0.05). This difference was statistically different after 9 months of storage for *b**, while for *a** and *L** the pressurized samples presented a higher increase at 12 months, leading to more pronounced orange-red hue in the samples. As consequence, an increment of the *C**$_{ab}$ and h_{ab} values in these samples was also observed (p<0.05) at 12 months of storage.

In order to establish whether the observed changes in the chromatic parameters were visually relevant, the colour differences (ΔE*) between pressurized and unpressurized samples were calculated. This parameter has been suggested to estimate in CIELab units how samples are different: ΔE* values higher than 3 CIELab units indicate that the differences can be perceived by the human eye (Martínez *et al.*, 2001). Until nine months of

storage, the ΔE* values for the wines pressurized at 425 MPa and 500 MPa were lower than 3 CIELab units (data not shown), but for nine months of storage the ΔE* values were 4 and 6 respectively, when compared with the unpressurized wines. At the end of 12 months of storage, the ΔE* values increased to 10 and 8, respectively. These results show that although in the early stages after pressurization, the difference in colour of the pressurized wines in relation to the unpressurized wines is not perceived by the human eye. However, after nine months of storage, a difference is noticed, increasing along the storage time.

Total Phenolic Compounds and Antioxidant Activity

The total phenolics (TP) content and the antioxidant activity (AOA) of the wine samples during bottle storage are shown in Figure II.2.2. At the beginning of the storage, the wine samples did not show any statistical differences on TP and AOA. However, along the time of storage, the wines showed different evolution of TP and AOA values. In relation to the TP (Fig. II.2.1), the pressurized wines presented a slight decrease (9%) of TP after 9 months of storage (p<0.05).

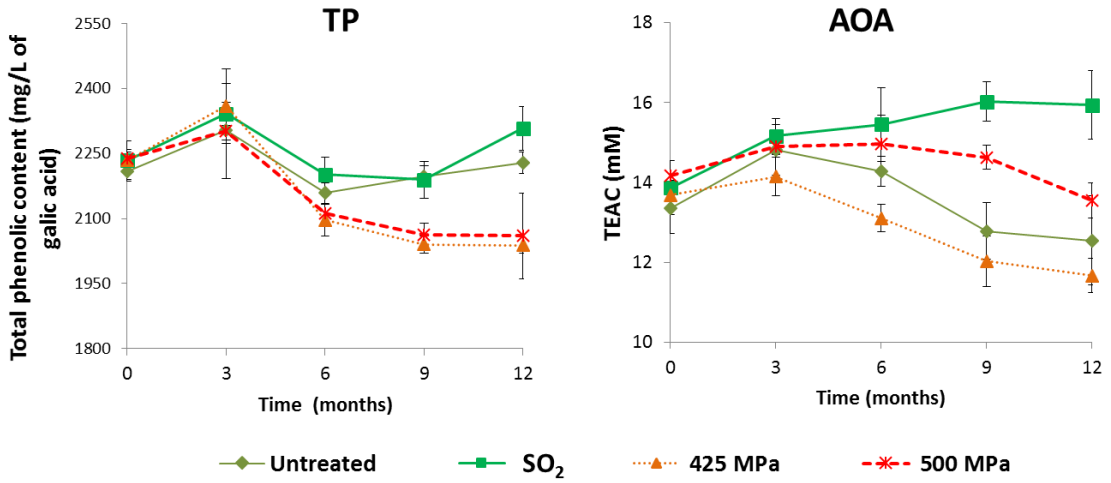

Figure. II.1.2. Evolution of total phenolic compounds content (TP), and antioxidant activity (AOA) of wine simples, 2010 vintage, during 12 months of aging in bottle**.**

Regarding the evolution of wines AOA, the untreated and pressurized samples showed a slight decrease in AOA along storage. After 12 months, the *untreated*, *425 MPa,* and *500 MPa* treated wine samples had, respectively, 21%, 27%, and 15% lower AOA than the wine with SO_2 (Fig. II.2.2). The wine with SO_2 exhibited at the end of 12 months of storage, 15% higher AOA compared to the value at the beginning of storage, which is due to the

antioxidant capacity that the SO_2 provides to the wine (Burin *et al.*, 2010b). During wine aging, different condensation reactions can occur in each wine, leading to different antioxidant activity of the compounds. Therefore, besides the possible occurrence of a higher rate of condensation reactions of phenolic compounds in the pressurized red wine, these reactions should be different to those occurring in untreated wine and wine with SO_2, explaining the different behaviour of the antioxidant activity and total phenolic content observed for these wines during aging.

Anthocyanins Composition

The pronounced orange-red hue and the lower TP and AOA of the pressurized wines points to a higher degree of pigmentation/polymerization of the anthocyanins in these samples (He *et al.*, 2006), when compared with the untreated wine and with the wine with SO_2. This indicates that the pressurized wines should present a higher degree of anthocyanins polymerization, and a lower content of monomeric anthocyanins (MA). To verify this assumption, the wines were analysed after 9 and 12 months of storage for their content in MA. All samples showed from 9 to 12 months of storage, a decrease in the individual anthocyanin content and, consequently, a decrease in total monomeric anthocyanins content (Table.II.1.1). This decrease was mainly due to the high decrease of malvidin 3-glucoside content, the MA present in high content in red wines.

After 9 months of storage, the pressurized wines presented a significant lower total MA content (54 - 68 mg/L) than the unpressurized wines (124 – 138 mg/L). The *425 MPa* wine presented a total MA content 56% and 61% lower than the *untreated* and *SO_2* wines, respectively, while the *500 MPa* wine presented a total MA content 45% and 51% lower. These results are in agreement with those reported by (Morata *et al.*, 2012) which showed that the final anthocyanin content of red wine is affected by HHP treatments. As the lower monomeric anthocyanin content in the pressurized samples was in agreement with the higher CIELab values found, it is possible that the high pressure treatments promote the acceleration of condensation reactions during the wine storage period, involving monomeric anthocyanins and possibly other phenolic compounds. Accordingly, Boido *et al.*, (2006) suggested that the increase in the h_{ab} values along wine aging could be attributed to the decrease of the amount of the monomeric anthocyanins. This is also reinforced by results

obtained by Corrales *et al.,* (2008), who described the formation of vitisin A-type derivative by degradation of cyanidin 3-glucoside after HHP treatment.

The higher content of monomeric anthocyanins of the wine with SO_2 (Table II.1.1) might be due to the reaction of SO_2 with the anthocyanins, as well as with several other constituents, reducing the rate of phenolic compounds polymerization and, consequently, the colour alteration usually observed during wine aging (Santos *et al.,* 2012).

Table I.1.1. Monomeric anthocyanins content of the wine samples, 2010 vintage, after 9 and 12 months of storage.

	Monomeric anthocyanins content (mg/L)[1]							
	9 Months				**12 Months**			
Anthocyanin[2]	**Untreated**	**SO_2**	**425 MPa**	**500 MPa**	**Untreated**	**SO_2**	**425 MPa**	**500 MPa**
Dp3Glc	2.01 ± 0.12^{a}	2.57 ± 0.26^{b}	0.25 ± 0.06^{c}	0.43 ± 0.01^{cd}	0.41 ± 0.10^{cd}	0.62 ± 0.07^{d}	0.32 ± 0.02^{cd}	0.39 ± 0.03^{cd}
Pt3Glc	5.04 ± 0.06^{a}	5.98 ± 0.14^{b}	0.95 ± 0.11^{c}	1.44 ± 0.18^{de}	1.76 ± 0.25^{e}	2.39 ± 0.29^{f}	0.94 ± 0.09^{c}	1.12 ± 0.05^{cd}
Pn3Glc	2.36 ± 0.16^{ab}	2.61 ± 0.12^{b}	0.81 ± 0.07^{c}	1.15 ± 0.13^{cd}	1.56 ± 0.17^{cde}	1.94 ± 0.20^{abd}	0.77 ± 0.42^{c}	1.07 ± 0.61^{c}
Mv3Glc	81.49 ± 1.55^{a}	89.08 ± 1.92^{b}	29.38 ± 1.42^{c}	36.18 ± 2.91^{d}	57.06 ± 7.72^{e}	69.70 ± 3.66^{f}	23.63 ± 0.30^{c}	23.29 ± 0.88^{c}
Dp3AcGlc	5.01 ± 0.35^{a}	5.91 ± 0.17^{a}	10.75 ± 0.29^{b}	12.49 ± 0.81^{b}	3.97 ± 1.13^{a}	5.91 ± 0.75^{a}	10.51 ± 0.34^{b}	11.11 ± 1.48^{b}
Cy3AcGlc	1.34 ± 0.06^{ab}	1.67 ± 0.13^{ab}	2.18 ± 0.61^{ac}	3.02 ± 0.24^{c}	0.85 ± 0.68^{b}	1.51 ± 0.34^{ab}	2.24 ± 0.30^{ac}	2.33 ± 0.24^{ac}
Pt3AcGlc	1.42 ± 0.25^{a}	1.96 ± 0.21^{b}	0.15 ± 0.01^{c}	0.20 ± 0.03^{c}	0.77 ± 0.13^{d}	0.69 ± 0.26^{de}	0.15 ± 0.01^{c}	0.29 ± 0.02^{ce}
Pn3AcGlc	2.84 ± 0.43^{ab}	3.23 ± 0.25^{b}	2.32 ± 0.19^{a}	2.83 ± 0.31^{ab}	0.48 ± 0.10^{c}	2.90 ± 0.54^{ab}	2.04 ± 0.10^{a}	2.16 ± 0.32^{a}
Mv3AcGlc	17.09 ± 0.90^{a}	18.78 ± 0.99^{a}	5.93 ± 0.29^{b}	8.27 ± 0.73^{c}	11.95 ± 0.49^{d}	12.40 ± 0.83^{d}	4.45 ± 0.12^{b}	4.40 ± 0.02^{b}
Pn3CmGlc	0.87 ± 0.05^{a}	1.01 ± 0.05^{a}	0.18 ± 0.02^{bc}	0.28 ± 0.02^{bc}	0.35 ± 0.13^{c}	0.57 ± 0.09^{d}	0.13 ± 0.02^{b}	0.11 ± 0.02^{b}
Mv3CmGlc	4.89 ± 0.36^{a}	5.57 ± 1.58^{a}	1.54 ± 0.12^{b}	2.03 ± 0.10^{c}	3.45 ± 0.40^{c}	4.41 ± 0.51^{a}	1.20 ± 0.14^{b}	1.05 ± 0.17^{b}
Total	124.38 ± 4.30^{a}	138.38 ± 5.86^{b}	54.44 ± 3.19^{c}	68.32 ± 5.48^{d}	82.90 ± 6.26^{e}	103.04 ± 7.53^{f}	46.43 ± 1.86^{c}	47.32 ± 3.84^{c}

[1] All data are expressed as mean value ± standard deviation (n = 3). In the same line, different letters indicate significant differences according to a Tukey test (p<0.05). [2]**Dp3Glc**-Delphinidin-3-glucoside, **Pt3Glc**-Petunidin-3-glucoside, **Pn3Glc**-Peonidin-3-glucoside, **Mv3Glc**-Malvidin-3-glucoside, **Dp3AcGlc**-Delphinidin-3-glucoside-acetaldehyde, **Cy3AcGlc**-Cyanidin-3-glucoside-acetaldehyde**,** **Pt3AcGlc**-Petunidin-3-glucoside-acetaldehyde, **Pn3AcGlc**-Peonidin-3-glucoside-acetaldehyde, **Mv3AcGlc**-Malvidin-3-glucoside-acetaldehyde, **Pn3CmGlc**-Peonidin-3-(6-*p*-coumaroyl)-glucoside, **Mv3CmGlc**-Malvidin-3-(6-*p*-coumaroyl)-glucoside

Principal component analysis

The principal component analysis (PCA) was performed on the normalized values of *a* *, *b** and *L** CIELab values, AOA, and TP for the pressurized and unpressurized wines at the beginning and after 12 months of storage (Figure II.1.3). At the beginning of storage, the PC1 explained 64% of the variation of the data. The scores scatter plot (Figure II.1.3A) showed that along PC1 axis the samples were separated according to the presence (PC1 positive) or absence (PC1 negative) of SO_2. Regarding to the loadings plot (Figure II.1.3B), it can be seen that the variables *a**, *L**, and AOA contributed to the samples located on PC1 positive, while *b** and TP contributed to the samples location in PC1 negative. These results suggest that at the beginning of storage the addition of SO_2 was the main factor to differentiate the wines and, comparatively, the high hydrostatic pressure treatments have a lower impact on the physicochemical parameters of the wine.

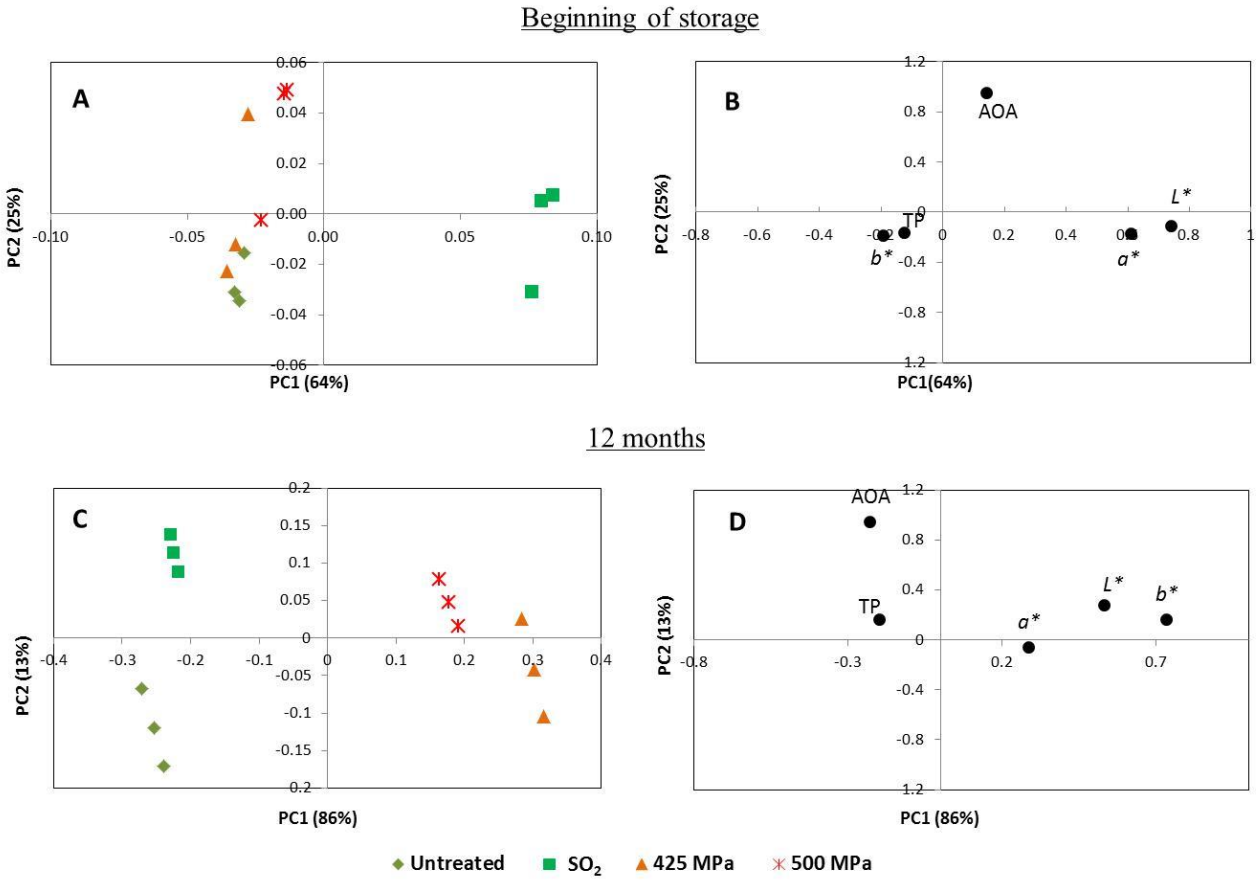

Figure II.1.3. PC1×PC2 scatter plots of the wine samples, 2010 vintage, at the beginning (A- scores; B- loadings) and after 12 months of storage (C- scores; D- loadings) related to *a**, *b**, *L**, AOA, and TP.

After 12 months of storage, the PCA showed a variation of 86% explained by PC1 (Figure II.1.3C), positively related to the CIELab parameters and negatively related to the AOA and TP (Figure II.1.3D). Contrarily to the beginning of storage, after 12 months the wines were separated according to the pressure treatments. The pressurized wine samples were located in PC1 positive, while the unpressurized wines were located in PC1 negative (Figure II.1.3C). Moreover, the treatment with SO_2, that at the beginning of storage was the main source of differentiation, was after 12 months of storage, explained by PC2 (13% variation). Regarding to the loadings plot (Figure II.1.3D), it can be seen that the parameters responsible for the differentiation of the pressurized wines from those unpressurized were the CIELab values, whereas the AOA was the parameter that influenced the distinction between untreated wine (PC2 negative) and wine with SO_2 (PC2 positive). These results confirm that the high pressure treated wines presented alterations in the physicochemical properties only after several months of storage, being colour the principal factor of difference comparing with unpressurized wines. This effect had only been reported for severe high pressure treatments (> 30 min and >600 MPa) that modify immediately the physicochemical properties of the wine (Tao *et al.*, 2012), while possible effects during the wine conservation in bottle were never reported.

II.1.3.2 Physicochemical analysis of the 2011 vintage wine

In order to confirm, in a different vintage, the effect of HHP treatments in red wine, wine samples were produced in 2011 using the same conditions and grapes origin of the 2010 vintage. The wines physicochemical properties were analysed along 6 months of bottle storage being shown in Table II.1.2 As was observed in the 2010 data set, the pressurized samples presented higher b^* values after six months of storage, when compared to the unpressurized wines, leading to the increment of the C^*_{ab} and h_{ab} values, indicating a more orange-red hue colour. The ΔE^* for the *425 MPa* and *500 MPa* wines were 8 and 6, respectively, when compared with the unpressurized wines after 6 months of storage, indicating that the difference of colour between unpressurized and pressurized wine samples of 2011 vintage was also perceptible by the human eye. None of these differences were perceived after 3 months of storage. In terms of AOA and TP, the behaviour was also similar to the 2010 vintage, since the wine samples presented no significant differences ($p<0.05$), at the beginning of storage or after 3 months, but began to show different AOA and TP content

after 6 months. The pressurized wines presented an AOA and TP around 20% and 10% lower, respectively, than the unpressurized wines. These results show that the effect of HHP treatments, namely the increase of the orange-red hue colour, as well as the decrease of the antioxidant activity and total phenolic content in the pressurized wine samples were similar for the two vintages (2010 and 2011) studied.

Table II.1.2. Evolution of the colour, antioxidant activity and total phenols content of the wine samples, 2011 vintage, during 6 months of storage.

Wine Samples		Parameter[1]						
		Colour					AOA[2] (TEAC mM)	TP[3] (mg/L)
		a^*	b^*	L^*	C^*_{ab}	h_{ab}		
Beginning of storage	**Untreated**	42.1	24.2	15.7	48.5	29.9	12.81 ± 0.59^{abc}	1701 ± 11^{a}
	SO_2	44.7	24.1	17.6	50.7	28.4	13.64 ± 0.89^{bc}	1900 ± 30^{b}
	425 MPa	41.4	29.7	15.6	50.9	35.7	13.09 ± 0.23^{abc}	1773 ± 22^{c}
	500 MPa	44.7	31.8	13.4	54.9	35.4	12.69 ± 0.16^{abc}	1675 ± 17^{ad}
3 months	**Untreated**	44.5	27.3	16.9	52.2	31.5	12.70 ± 0.44^{abc}	1852 ± 79^{b}
	SO_2	47.6	29.3	18.9	55.9	31.6	14.22 ± 0.71^{c}	1918 ± 57^{b}
	425 MPa	47.1	32.9	20.3	57.4	35.0	11.35 ± 0.51^{ad}	1633 ± 48^{ad}
	500 MPa	44.8	29.9	17.3	53.9	33.8	11.58 ± 1.12^{a}	1660 ± 11^{d}
6 months	**Untreated**	47.2	30.2	21.2	56.1	32.6	12.00 ± 0.81^{ab}	1705 ± 38^{ad}
	SO_2	49.8	30.8	21.4	58.5	31.7	13.11 ± 0.66^{abc}	1752 ± 21^{a}
	425 MPa	48.3	39.4	21.8	62.3	39.2	9.63 ± 0.58^{d}	1528 ± 18^{e}
	500 MPa	47.1	36.0	19.9	59.3	37.4	9.62 ± 0.49^{d}	1587 ± 57^{e}

[1] *AOA* and *TP* data are expressed as mean value ± standard deviation (n = 3). In the same column, different letters indicate significant differences according to a Tukey test (p<0.05).
[2] *AOA* Antioxidant activity data are expressed as nM Trolox equivalent antioxidant capacity (TEAC).
[3] *TP* Total Phenolic Compounds data are expressed as mg/L acid gallic equivalents.

II.1.3.3 Phenolic characterization of wine and deposits

During storage it was observed in the pressurized wine bottles a lower amount of TP in wine and also a higher formation of deposits when compared with the unpressurized wines. Therefore, the amount of TP, anthocyanins, and proanthocyanidins present in the deposits formed in the wine bottles of 2011 vintage were quantified after 6 months of storage. The anthocyanins and proanthocyanidins content were also quantified in the wine samples. These results are presented in Table II.1.3, expressed as amount (mg) per bottle (250 mL) for better comparison between the amount of phenolic content in the wines and deposits of each bottle.

Table II.1.3. Total phenols, anthocyanins and proanthocyanidins content of the wine and wine deposits samples, 2011 vintage, after 6 months of storage.

		Parameter[1]				
		Total Phenolic Compounds (mg/bottle)[2]	Anthocyanins[3] (mg/bottle)			Total proanthocyanidins (mg/bottle)
			Free	Polymeric	Total	
Wine	**Untreated**	426.26 ± 9.54^{a}	11.28 ± 0.61^{ac}	16.15 ± 1.62^{ab}	27.43 ± 1.23^{a}	397.34 ± 68.55^{a}
	SO_2	438.13 ± 5.27^{a}	21.16 ± 2.17^{b}	18.27 ± 0.41^{b}	39.42 ± 2.19^{b}	372.10 ± 58.89^{a}
	425 MPa	381.93 ± 4.50^{b}	9.61 ± 0.46^{c}	14.79 ± 0.46^{a}	24.40 ± 0.92^{c}	421.50 ± 129.91^{a}
	500 MPa	396.70 ± 13.92^{b}	11.31 ± 0.27^{a}	15.49 ± 1.33^{a}	26.80 ± 1.13^{ac}	380.69 ± 47.89^{a}
Wine deposit	**Untreated**	3.02 ± 0.43^{A}	0.16 ± 0.01^{A}	0.84 ± 0.01^{A}	1.00 ± 0.01^{A}	9.17 ± 0.84^{A}
	SO_2	0.05 ±0.01^{B}	0.01 ± 0.01^{B}	0.04 ± 0.01^{B}	0.05 ± 0.01^{B}	0.61 ± 0.01^{B}
	425 MPa	10.81 ± 0.93^{C}	0.24 ± 0.01^{C}	0.83 ± 0.02^{A}	1.07 ± 0.01^{A}	31.40 ± 1.47^{C}
	500 MPa	38.90 ± 3.38^{D}	0.25 ± 0.01^{C}	0.92 ± 0.01^{C}	1.18 ± 0.02^{C}	95.21 ± 4.00^{D}

[1] All data are expressed as mean value ± standard deviation (n = 3). In the same column, different letters (non-capitals for wine and capitals for bottle deposit) indicate significant differences according to a Tukey test (p<0.05).
[2] Anthocyanins are data expressed as mg of malvidin 3-glucoside equivalents.
[3]Total Phenolic Compounds data are expressed as mg of acid galic equivalents.

The pressurized samples presented deposits with higher TP content, 4-fold and 13-fold higher content in the 425 MPa and 500 MPa samples, respectively, when comparing with the untreated sample. These results are in accordance with the lower TP content present in the pressurized wines (around 10%). In terms of total anthocyanins content, the wines

without SO_2 (*untreated*, *425 MPa*, and *500 MPa*) presented the lower amount in wine (mainly *425 MPa* sample), but the higher amount in the deposits (mainly *500 MPa* sample). The deposit of the wine with SO_2 presented a very low amount of phenolic compounds when compared with the pressurized and untreated sample, pointing again to a lower rate of phenolic polymerization and precipitation due to the presence of SO_2. All wine samples presented a similar proanthocyanidins content in wine (p<0.05). However, considering that the amount of proanthocyanidins in the deposits were 3 and 10-fold higher for the samples pressurized at 425 MPa and 500 MPa, respectively, when compared with the untreated one (Table II.1.3), it seems that the pressure treatments accelerated the polymerization of the wine phenolic compounds, forming compounds with higher degree of polymerization that became insoluble in wine along the storage time. These results are in accordance with the transformations reported to occur to anthocyanins during wine ageing (de Freitas and Mateus, 2011; González-Manzano *et al.*, 2008; Monagas *et al.*, 2006). Therefore, the alterations that occur in the pressurized red wine characteristics, such more orange-red colour, the lower antioxidant activity, total phenolic content, and anthocyanins content, may be due to the increase of condensation reactions of phenolic compounds, resulting in compounds with higher polymerization degree, namely anthocyanins and proanthocyanidins, along the wine aging.

II.1.2.4 Sensorial properties

The sensorial properties of the wines of 2010 vintage were analysed after 9 months of storage in order to assess the organoleptic characteristics of the pressurized wines in terms of aroma, colour and taste. The results of the average scores of the panellists are displayed in Figure II.1.4.

The wines pressurized at 425 MPa presented a very similar aroma compared to the wines with SO_2 (Figure II.1.4A). However, the wine pressurized at 500 MPa presented more scents of cooked fruit and spices aroma. The untreated wines presented less perceived fruity and floral aroma and had a more pronounced metallic and leather aroma than the other wines. Comparing the taste assessment of the different wine samples, the pressurized wines presented a similar taste assessment than the wine with SO_2 (Figure II.1.4B). Untreated wines showed a higher acidity and lower balance. In terms of colour (Figure II.1.4C), the pressurized wines presented higher values of brown and limpidity and lower values of violet

than unpressurized wines. These results are in agreement with the colour analysis by CIELab parameters, since the pressurized wines presented higher values of b^*, L^*, and h_{ab}. Therefore, the pressurized wines showed, after 9 months of storage, a similar global assessment when compared with the wines with SO_2, but a better global assessment than the untreated wine (Figure II.1.4D).

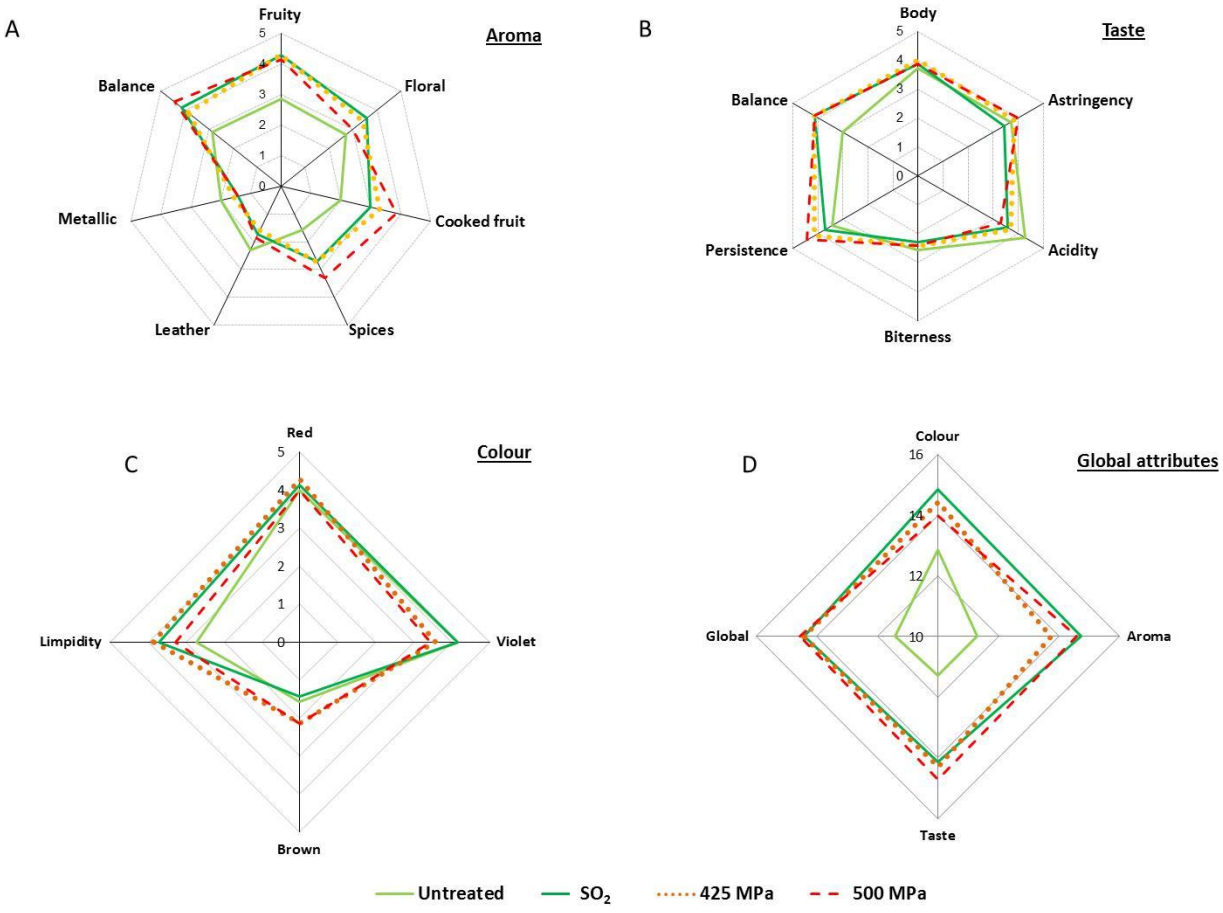

Figure II.1.4. Descriptive sensory analysis of the aroma (A), taste (B), colour (C), and global attributes (D) of wines, 2010 vintage, after 9 months of storage.

The high pressure treatments seems to alter significantly the colour, aroma, and taste of the wine after several months of storage. Although no differences were observed in the aroma, taste, mouth-feel, and overall sensorial quality between HHP treated and untreated wine samples, immediately after the HHP treatment (Mok *et al.*, 2006).

II.1.4.Concluding remarks

This work demonstrates that high pressure treatments with processing time of 5 min and pressures between 400 and 500 MPa can influence red wine physicochemical and sensorial characteristics. However, the effects are only perceptible after, at least, 6 months of storage.

The alterations that occur on the pressurized red wine characteristics, such as the more orange-red colour and the lower antioxidant activity, total phenolic content, and anthocyanins content, are due to an increase of condensation reactions of phenolic compounds. These changes lead to aged wine-like characteristics and do not affect negatively the global sensorial appreciation of wines.

This is the first report where physicochemical and sensorial characteristics of pressurized wines of two consecutive vintages were evaluated during one year of bottle aging. The data presented showed that the use of HHP to pasteurize wine needs to be applied with care to minimize the impact on long term wine quality. More studies concerning the chemical reactions promoted by HHP treatments in wine are needed to identify the optimal conditions to preserve wine by this new technology.

CHAPTER II.2

Impact of high pressure treatments on the physicochemical properties of a sulphur dioxide-free white wine during bottle storage: Evidence of Maillard reaction acceleration

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Impact of high pressure treatments on the physicochemical properties of a sulphur dioxide-free white wine during bottle storage: Evidence for Maillard reaction acceleration

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Keywords: Colour; antioxidant activity; Maillard reaction; sensorial analyses; high pressure; amino acids

II.2.1 Overview

As reported in chapter II.1, pressure treatments of 400-500 MPa for 5 min have shown to influence long term red wine physicochemical and sensorial characteristics, namely more orange-red colour, and lower antioxidant activity, total phenolic content, and anthocyanins content due to an increase of condensation reactions of phenolic compounds along the wine aging. These changes led to aged wine-like characteristics, reflected not only on wines physicochemical properties, but also on sensorial appreciation of wines. In order to use high hydrostatic pressure to preserve also white wine more studies are needed, namely the effects of HHP on the white wines physicochemical properties during storage. Therefore, the aim of this work was to study the effects of HHP treatments on colour, antioxidant activity and total phenolic compounds of a sulphur dioxide-free white wine during one year of bottle storage. For this purpose, a white wine was produced without the addition of SO_2 and was pressurized at two pressure conditions, 500 and 425 MPa, for 5 min at 20 ºC. A wine with 40 ppm of SO_2 and a wine with no preservation treatment were used as controls.

II.2.2 Materials and methods

II.2.2.1 Chemicals

Absolute ethanol p.a, tartaric (99%) acid and gallic acid (99%) were purchased from Panreac (Barcelona, Spain) Sodium carbonate anhydrous (99%) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic) (ABTS, 99%) were purchased from Fluka (St. Louis, MO). Potassium persulfate (99%), Folin-Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 99%), ethyl acetate, isobutanol, 3,5-di-tert-butyl-4-hydroxytoluene (BHT) of gas chromatography (GC) grade quality, sodium chloride (99.5%, foodstuff grade), heptafluorobutyric anhydride of derivatization grade quality, and also the *L*-amino acids standards (alanine, glycine, valine, threonine, serine, leucine, isoleucine, proline, aspartic acid, phenylalanine, glutamic acid, lysine, tyrosine, and arginine) and the internal standard (*L*-norleucine) were purchased from Sigma (Seelze, Germany).

II.2.2.2 Wine samples and high pressure treatments

Wine samples without the addition of SO_2 were produced by Dão Sul SA (Carregal do Sal, Portugal) using Encruzado white grape variety from Dão Appellation from 2010 harvest. An industrial batch fermenter of 200 L was used. After alcoholic fermentation, the wine was transferred to 250 mL polyethylene bottles, stoppered, and were pressurized during 5 min at 20 °C at 425 MPa or 500 MPa, conditions that assured microbiologically safe wines (Buzrul *et al.*, 2004), in a hydrostatic press from Avure Technologies (Model 215L-600, USA), giving origin to the samples 425 MPa and 500 MPa, respectively. Pressurizing water was used at a controlled temperature of 15 °C. Pressure build-up took place at a compression rate of about 300 MPa/min (adiabatic heating caused an increased in temperature of about 4.0 °C), while decompression was nearly instantaneous. Two lots of the same wine were also bottled in the polyethylene bottles, one with an addition of 40 ppm of SO_2, the typical amount used in the wine industry (sample named as SO_2), and other with no addition of SO_2 nor submitted to any high pressure treatment (untreated). As polyethylene bottles can have a little impact on the sensorial properties of the white wine (Ghidossi *et al.*, 2012), all wine samples (pressurized and unpressurized) were bottled in polyethylene bottles. The oenological parameters of the wines at the beginning of storage are shown in Table II.2.1. All the oenological parameters were determined by the methods described by the Office

International de la Vigne et du Vin (1990). All wines were stored at 80% relative humidity in the absence of light at a temperature ranging between 10 °C

Table II.2.1. Oenological parameters of the wine samples at the beginning of bottle storage.

	Wine samples			
	Untreated	***SO_2***	***425 MPa***	***500 MPa***
Alcohol level (% v/v)	13.21	13.15	13.18	12.99
Volatile acidity (g/L, expressed as acetic acid)	0.38	0.39	0.39	0.40
Total acidity (g/L, expressed as tartaric acid)	6.49	6.55	6.52	6.55
pH	3.41	3.37	3.43	3.38
Reducing sugars (g/L)	1.37	2.02	1.88	1.79

II.2.2.3 Physicochemical analysis of wine

Colour

The measurement of the wine colour was carried out using the CIELab space. The absorption spectra were recorded using a PerkinElmer Instruments Lambda 35 spectrophotometer (USA) and a 10 mm optical path glass cell. The whole visible spectrum (380-780 nm) was recorded and Illuminant D65 and 10° Observer were used for the calculations. The CIELab parameters were determined using the original PerkinElmer UV WinLab® Software according to regulations by the International commission on Illumination: red/green colour (a^*) and yellow/blue colour (b^*) components, and luminosity (L^*). The parameters correlated with the colour perception, namely the polar coordinates chroma (C^*_{ab}) and hue angle (h_{ab}) were determined according to the equations $C^*_{ab} = (a^{*2} + b^{*2})^{0.5}$ and $h_{ab} = \tan^{-1}(b^*/a^*)$. Colour differences ($\Delta E^*$) between wines were calculated from the equation $\Delta E^* = (\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2})^{0.5}$, where $\Delta L^* = L^*_{(sample)} - L^*_{(ref)}$, $\Delta C^* = C^*_{ab(sample)} - C^*_{ab(ref)}$ and ΔH^* (hue difference) = $2 \sin(\Delta h_{ab}/2)(C^*_{ab(ref)} \times C^*_{ab(sample)})$, with $\Delta h_{ab} = h_{ab(sample)} - h_{ab(ref)}$ (hue angle difference) (Berké and de Freitas, 2007).

Total phenolic content and antioxidant activity

The total phenolic (TP) content of the samples was determined by the Folin–Ciocalteu method as described by Singleton (1985). The samples were a diluted 1:2 in a solution of 10% ethanol. The calibration curve was performed using gallic acid as

standard in a concentration range between 50 and 500 mg/L. The results were expressed as gallic acid equivalents.

The antioxidant activity (AOA) was determined by the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) method (Pellegrini *et al.,* 2000). The samples were diluted 1:5 in a solution of 10% ethanol. The calibration curve was performed using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard in a concentration range between 50 and 400 µM. The results were expressed as Trolox equivalent antioxidant capacity (TEAC). All samples were analysed in triplicate for TP and AOA methods.

II.2.2.4 Free amino acids analysis

Amino acids analysis was carried out by derivatisation and subsequent separation and analysis by gas chromatography according to the methodology described by (Coimbra *et al.,* 2011).

Two milliliters of each wine sample were added to test tubes. The wine samples were dealcoholized under vacuum using a centrifugal evaporator. After, 500 µL of the internal standard solution (L-norleucine 5.0 mM in HCl 0.1 M) were added and the tubes content was evaporated to dryness under vacuum. The resulting material was dissolved in 1 mL HCl 0.1 M, filtered with 0.45 µm filters and dried under vacuum using a centrifugal evaporator. 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 on 4 Å molecular sieves. The mixture was heated to 120 ºC for 10 min using test tubes with a screw cap with PTFE. After shaking in a vortex, the tubes were 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 0.2 mg/mL BHT prepared in ethyl acetate was added and the solvent was removed under vacuum. 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.

The 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, USA) 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 14 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 forms. As such, those amino acids were quantified together as Asx and Glx, respectively. Also, the methodology used did not allow the detection of histidine. The limit of quantification of the analysed amino acids was determined to be ten times the value of the residual signal peaks.

II.2.2.5 Furans composition analysis

The furans composition of the white wine samples was analysed by headspace solid phase microextraction (HS-SPME) combined with comprehensive two-dimensional gas chromatography with time of flight mass spectrometry (GC × GC–ToFMS).

The SPME holder for manual sampling and fibre were purchased from Supelco (Aldrich, USA). The SPME device included a fused silica fibre coating partially cross-linked with 50/30 µm divinylbenzene/carboxen/ polydimethylsiloxane coating (DVB/CAR/PDMS). Prior to use, the SPME fibre was conditioned at 270 ºC for 60 min in the GC injector, according to the manufacturer's recommendations. Then, the fibre was daily conditioned for 10 min at 250 ºC. For the HS-SPME assay, aliquots of 3 mL of the sample were placed into a 9 mL glass vial. After the addition of 0.6 g of NaCl at 400 rpm the vial was capped with a PTFE/Silicone Septa (Supelco, USA). The vial was placed in a thermostated bath adjusted to 40.0 ± 0.1 C and stirring (1.5 x 0.5 mm bar), and the SPME fibre was manually inserted into the sample vial headspace for 20 min. Each sample was analysed in triplicate. Blanks, corresponding to the analysis of the coating fibre not submitted to any extraction procedure, were run between sets of three analyses.

The GC × GC-ToFMS methodology was based on a previous study (Perestrelo *et al.*, 2011), with the exception that the modulation time was 5 s. Total ion chromatograms (TIC) were processed using the automated data processing software ChromaTOF®

(LECO) at a signal-to-noise threshold of 6. Contour plots were used to evaluate the separation general quality and for manual peak identification; a signal-to-noise threshold of 100 was used. The ion extraction chromatography (IEC) mode was used to increase the specificity and sensitivity detection of the target analyte. IEC allows the analysis of a global volatile profile by combining the spectral evidence with a target ion selection and retention time, thus minimizing the contribution of co-eluted compounds and increasing the peak area of the targeted compound (Coelho *et al.*, 2009). The ions at *m/z* 118, 110, 95, 81, and 43 were used for furans detection. Two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0, Mainlib and Replib) were used. A mass spectral match factor, the identified compounds showed similarity matches >850, was set to decide whether a peak was correctly identified or not. Furthermore, a manual inspection of the mass spectra was done, combined with the use of additional data, such as the retention index (RI) value, which was determined according to the van den Dool and Kratz RI equation (van Den Dool and Kratz, 1963). For the determination of the RI, a $C_8 - C_{20}$ *n*-alkanes series was used, and these values were compared with values reported in the literature for chromatographic columns equivalents to that used in the first dimension (Ansorena *et al.*, 2000; Cardeal *et al.*, 2008; Engel *et al.*, 2002; Eyres *et al.*, 2005; Fan and Qian, 2006; Leffingwell and Alford, 2005; Perestrelo *et al.*, 2011; Robinson *et al.*, 2011b). The GC × GC area data was used as an approach to estimate the relative content of each component. Reproducibility was expressed as relative standard deviation (RSD).

II.2.2.6 Sensorial analysis

A blind tasting test was done to the wines after 9 months of bottling by 7 expert panellists of the wine producer (5 men and 2 women). Wines (30 mL) were presented in transparent glasses coded with a three-digit random code and distributed in a completely randomized order. In each session a descriptive analysis of each wine was conducted.

All tasters were informed that the wines had different treatments, but the panellists did not have any details of the experimental design. Each panellist was presented with the four samples: *untreated*, *SO_2*, *425 MPa* and, *500 MPa*. Wines were evaluated on a predefined score sheet (scale from 0 to 5) that included 13 descriptors in three categories: colour (limpidity, yellow, green, and brown), aroma attributes (balance, fruity, floral, and cooked fruit) and taste attributes (body, bitterness, acidity, balance, and persistence). The score sheet was drawn up by the project team in accordance with the objectives of the

trials. Also a global evaluation was done on a scale from 0 to 20 in 4 categories: colour, aroma, taste and global attributes. Averages of the scores for each descriptor were calculated.

II.2.2.7 Statistical analysis

Statistical data analysis was performed using Analysis of Variance (ANOVA). Tukey's HSD Test was used for free amino acids data as comparison test when samples analyses gave significantly different after ANOVA (p<0.05).

II.2.3 Results and discussion

II.2.3.1 Physicochemical characteristics

Colour

The CIELab (a^*, b^*, and L^*) parameters calculated for the four white wine samples: without the addition of SO_2 (*Untreated*) with the addition of 40 ppm of SO_2 (*SO_2*), pressurized at 425 MPa for 5 min (*425 MPa*), and pressurized at 500 MPa for 5 min (*500 MPa*) are presented in Figure II.2.1. An increase in the a^* and b^* parameters and a decrease in the L^* parameter (p<0.05) was observed for all wines along storage, indicating a change in the wine colour, shifting to more yellow and red and less luminous, respectively. This change was also verified through the increment in the chroma (C^*_{ab}) and a decrease in the hue (h_{ab}) values. These results are globally in agreement with others reported in the literature which showed that the increase of the C^*_{ab} and decrease of the hab are the characteristic colour changes in white wines during storage, resulting in a change in the colour of the wine from pale yellow to yellow-brown (Recamales *et al.*, 2006).

Although with the same trends, the pressurized samples presented different values of the CIELab parameters, when compared with the unpressurized wines (Figure II.2.1). For a^* and b^* parameters, a higher increase of red (reaching positive a^* values) and yellow colours in the pressurized samples was observed at 6 and 12 months of storage, respectively, leading to the increment of the C^*_{ab} and the decrease of the h_{ab} values in these samples (p<0.05). L^* value is similar for all the wine samples until 3 months of storage, but after 6 months the pressurized wine samples presented a significant lower luminosity when compared with the unpressurized wine samples. Therefore, the

pressurized wine samples presented a more brownish colour when compared to the unpressurized wines.

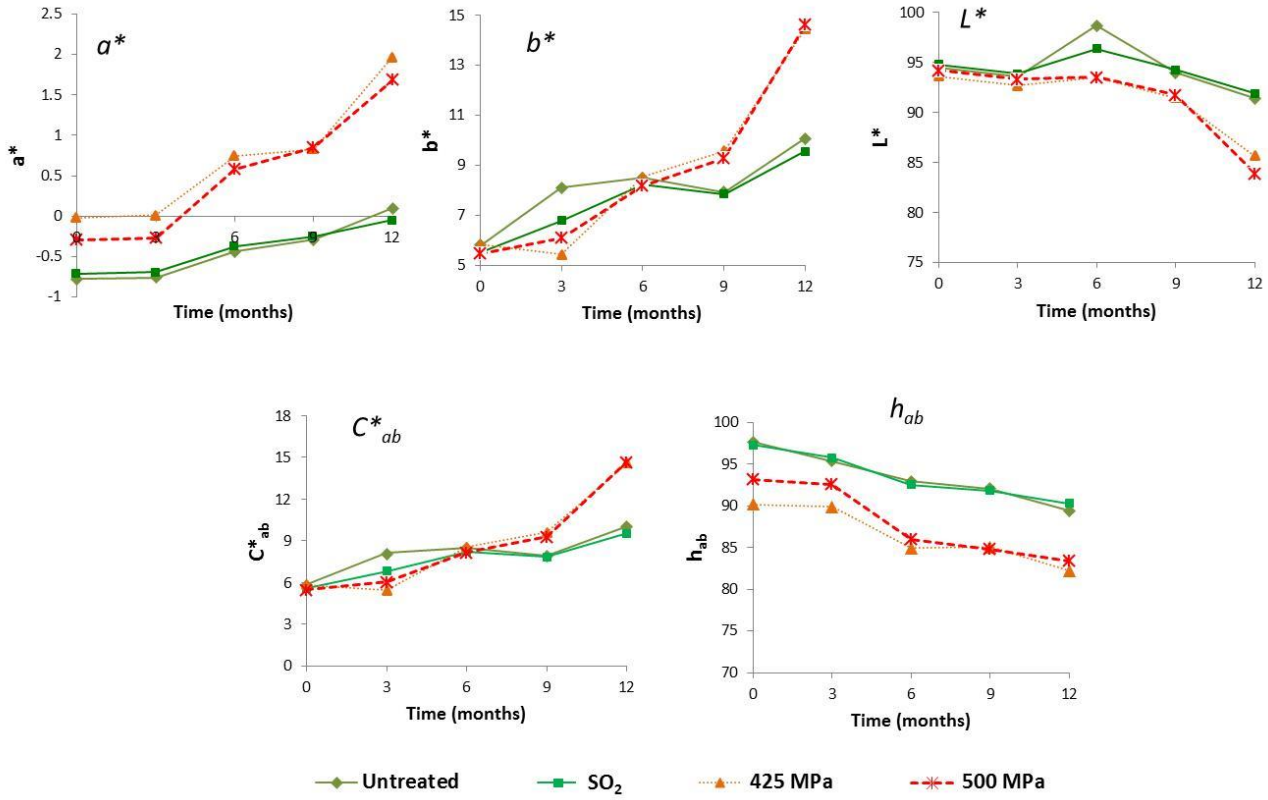

Figure II.2.1. Evolution of CIELab parameters (*a**, *b**, *L**, *C**$_{ab}$ and *h*$_{ab}$) of wine samples, during 12 months of aging in bottle.

In order to establish whether the observed changes in the chromatic parameters were visually relevant, the colour differences (ΔE*) between pressurized and unpressurized samples, were calculated. This parameter has been used to evaluated if two wines are different as when ΔE* values are higher than 3 CIELab units the differences can be perceived by the human eye (Martínez *et al.*, 2001). Until 3 months of storage the ΔE* values were all lower than 3 CIELab units (data not shown). However, after 3 months of storage, the ΔE* values between the unpressurized and pressurized wines are all higher than 3 CIELab units (5.5, 3.5, and 7.5 CIELab units for 6, 9, and 12 months, respectively). Therefore, after 3 months of storage the differences in the CIELab parameters, such the increase of the red colour (higher *a**) and the decrease in the luminosity (lower *L** values) for the pressurized wines, become perceived by the human eye and increase with storage time.

Total phenolic compounds and antioxidant activity

The total phenolic compounds (TP) content and the antioxidant activity (AOA) of the wine samples during bottle storage are presented in Figure II.2.2. The wine samples presented, at the beginning of storage, no TP and AOA differences. However, along the storage time, the pressurized samples showed a slight decrease in TP and AOA at 9 months of storage, presenting at 12 months of storage an TP and AOA 10% and 15% lower (p<0.05), respectively, comparing with the untreated wines (Figure II.2.2). The decrease of the TP and AOA in the pressurized wines after 6 months of storage seems to be related with the higher increase of b^* parameter (Figure II.2.1). The decrease of the total phenolic content and antioxidant activity in the pressurized wines may be associated with the generation of high-reactive radicals during pressurization and the enhancement of chemical oxidation of polyphenols during storage (Clariana *et al.*, 2011).

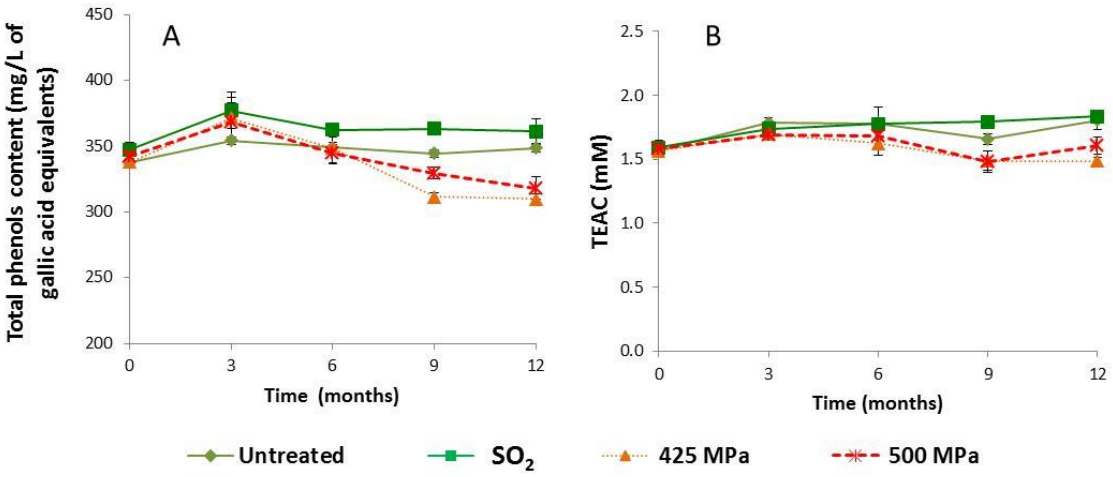

Figure II.2.1. Evolution of the total phenols content (A) and antioxidant activity (B) of the wine samples, during 12 months of aging in bottles.

The increase of yellow colour in white wines is reported to be due to the oxidation of polyphenols forming new compounds with a typical yellow-brown hue (Clark 2008; Recamales *et al.*, 2006). This was also observed in red wines, where chemical reactions among phenolic compounds can be accelerated by HHP, namely phenolic compound polymerization, leading to a decrease of the polyphenol and antioxidant activity (Chapter II.1; Tao *et al.*, 2012). The increase of the a^* parameter in the pressurized wines (Figure

II.2.1), together with ΔE* values higher than 3 CIELab units at 6 months of storage, may not be only due to the possible higher oxidation rate of phenols, and the respective decrease of the TP and AOA in these wines (Figure II.2.2), since this decrease is only significantly different at 12 months of storage. In fact, additionally to the major chemical browning reactions involving wine phenols, the main browning reactions occurring during wine storage are Maillard reaction (Oliveira *et al.,* 2011). The Maillard reaction involves condensation of reducing sugars with amino acids and proteins can occurs in foods during processing and cooking or even during storage (Jaeger *et al.,* 2010; Oliveira *et al.,* 2011), giving rise to different compounds that include reductones, furfurals, and a variety of other cyclic substances (Glomb and Monnier, 1995; Moreno *et al.,* 2003). The results obtained infer the possibility of Maillard reactions acceleration in the pressurized wine samples, however to confirm this hypothesis, at 9 months of storage, the analysis of free amino acids and volatile furans content of all wine samples was performed.

II.2.3.2 Investigation of the occurrence of Maillard reaction

Amino acids analysis

The free amino acid content of the white wines was quantified at 9 months of storage (Table II.2.2). Pressurized wines presented around less 20 and 15% of free amino acids content than the untreated and SO2 samples, respectively ($p<0.05$). The pressurized wine samples presented lower content of serine (81 to 87%), valine (19 to 42%), phenylalanine (25 to 28%), glutamic acid (including glutamine) (20 to 30%), and arginine (9 to 24%), when compared with the two unpressurized wines ($p<0.05$). The pressurized samples did not show significantly different lysine content when compared with the SO_2 sample, but they showed 85% less lysine content than untreated sample.

The lower content of amino acids indicates that reactions involving amino acids occurred to a higher extent in the pressurized wine samples. These results, together with the higher brownish colour presented in the pressurized wines at 9 months of storage, lead to infer a possible effect of HHP treatments in the acceleration of Maillard reactions during the wine storage period. These observations are in accordance with the studies conducted in model systems containing amino acids and sugars that demonstrated that high pressure treatments can accelerate the formation of Amadori rearrangement compounds (Hill *et al.,* 1996; Jaeger *et al.,* 2010; Moreno *et al.,* 2003; Schwarzenbolz *et al.,* 2002).

Table II.2.2. Free amino acid content of the wine samples at 9 months of storage.

Free amino acids content (mg/L)[1]				
Amino acids[2]	***Wine samples***			
	Untreated	***SO_2***	***425 MPa***	***500 MPa***
Ala	14.49 ± 0.12^{a}	13.49 ± 0.38^{b}	12.86 ± 0.59^{bc}	12.13 ± 0.69^{c}
Gly	7.17 ± 0.21^{a}	5.66 ± 0.98^{b}	4.94 ± 0.79^{b}	4.48 ± 0.69^{b}
Val	5.90 ± 0.28^{a}	5.12 ± 0.50^{a}	4.15 ± 0.22^{b}	3.41 ± 0.14^{c}
Thr	2.61 ± 0.53^{a}	1.96 ± 0.36^{ab}	1.55 ± 0.16^{b}	1.32 ± 0.36^{b}
Ser	8.17 ± 1.31^{a}	7.04 ± 1.52^{a}	1.36 ± 0.44^{b}	1.08 ± 0.55^{b}
Leu	6.08 ± 0.59^{a}	8.25 ± 0.96^{b}	10.30 ± 0.99^{b}	8.30 ± 0.95^{b}
Ile	2.06 ± 0.10^{a}	2.75 ± 0.34^{b}	1.74 ± 0.52^{ac}	1.62 ± 0.21^{c}
Pro	44.56 ± 2.72^{a}	43.87 ± 3.60^{a}	34.39 ± 7.15^{a}	35.06 ± 6.12^{a}
Asx	24.36 ± 0.30^{a}	24.06 ± 2.40^{a}	25.10 ± 0.46^{a}	23.80 ± 1.43^{a}
Phe	7.70 ± 0.16^{a}	7.88 ± 0.47^{a}	5.76 ± 0.44^{b}	5.65 ± 0.53^{b}
Glx	30.14 ± 0.64^{a}	29.74 ± 1.43^{a}	23.81 ± 2.81^{b}	21.16 ± 2.20^{b}
Lys	8.58 ± 0.74^{a}	1.50 ± 0.32^{b}	1.39 ± 0.36^{b}	1.33 ± 0.17^{b}
Tyr	1.33 ± 0.09^{a}	1.52 ± 0.18^{ac}	2.32 ± 0.27^{b}	2.15 ± 0.43^{bc}
Arg	1.63 ± 0.10^{a}	1.55 ± 0.16^{a}	$1.41 \pm 0.18a^{b}$	1.22 ± 0.05^{b}
Total content	162.84 ± 2.51^{a}	152.15 ± 4.20^{b}	133.02 ± 11.02^{c}	124.96 ± 4.35^{c}

[1] All data are expressed as mean value ± standard deviation (n = 3). In the same line, different letters indicate significant differences according to a Tukey test (p<0.05).
[2] **Gly**-glycine, **Ala**-alanine, **Ser**-serine, **Pro**-proline, **Val**-valine, **Thr**-threonine, **Leu**-leucine, **Ile**-isoleucine, **Asx**-asparagine + aspartic acid, **Glx**-glutamic acid + glutamine, **Lys**- lysine, **Phe**-phenylalanine, **Tyr**-tyrosine and **Arg**-arginine.

Furans composition analysis

At 9 months of storage it was also investigated the presence of furans in the white wine samples by HS-SPME/GC × GC-ToFMS. Table II.2.3 gives detailed information for each compound, including GC peak area, RSD, and RI experimentally calculated as well as reported in the literature.

Table II.2.3. Furans identified by HS-SPME/GC × GC-ToFMS in the white wines at 9 months of storage.

^1t_R [a] (s)	^2t_R [a] (s)	Furans	$RI_{lit.}$ [b]	RI_{cal} [c]	*Wine samples*			
					Untreated	*SO_2*	*425 MPa*	*500 MPa*
					Peak Area[d] ($x10^5$) and RSD [e] (%)			
110	0.416	Tetrahydro-furan	623	648	26.16 (13)	27.02 (9)	22.07 (11)	17.48 (4)
205	1.808	2-Furanylmethanal (2-furfural)	830	840	20.97 (34)	31.28 (12)	280.13 (1)	166.46 (7)
240	3.816	2-Furanylmethanol (Furfuryl alcohol)	866	867	44.87 (16)	42.02 (24)	41.90 (25)	29.89 (21)
275	1.536	1-(2-Furanyl)-ethanone (2-Acetylfuran)	910	917	3.51 (29)	2.94 (19)	3.54 (4)	3.33 (21)
325	1.504	5-Methylfuran-2-carbaldehyde (5-Methylfurfural)	962	965	-	-	4.16 (26)	6.03 (52)
325	1.536	Methyl furan-2-carboxylate (Methyl 2-furoate)	983	975	1.11 (31)	1.03 (14)	-	-
345	0.568	2-Pentyl-furan	992	990	0.73 (41)	0.49 (34)	0.41 (50)	0.30 (7)
350	1.112	Benzofuran	1006	996	1.26 (15)	1.23 (7)	1.22 (8)	1.27 (5)
365	1.272	1-(2-Furanyl)-propan-1-one (2-Propionylfuran)	1008	1007	0.54 (7)	0.68 (8)	0.85 (11)	0.95 (7)
390	1.288	2-Acetyl-5-methylfuran	1039	1046	-	-	1.12 (6)	1.18 (7)
405	1.224	Ethyl 2-furancarboxylate (Ethyl 2-furoate)	1062	1051	14.69 (18)	15.20 (18)	20.72 (6)	18.94 (11)
485	0.696	2-Heptylfuran	1196	1154	1.88 (11)	2.11 (8)	1.50 (15)	1.82 (10)
		Total (GC Peak Area)			115.72 (20)	114.75 (29)	377.49 (3)	247.22 (7)
		Total (Number of Identified Furans)			10	10	11	11

[a] Retention times for first (^1t_R) and second (^2t_R) dimensions.
[b] RI, Retention Index reported in the literature for HP-5 GC column or equivalents (Ansorena et al., 2000; Cardeal et al., 2008; Engel et al., 2002; Eyres et al., 2005; Fan & Qian,2006; Leffingwell & Alford, 2005; Perestrelo et al., 2011; Robinson et al., 2011a).
[c] RI: retention index obtained through the modulated chromatogram.
[d] Mean of three replicates.
[e] Relative standard deviation, expressed in percentage.

The pressurized wines showed higher content of furans (3 and 2 fold higher for *425 MPa* and *500 MPa*, respectively) than the unpressurized wine samples. The higher content of furans in pressurized wines was mainly due to the higher content of 2-furfural, 10 and 5-fold higher content for *425 MPa* and *500 MPa* samples, respectively. Moreover, 5-methylfurfural and 2-acetyl-5-methylfuran were only detected in the pressurized wine samples.

Furans, namely 2-furfural, are considered Maillard volatile compounds, since they can be formed by the dehydration of sugars through Maillard reaction (Oliveira e Silva *et al.*, 2008). These results reinforce the idea that the HHP treatments accelerated the Maillard reaction, leading to the decrease of amino acids content and the increase of

volatile Maillard compounds formation. The pressurized wines presented a furans composition characteristic of a faster aged/thermally treated wine as the furans have a tendency to increase linearly during wine aging (Perestrelo *et al.*, 2011).

II.2.3.3 Sensorial characteristics

The sensorial properties of the wines were analysed, at 9 months of storage, to assess the organoleptic characteristics of the wine in terms of colour, aroma, and taste. The results of the average scores of the panelists are displayed in Figure II.2.3.

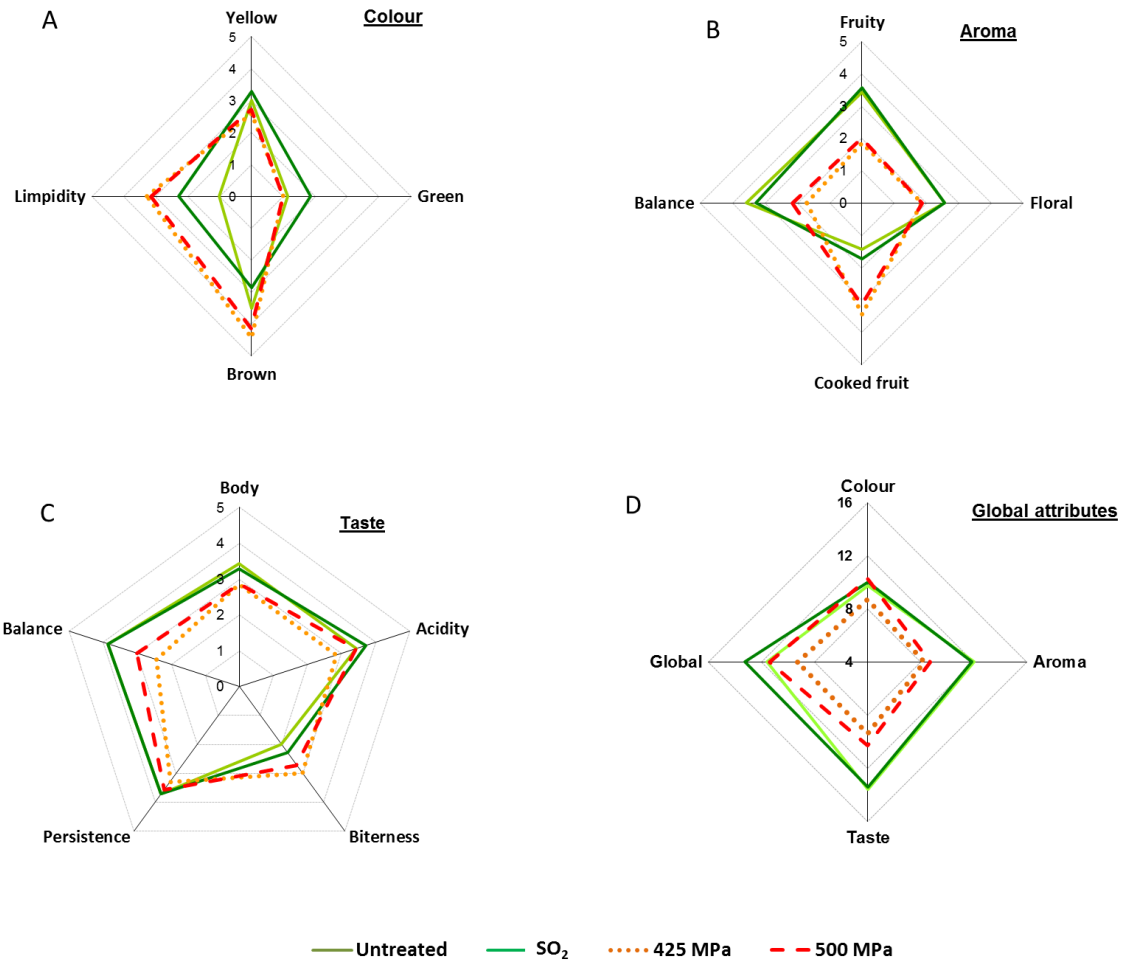

Figure II.2.3. Descriptive sensory analysis of the colour (A), aroma (B), taste (C), and global attributes (D) of wines, at 9 months of storage.

Regarding the colour evaluation (Figure II.2.3A), it can be observed that the pressurized wines presented higher values of brown colour and limpidity, and lower values of green colour than unpressurized ones. These values are in agreement with the CIELab parameters obtained for the pressurized wines, since these wines presented higher

values of *a** and *b**. In terms of aroma (Figure II.2.3B), the pressurized wine samples presented a higher scents of cooked fruit aroma and lower scents of fruity and floral aromas than the unpressurized wines. The higher cooked fruit aroma detected in the pressurized wines could be explained by the higher content of Maillard volatile compounds, namely 2-furfural, in these samples, since these compounds are described to have "roasty" fruit notes (Castro-Vázquez *et al.*, 2011; Jeleń *et al.*, 2011; Pripis-Nicolau *et al.*, 2000). The perception threshold of 2-furfural (around 20 mg/L) is normally higher than the concentrations found in non-sherry wines (Prida and Chatonnet, 2010). However, the sensorial evaluation of pressurized wines allows to infer that the 5 and 10-fold higher content of 2-furfural in the pressurized wines could exceed its perception threshold. Comparing the taste assessment of the different wine samples (Figure II.2.3C), the pressurized wines showed a slightly higher bitterness level and a lower body and balance level than the other wines. No significant differences were observed in terms of taste and aroma between the two pressurized wine samples. The pressurized wines showed at 9 months of storage a lower global assessment, namely in terms of aroma and taste quality, when compared with the unpressurized wines samples (Figure II.2.3D). In general, regarding sensorial analysis, it seems that the physicochemical changes caused by the acceleration of Maillard reactions due to the HHP treatments alter significantly the colour, aroma, and taste of the wine. In a sensorial point of view, the pressurized wines are not considered suitable for commercialization as table white wines due to the higher brownish colour and cooked fruit aroma characteristic of an aged or thermally treated wine (Chaves *et al.*, 2007; López de Lerma *et al.*, 2010).

II.2.4.Concluding remarks

The results obtained in this work demonstrate that high hydrostatic pressure treatment with 5 min of processing time and pressures of 425 and 500 MPa influence the physicochemical characteristics of the wines. These effects are perceptible only after, at least, 6 months of storage, and leading to alterations in the wine sensorial characteristics. The higher brownish colour and cooked fruit aroma, and also the lower free amino acid content and higher furans content in the pressurized wines indicate that the HHP treatments accelerates the Maillard reaction, leading to wine physicochemical and sensorial characteristics of an aged white wine. This work is in agreement with the results obtained for red wine (Chapter II.1) that showed that high pressure treatments (400-500

MPa for 5 minutes) do not alter immediately the wine physicochemical and sensorial properties, but influence/accelerate the wine aging characteristics, leading to alterations in the wine during storage. These aspects should be taken into consideration in the implementation of HHP treatments to wine conservation as an alternative to SO_2.

CHAPTER II.3

High pressure treatments accelerate changes in the volatile composition of sulphur dioxide-free wine during bottle storage

High pressure treatments accelerate changes in the volatile composition of sulphur dioxide-free wine during bottle storage

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KEYWORDS: *Sulphur dioxide-free wines; High pressure; Volatile compounds; Maillard reaction; fatty acids oxidation*

II.3.2 Overview

In chapters II.1 and II.2 it was shown that moderate HHP treatments, 425 and 500 MPa during 5 min, influenced long term sensorial characteristics of red and white wines, respectively, since the pressurized wines presented higher cooked fruit aroma and lower fruity and floral aromas than the unpressurized wines. Furthermore, pressurized white wines presented, after 9 months of storage higher content of furans when compared with the unpressurized wines (chapter II.2). These results demonstrate that the HHP treatments influence the volatile composition of the wines. As the aroma is one of the most important quality parameters of the wine for consumers' acceptance, the aim of this work was to study the effect of high hydrostatic pressure treatments on the volatile composition of the sulphur dioxide free-red and white wines.

In order to obtain a deeper characterization of the chemical groups potentially affected by HHP treatments, comprehensive two-dimensional gas chromatography coupled to mass spectrometry with a high resolution time of flight analyzer (GC×GC-ToFMS) combined with headspace solid-phase microextraction (HS-SPME) was used. This technique is the most suitable gas chromatography technique for untargeted analysis of complex samples, such as wine (Welke *et al.*, 2014). GC×GC-ToFMS offers superior separation capabilities afforded by high peak capacity, selectivity, structural chromatographic peak organization, and sensitivity enhancement in comparison to 1D-GC (Marriott and Shellie, 2002; Rocha *et al.*, 2013). GC×GC has been used in the determination of volatile compounds in different grape and wine varieties, including Cabernet Sauvignon (Robinson *et al.*, 2011a), Fernão-Pires (Rocha *et al.*, 2007), Madeira

(Perestrelo *et al.*, 2011), Pinotage (Weldegergis *et al.*, 2011b), Muscat (Bordiga *et al.*, 2013), and Marsala (Dugo *et al.*, 2014) wines.

II.3.2 Materials and methods

II.3.2.1 Wine samples and high pressure treatments

Wine samples without the addition of SO_2 were produced by Dão Sul SA (Carregal do Sal, Portugal) using Encruzado white grape variety and Touriga Nacional red grape variety from Dão Appellation of 2010 harvest. An industrial batch fermenter of 200 L was used. After alcoholic fermentation, the wines were transferred to 250 mL polyethylene bottles, stoppered, and pressurized during 5 min at 20 °C at 425 MPa or 500 MPa, conditions that assured microbiologically safe wines (Buzrul 2012), in a hydrostatic press from Avure Technologies (Model 215L-600, USA), giving origin to samples *425 MPa* and *500 MPa*, respectively. Pressurizing water was used at a controlled temperature of 15 °C. Pressure build-up took place at a compression rate of about 300 MPa/min (adiabatic heating caused an increased in temperature of about 4.0 °C), while decompression was nearly instantaneous. Two lots of the same wines were also bottled in the polyethylene bottles, one with an addition of 40 ppm of SO_2, the typical amount used in the wine industry (sample named as *SO_2*), and other with no addition of SO_2 nor submitted to any high pressure treatment (*untreated*). As polyethylene bottles can have a little impact on the sensorial properties of the white wine (Ghidossi et al. 2012), all wine samples (pressurized and unpressurized) were bottled in polyethylene bottles. All the oenological parameters were determined by the methods described by the Office International de la Vigne et du Vin (1990). Therefore, the oenological parameters of the wines at the beginning of storage was not altered by the pressure treatments (Table II.3.1). All wines were stored at 80% relative humidity in the absence of light at a temperature around 10 °C.

Table II.3.1. Oenological parameters of the wine samples at the beginning of bottle storage.

	White wines				*Red wines*			
	Untreated	***SO_2***	***425 MPa***	***500 MPa***	***Untreated***	***SO_2***	***425 MPa***	***500 MPa***
Alcohol level (% v/v)	13.21	13.15	13.18	12.99	14.41	14.43	14.38	14.34
Volatile acidity (g/L, expressed as acetic acid)	0.38	0.39	0.39	0.40	0.48	0.60	0.48	0.48
Total acidity (g/L, expressed as tartaric acid)	6.49	6.55	6.52	6.55	6.49	6.88	6.47	6.06
pH	3.41	3.37	3.43	3.38	3.96	3.95	3.97	3.92
Reducing sugars (g/L)	1.37	2.02	1.88	1.79	1.72	1.98	2.12	2.02

II.3.2.2 Volatile composition analyses

The volatile composition of the red and white wines samples was analysed (three independent aliquots) by HS-SPME combined with a GC×GC–ToFMS after 2 and 9 months of storage.

The solid-phase microextraction (SPME) holder for manual sampling and fiber were purchased from Supelco (Aldrich, USA). The SPME device included a fused silica fiber coating partially cross-linked with 50/30 μm divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) coating. SPME fibres were preconditioned in the GC injector, according to the recommendation of the manufacturer and daily conditioned for 10 min at 250 ºC. Then, the fiber was daily conditioned for 10 min at 250 ºC. For the HS-SPME assay, aliquots of 3.0 mL of the sample were placed into a 9 mL glass vial. After the addition of 0.6 g of NaCl each vial was capped with a PTFE/Silicone Septa (Supelco, USA). The vial was placed in a thermostated bath adjusted at 40.0 ± 0.1 ºC with stirring (1.5x0.5 mm bar) at 400 rpm, and the SPME fiber was manually inserted into the sample vial headspace for 20 min. Blanks, corresponding to the analysis of the coating fiber not submitted to any extraction procedure, were run between sets of three analyses.

After the extraction/concentration step, the SPME coating fiber was manually introduced into the GC×GC-ToFMS injection port at 250 ºC and kept for 30 s for the compounds desorption. The injection port was lined with a 0.75 mm I.D. splitless glass liner. The LECO Pegasus 4D (LECO, St. Joseph, MI, USA) GC×GC-ToFMS system consisted of an Agilent GC 7890A gas chromatograph (Agilent Technologies,

Inc., Wilmington, DE), with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven, and mass spectrometer equipped with a high resolution ToF analyzer. The detector was a highspeed ToF mass spectrometer. A HP-5 column (30 m x 0.32 mm I.D., 0.25 μm film thickness, J&W Scientific Inc., Folsom, USA) was used as first-dimension column, and a DB-FFAP (0.79 m x 0.25 mm I.D., 0.25 μm film thickness, J&W Scientific Inc., Folsom, USA) was used as a second-dimension column. The carrier gas was helium at a constant flow rate of 2.5 mL/min. The primary oven temperature was programmed from 40 (1 min) to 230 ºC (2 min) at 10 ºC/min. The secondary oven temperature was programmed from 70 (1 min) to 250 ºC (3 min) at 10 ºC/min. The MS transfer line temperature was 250 ºC, and the MS source temperature was 250 ºC. The modulation time was 5 s; and the modulator temperature was kept at 20 ºC offset (above primary oven). The ToFMS was operated at a spectrum storage rate of 125 spectra/s. The mass spectrometer was operated in the EI mode at 70 eV using a range of *m/z* 33-350 and the detector voltage was -1786 V.

Total ion chromatograms (TIC) were processed using the automated data processing software ChromaTOF® (LECO) at a signal-to-noise threshold of 100. Two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0, Mainlib and Replib) were used. A mass spectral match factor, the majority (86%) of the tentatively identified compounds showed similarity matches >850, was set to decide whether a peak was correctly identified or not. Furthermore, a manual inspection of the mass spectra was done, combined with the use of additional data, such as the retention index (RI) value, which was determined according to the van Den Dool and Kratz RI equation (van Den Dool and Kratz, 1963). For the determination of the RI, a C_8 – C_{20} *n*-alkanes series was used, and these values were compared with values reported in the literature for chromatographic columns similar to that used in the present work (Ansorena *et al.,* 2000; Campeol *et al.,* 2003; Cardeal *et al.,* 2008; Engel *et al.,* 2002; Eyres *et al.,* 2005; Fan and Qian, 2006; Högnadóttir and Rouseff, 2003; Jalali *et al.,* 2012; Jordán *et al.,* 2002; Leffingwell and Alford, 2005; Perestrelo *et al.,* 2011; Petronilho *et al.,* 2011; Pino *et al.,* 2005; Robinson *et al.,* 2011b; Rocha *et al.,* 2007; Salvador *et al.,* 2013; Silva *et al.,* 2010, 2015). The DTIC (Deconvoluted Total Ion Current) GC×GC area data were used as an approach to estimate the relative content of each volatile component in wine, and were expressed as arbitrary units (a. u.). Reproducibility was expressed as relative standard deviation (RSD).

II.3.2.2 Statistical analysis

Statistical data analysis was performed using Analysis of Variance (ANOVA) using Statistica[6.1] (Statsoft Inc., Tulsa, OK, USA).

Principal components analysis (PCA) was applied to the auto-scaled areas of all volatile compounds identified by HS-SPME/GC×GC–ToFMS presented in the the pressurized and unpressurized wines after 2 and 9 months of storage. The goal of this approach was to extract the main sources of variability and hence to help on the characterisation of the dataset.

II.3.3 Results and discussion

All the wine samples were analysed after 2 and 9 months of bottle storage in order to observe a possible effect of the high pressure treatments on the volatile composition of the wines.

Tables S1 and S2 shown as *Supplementary data*, in the thesis annex, gives detailed information for each compound, including GC peak area, RSD, and RI experimentally calculated as well as reported in the literature, for the white and red wines, respectively. The reproducibility, expressed as RSD, of the different identified volatile compounds ranged from 1% to 58%, which is a common range for natural products. The highest variability was usually observed for the compounds identified in trace amounts.

II.3.3.1 Volatile composition of the wines after 2 months of storage

The volatile composition analysis, after 2 months of storage, revealed for white wine samples the presence of 167, 172, 163, and 157 compounds in the *untreated*, *SO_2,* and pressurized at *425 MPa* and *500 MPa*, respectively. In the red wine samples, 157, 163, 166 and 167 compounds were detected in the *untreated*, *SO_2*, *425 MPa* and *500 MPa* samples, respectively. These compounds belong to 12 chemical families, such as acids, esters, alcohols, volatile phenols, aldehydes, ketones, furans, lactones, acetals, thiols and others sulphur compounds, norisoprenoids, and terpenic compounds. Among all the chemical groups, the esters presented the higher number of identified compounds (62/63 in white/red wines), followed by alcohols (30/35 in white/red wines), and terpenic compounds (15 in white wines and 23 in red wines) (Tables S1 and S2). These results are in accordance with the studies conducted in Pinotage wines (Weldegergis *et al.*, 2011b),

South Africa red wines (Weldegergis *et al.*, 2011a), and Brazilian Merlot wines (Welke *et al.*, 2012).

The total peak areas for each chemical group identified in the white and red wine samples, after 2 months of storage, are presented in Figures II.3.1 and II.3.2, respectively.

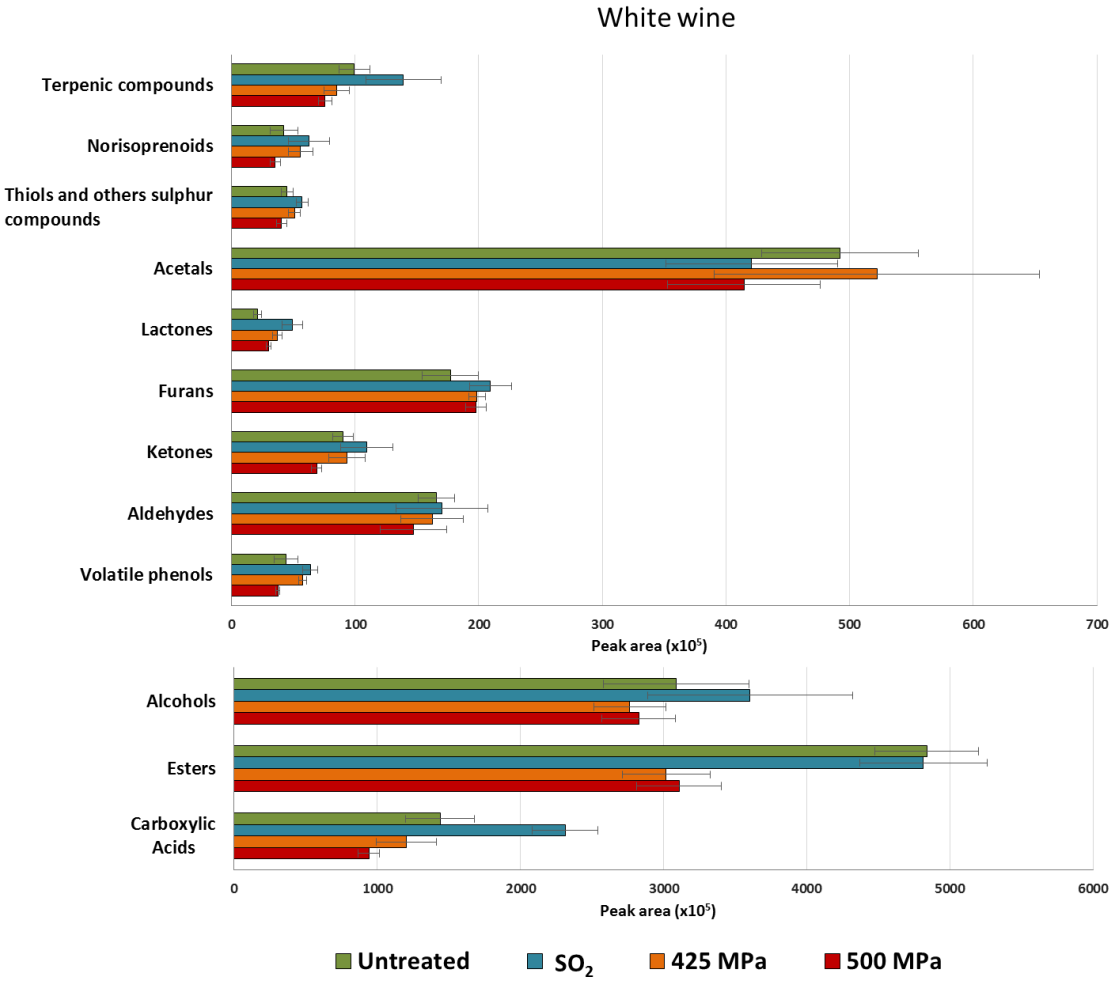

Figure II.3.1. GC×GC–ToFMS peak area of the chemical groups identified in all white wines after 2 months of storage.

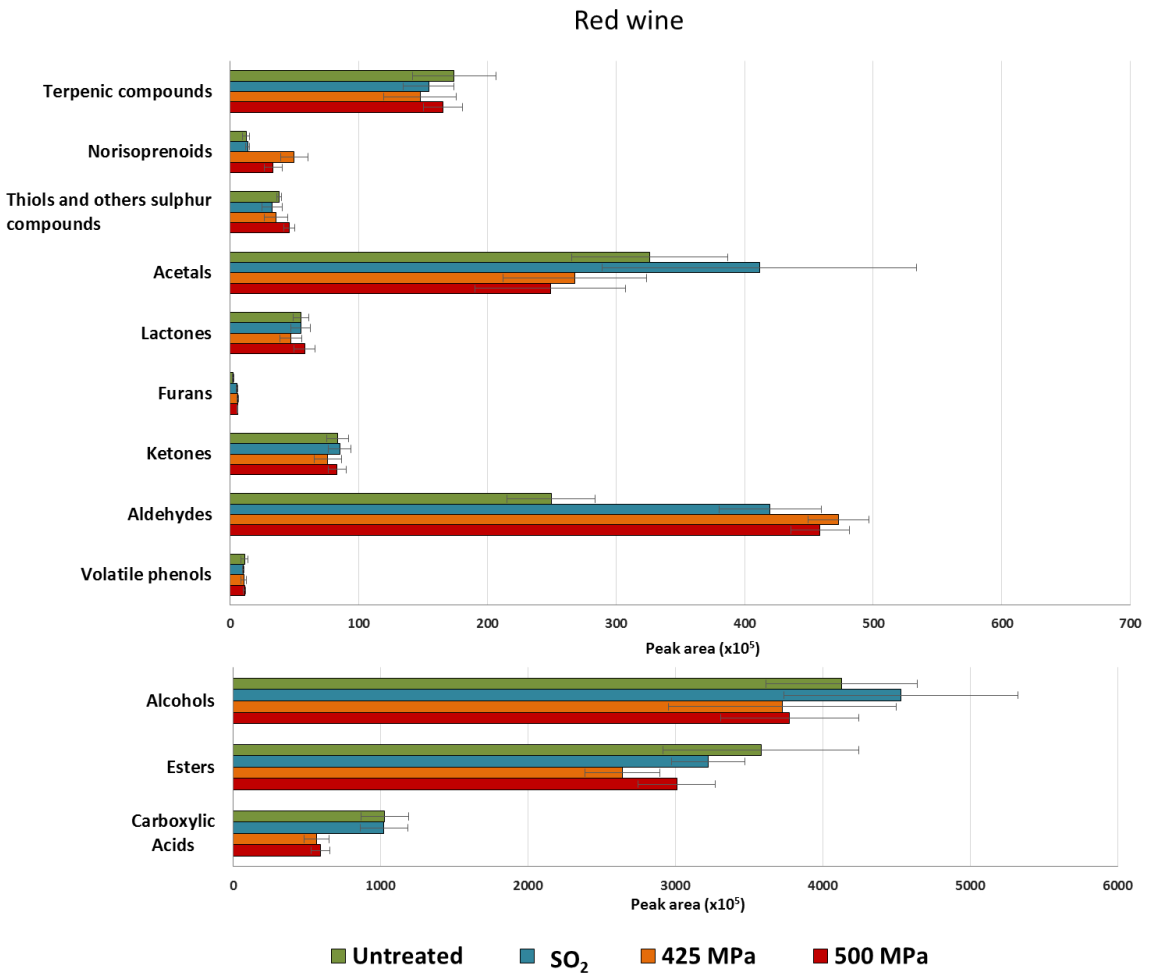

Figure II.3.2. GC×GC–ToFMS peak area of the chemical groups identified in all red wines after 2 months of storage

After 2 months of storage, the impact of the two pressure treatments on the volatile composition of both white and red wines, are minimal, but statistically significant for some chemical groups (p<0.05), namely for esters and acids in the case of white wine, and acids and norisoprenoids for red wine.

After 2 months of storage the pressurized white wines presented lower content of esters than the unpressurized white wines (p<0.05) (Figure II.3.1). This lower content of esters is mainly due to the lower content of the aliphatic ethyl esters, such as a 2-fold lower content of ethyl octanoate (peak number 27) and 6 to 9-fold lower amount of ethyl decanoate (peak number 32, Table S1) than the unpressurized white wines. These two esters are frequent products of fermentation, and described to have fruity and floral odours (Weldegergis *et al.*, 2011b).

In terms of red wine samples, both pressurized red wines presented a lower content of carboxylic acids (Figure II.3.2), mainly due to the 2 to 3-fold lower area of the acetic acid peak (peak number 1, Table S2) than the unpressurized wines. Acetic acid was one of the dominant acids in red wines, based on their peak area, in agreement with previous reports (Weldegergis *et al.,* 2011a; Weldegergis *et al.,* 2011b), and is known to contribute to a vinegar odour, contributing negatively to the wine bouquet (Fang and Qian, 2005). Since this compound is produced during fermentation, the lower content of acetic acid in pressurized wines could indicate that the pressure treatments stopped the fermentation of the wine in a more effective way than the addition of SO_2. The pressurized red wines presented also higher content of norisoprenoids ($p<0.05$) when compared with the *SO_2* and *untreated* samples. The higher content of norisoprenoids in pressurized wines, after 2 months of storage, was mainly due to the presence of geranyl acetone (peak number 117, Table S2) that was only identified in the pressurized wines samples. The C_{13} norisoprenoids have been related to complex wine flavours, described as grassy, tea, lime, honey, and pineapple, and rose for the case of geranyl acetone (Pino *et al.,* 2005; Weldegergis *et al.,* 2011a; Weldegergis *et al.,* 2011b). These compounds, similar to the monoterpenes, occur in grapes largely as non-bound carotenoid precursors (Ribéreau-Gayon *et al.,* 2006), while geranyl acetone may result from the oxidative cleavage of squalene (Ikeguchi *et al.,* 1988)

Overall, despite some differences observed in the volatile composition of the pressurized wines, the impact of the pressure treatments was minimal after 2 months of storage. This result is in agreement with the previous results showing that high pressure treatments (400–500 MPa for few minutes) do not alter significantly the white (Chapter II.2) and red (Chapter II.1) wine physicochemical and sensorial properties in the first months of storage.

II.3.3.2 Volatile composition of the wines after 9 months of storage

After 9 months of storage a lower number of compounds were detected (up to 15% less) in both white and red wines when compared with the same wine samples after 2 months of storage (Tables S1 and S2). This behavior is explained by the increase of the interaction between volatile compounds and other compounds present in wine, namely polyphenols, along wine aging (Ribéreau-Gayon *et al.,* 2006). It were detected in white wine samples 148, 146, 148 and 148 compounds in the *unpressurized*, *SO_2* and pressurized *425 MPa* and *500 MPa*, respectively. In the red wine samples, it were detected

150, 151, 141 and 142 in the samples *untreated*, *SO_2*, *425 MPa*, and *500 MPa*, respectively. As observed in the wines with 2 months of bottle aging, esters presented the higher number of identified compounds (59/69 in white/red wines), followed by alcohols (26/24 in white/red wines), and terpenic compounds (13/16 in white/red wines) for 9 months of bottle aging (Tables S1 and S2).

The total peak areas for each chemical group identified in the white and red wines, after 9 months of storage, are presented in Figures II.3.3 and II.3.4, respectively. It can be notice that, contrary to the wine samples with 2 months of bottle aging, the pressurized wine samples presented a volatile composition remarkably different than the unpressurized, indicating a large impact of the pressure treatments on the volatile composition of both white and red wines. Particularly, the pressurized wine samples presented higher content of acetals, ketones, furans, and aldehydes.

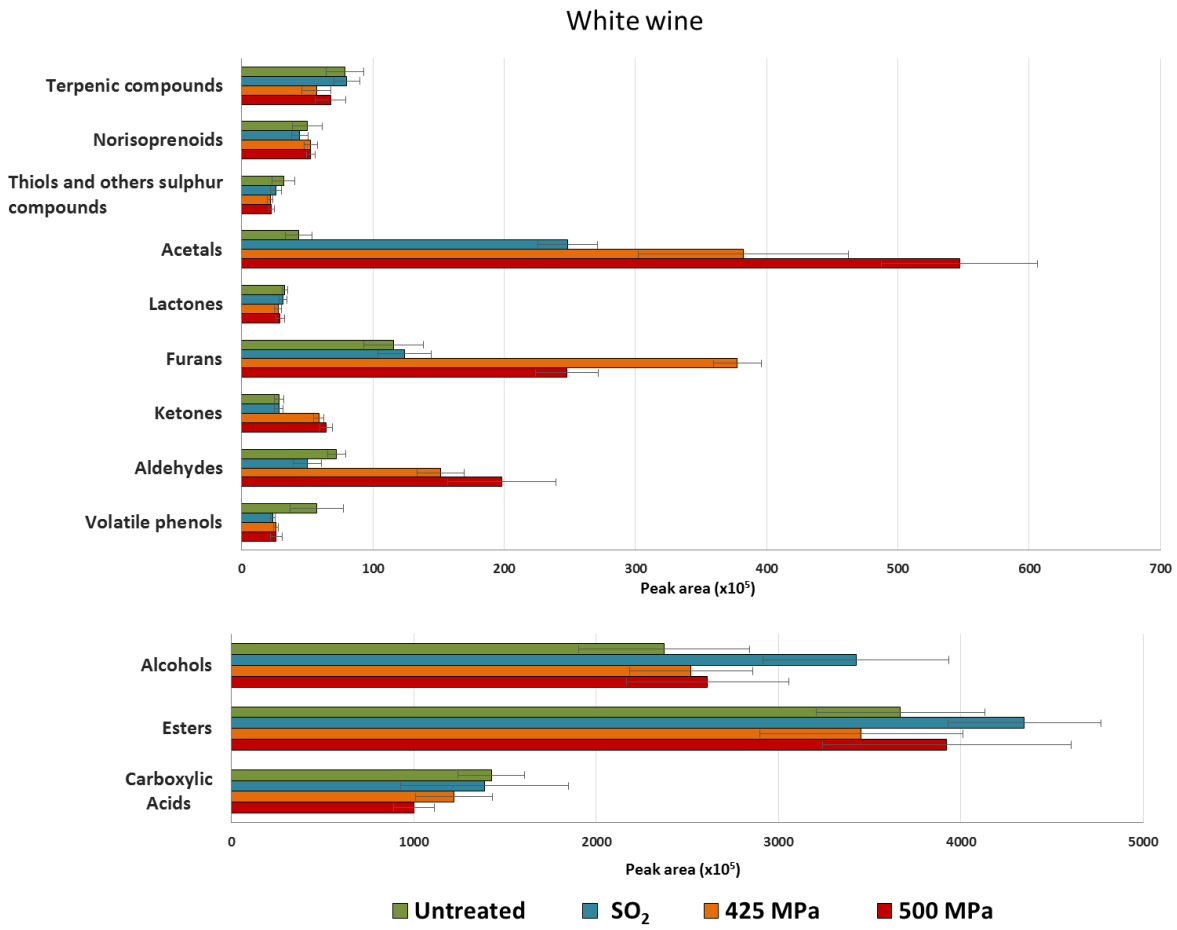

Figure II.3.3. GC×GC–ToFMS peak area of the chemical groups identified in all white wines after 9 months of storage.

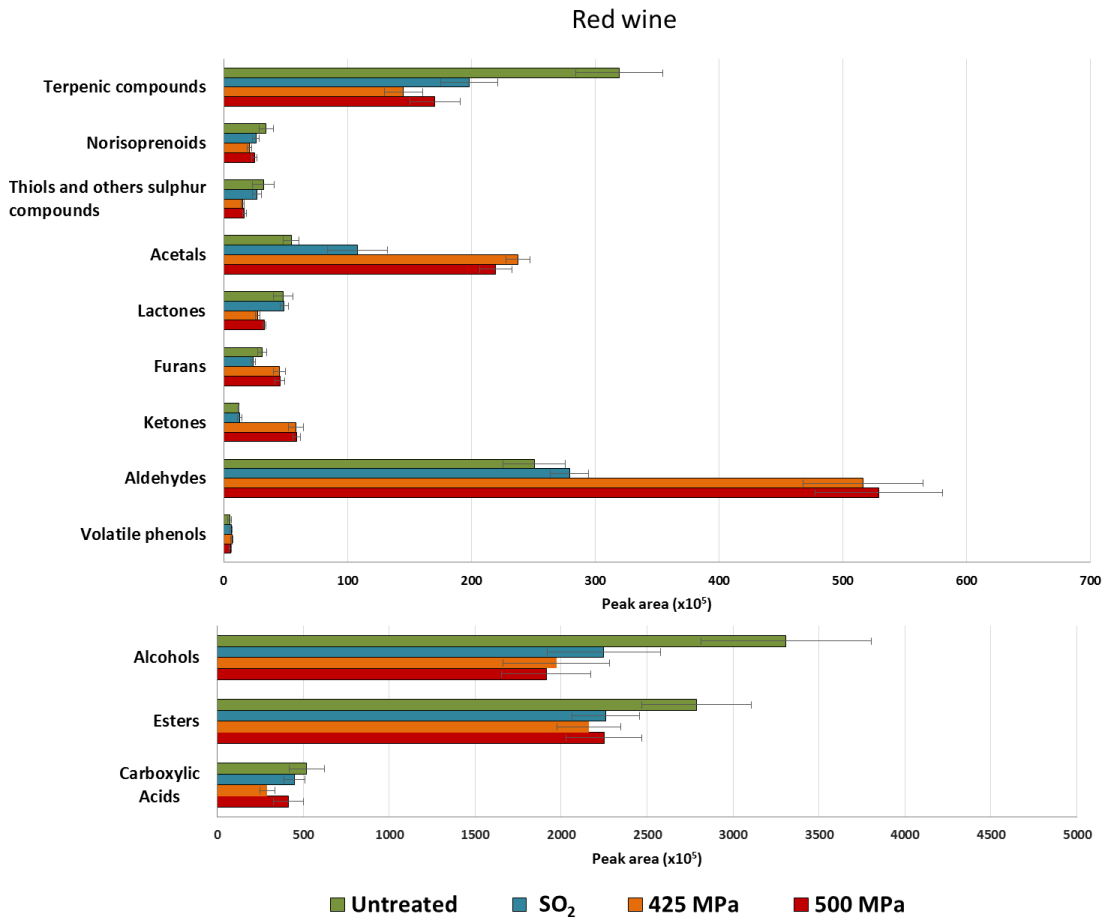

Figure II.3.4. GC×GC–ToFMS peak area of the chemical groups identified in all red wines after 9 months of storage.

In order to reduce the dimensionality of the data set, allowing to study the main sources of variability of the data set and detect differences/similarities among wine samples, a principal component analysis (PCA) procedure was performed using, as analytical variables, the GC peak area of all volatile compounds of white and red wine samples with 9 months of bottle storage. This allowed to study the effect of the different treatments in the wines volatile composition along storage and to establishment the relationships/correlations between wine samples and compounds.

White wine

Figure. II.3.5 shows a biplot reporting the score plots combined with the loadings plots of the two first principal components (which explains 77% of the total variability of the data set) for the white wine samples. The loadings establish the relative importance of each volatile compound for the observed sample distribution. PC1, which explains 60% of the total variability, allowing to distinguish wines treated with high pressure (*425 MPa*

and *500 MPa*) from the *untreated* and SO_2 ones. PC2, explaining 17% of the total variability, shows the distribution of the wines according to the presence of sulphur dioxide. The pressurized wines were negatively located in relation to PC1 and positively located in relation to PC2. These samples are characterized mainly by ketones, acetals, furans, and aldehydes.

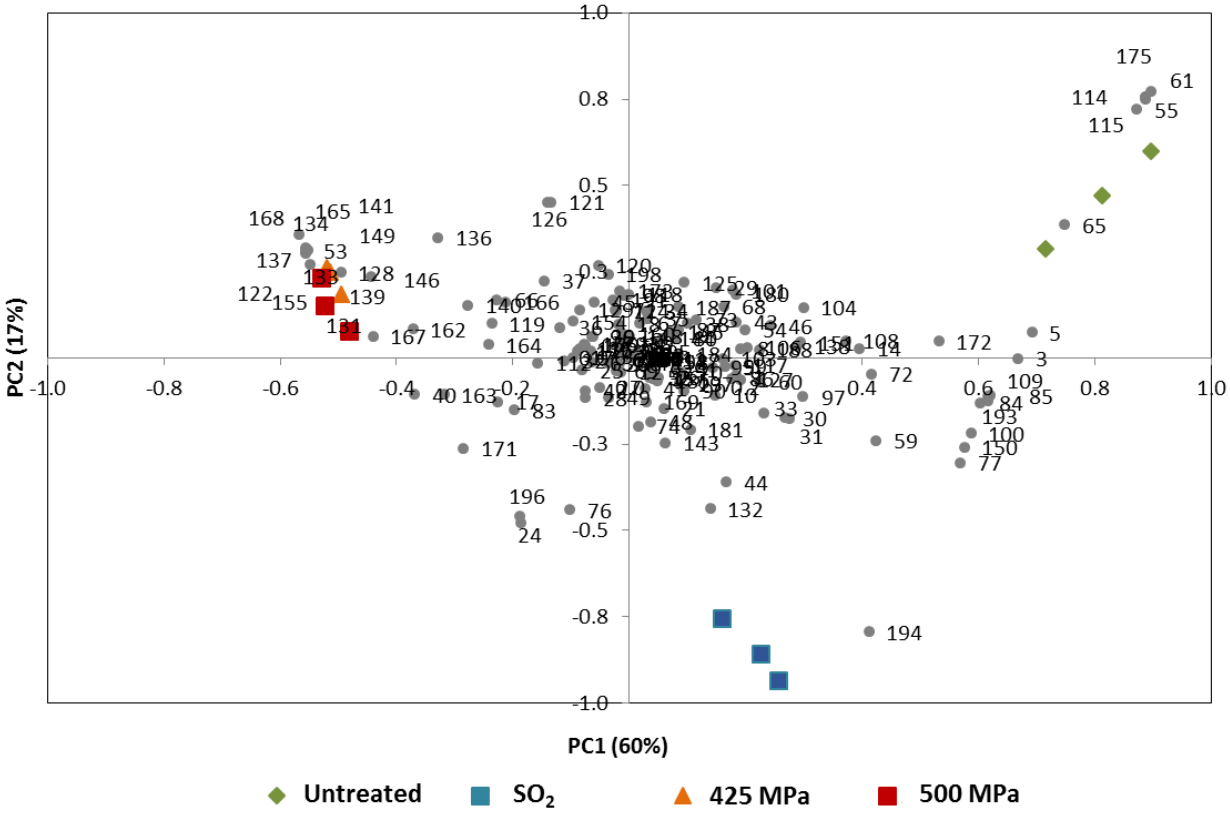

Figure. II.3.5. Biplots in the PC1×PC2 plane combining score plots and loadings plots of the different white wines, after 9 months of storage, related to the volatile compounds. Attribution of the peak number is shown in Table S1 in Supplementary Material.

The ketones 3-pentanone (peak number 131), 3-penten-2-one (peak number 133), 1-(ethenyloxy)-3-methyl-butane (peak number 134), octan-3-one (peak number 137), nonan-3-one (peak number 139), and octan-2,5-dione (peak number 141) were only identified in the pressurized wine samples (Table S1). Ketones are reported to result from the direct oxidation of fatty acids (Campo *et al.*, 2006; Weldegergis *et al.*, 2011b) and are mainly described to have "buttery" and "fatty" odours (Jiang and Zhang, 2010; Schneider *et al.*, 1998). The presence of these ketones in the pressurized wines indicate the occurrence of fatty acids oxidation with the pressure treatments. These results are in

agreement with the literature, since some studies show that HHP treatments enhance lipid oxidation in foods (Bolumar *et al.*, 2012a).

In the acetal family, the 1,1-diethoxy-pentane (peak number 168) and 1-(1-ethoxyethoxy)-butane (peak number 165) were only identified in the pressurized wines and the content of 1,1-diethoxy-ethane (peak number 162) and 1-(1-ethoxyethoxy)-pentane (peak number 167) were 40 to 49% and 65 to 68% higher, respectively, in these samples when compared with the *SO_2* wine. These acetals are reported to have "caramel" and "dried fruit" odours and their presence is common in wines submitted to oxidative aging, as well as in Sherry wines (Schneider *et al.*, 1998). These results are in agreement with the results of Chapters II.1 and II.2, since in this chpaters it was shown that both sulphur dioxide-free red and white wines presented higher "cooked fruit" aroma after pressure treatments. Since the acetals are formed by the reaction of aldehydes (mainly acetaldehyde) with alcohols, it seems that the HHP treatments accelerated the occurrence of this reaction during the wine storage.

The importance of these furans and aldehydes in the differentiation of the pressurized wines from unpressurized (Figure II.3.5) is due to the higher content of these compounds in the *425 MPa* and *500 MPa* wines (Table S1). The higher content of furans in pressurized wines samples was mainly due to the 10- and 5-fold higher content of 2-furfural (peak number 146) in the samples pressurized at 425 MPa and 500 MPa, respectively. Moreover, the 5-methylfurfural (peak number 149) and the 2-acetyl-5-methylfuran (peak number 155) were only detected in the pressurized wine samples. The higher content of aldehydes in the pressurized wine samples was mainly due to the higher content of benzaldehyde (peak number 128), 10- and 15-fold higher content in the sample pressurized at 425 MPa and 500 MPa, respectively, when compared with the *untreated* and *SO_2* white wines. Both 2-furfural and benzaldehyde are considered Maillard reaction-derived volatile compounds, as the 2-furfural can be formed by the dehydration of sugars through Maillard reaction (Oliveira *et al.*, 2011; Perestrelo *et al.*, 2011) and benzaldehyde by the Strecker degradation of amino acids as a result of the Maillard reaction (Pripis-Nicolau et al. 2000). Nevertheless, benzaldehyde may be formed through the shikimic acid, having phenylalanine as intermediate (Ribéreau-Gayon et al. 2006). In the Strecker degradation, the amino acid in the presence of α-dicarbonyl compounds is decarboxylated and deaminated, forming an aldehyde with one carbon atom less than the amino acid, known as "Strecker aldehyde" (Keim *et al.*, 2002; Oliveira *et al.*, 2011). The results obtained lead to infer that the HHP treatments accelerated Maillard reactions during the

wine storage period. These conclusions are also supported by the studies of Chapter II.2 that showed that pressurized white wines presented, at least after 6 months of storage, a more brownish colour, lower content of free amino acids and higher content of furans.

According to Figure II.3.5, the untreated white wine is characterized (PC1 and PC2 positive) by 4-ethyphenol (peak number 114), 4-ethylguaiacol (peak number 115), isobutyl butyrate (peak number 55), propyl hexanoate (peak number 61), hexyl 2-methyl-butyrate (peak number 65), and isophorone (peak number 175) (Table S1). The ethylphenols are normally produced by spoilage of *Brettanomyces/Dekkera* spp. yeasts involving cinnamic, coumaric, and ferulic acids, free or esterified with tartaric acid (Larcher *et al.,* 2012). These compounds are responsible for a particularly unpleasant sensory defect known as 'mousy off-flavour' (Romano *et al.,* 2008). Therefore, these compounds indicate wine spoilage in the untreated samples (Chatonnet *et al.,* 1995). In fact, due to the absence of any inhibitor of microorganisms growth in the untreated wine (contrary to the pressurized wines and the wine with SO_2) it was expected a possible contamination of these samples by microorganisms. The presence of the esters isobutyl butyrate, propyl hexanoate, and hexyl 2-methyl-butyrate may also be due to the presence of microorganisms in the untreated wine, since these compounds can result from fermentation occurring during wine ageing (Schulz and Dickschat, 2007; Weldegergis *et al.,* 2011b).

In the wine sample with addition of sulphur dioxide, geraniol (peak number 194, Table S1) is the principal contributor to its location in PC1 positive and PC2 negative (Figure II.3.5). The content of geraniol in this sample was 63% higher when compared with the untreated samples and was not identified in the pressurized wines (Table S1). Monoterpene alcohols, such geraniol, which contribute to the wine varietal characteristics, belong to the most relevant flavour compounds of several white wine varieties and are responsible for their characteristic floral aroma (Ribéreau-Gayon *et al.,* 2006). The sulphur dioxide was reported to have a protective effect on these volatiles (Roussis and Sergianitis, 2008), which explain the higher concentration of geraniol in the SO_2 wine sample, when compared with the other samples. In addition, geraniol content decreases with wine ageing and is usually present in trace amounts after two or three years in the bottle (Pedersen *et al.,* 2003). This compound can undergo several reactions during wine storage (easily isomerizes and oxidizes, forming oxides and aldehydes), induced by the time of storage and relatively low pH (Dziadas and Jeleń, 2010).

Red wines

Figure. II.3.6 shows the biplot reporting the score plots combined with the loadings plots of the two first principal components (which explain 65% of the total variability of the data set) of the PCA performed for the red wine samples. As observed for the white wine samples, also for the red wines the PC1, which explains 55% of the total variability, allowed to distinguish the wines as a function of the pressure treatments, and the PC2, explaining 10% of the total variability, differentiated the wines according to the presence of sulphur dioxide.

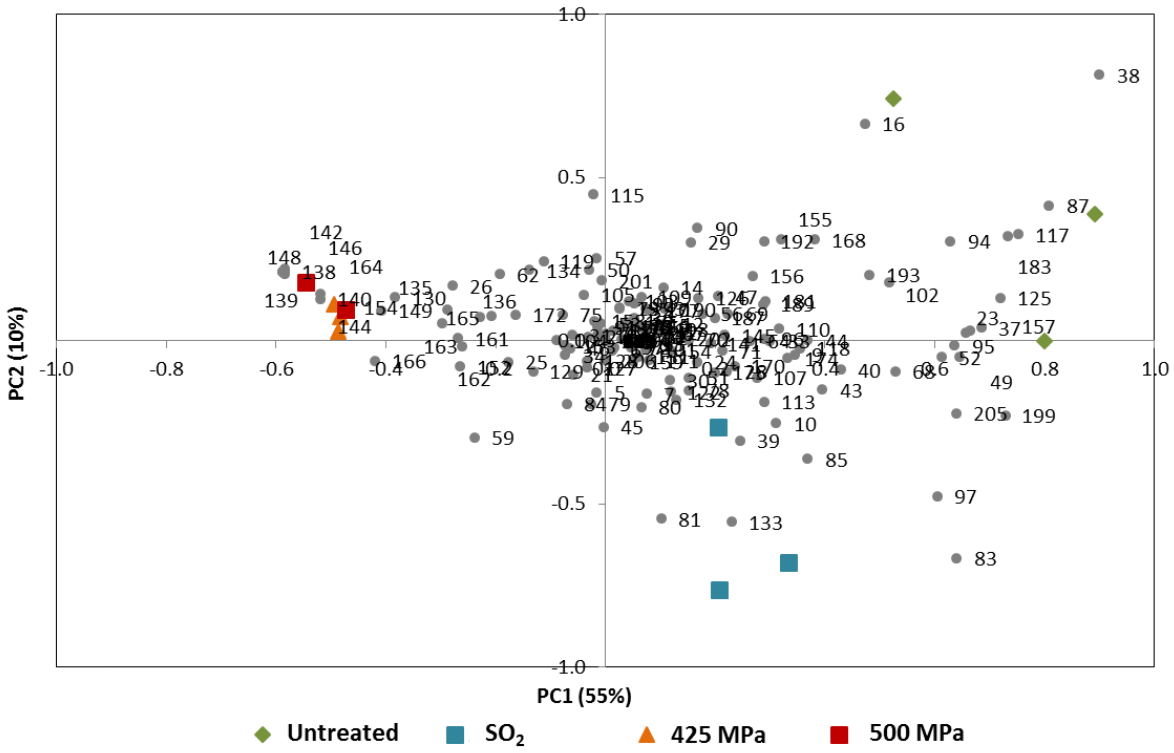

Figure. II.3.6. Biplots in the PC1×PC2 plane combining score plots and loadings plots of the different red wines, after 9 months of storage, related to the volatile compounds. Attribution of the peak number is shown in Table S2 in Supplementary Material.

The pressurized wines were negatively located in relation to PC1 and positively located in PC2, and no differences were observed between the samples *425 MPa* and *500 MPa* (Figure. II.3.6). These results show that the difference in the pressure value between the two pressures applied (425 MPa and 500 MPa during 5 min) had no significant effect on the volatile composition of the red wines. As observed for the white wines, the pressurized red wines were also characterized mainly by ketones, acetals, furans, and aldehydes.

The ketones responsible for the pressurized wine discrimination were 3-pentanone (peak number 138), 2,3-pentanedione (peak number 139), hexane-2,3-dione (peak number 142), octan-3-one (peak number 146) and nona-3-one (peak number 148), since these compounds have been only identified in these wines (Table S2). In addition, acetoin (peak number 140), heptan-2-one (peak number 144), and nona-2-one (peak number 149) are presented in higher concentration in the pressurized wines (up to 78%, 82%, and 86%, respectively), compared to both unpressurized red wine samples (Table S2). These ketones are described to have "buttery" and "fatty" odours (Schneider *et al.,* 1998) resultant from fatty acids oxidation.

The acetals that characterized the pressurized red wines (Figure. II.3.6) are 1,1-diethoxy-2-methyl-propane (peak number 163), 1-(1-ethoxyethoxy)-butane (peak number 164), and 1,1-diethoxy-3-methyl-butane (peak number 165) (Table S2). 1-(1-Ethoxyethoxy)-butane was only identified in the pressurized wines and the content of 1,1-diethoxy-2-methyl-propane and 1,1-diethoxy-3-methyl-butane were up to 70% and 68% higher, respectively, in these samples when compared with the SO_2 wine. These results show that, as observed for white wines, the formation of acetals are accelerated with the pressure treatments, increasing the content of compounds with "dried fruit" odours.

The hexanal (peak number 130) is one aldehyde that characterized the pressurized red wines, since these wines presented around 5-fold higher content of this compound than unpressurized wines. This result lead to infer that the oxidation of some alcohols, such hexanol, can also be accelerated by the pressure treatments.

The pressurized red wines, as observed for white wines, were also characterized by the presence of a higher content of Maillard volatile compounds, namely 2-furfural (peak number 154), benzaldehyde (peak number 135), and phenyl acetaldehyde (peak number 136) (Table S2). In fact, the pressurized wines presented 5- to 11-fold higher furfural content, and 2-fold higher benzaldehyde and phenyl acetaldehyde content when compared with the unpressurized samples. These results lead to infer that the pressure treatments accelerated Maillard reactions also during the storage period of red wine. However, the difference of Maillard volatile compounds between pressurized and unpressurized wines was lower in red wines in comparison with white wines. This behaviour can be due to the higher content of polyphenols in red wine, when compared with white wine, that reduce the rate of Maillard reactions, due to their higher antioxidant activity, and consequently decrease the formation of Maillard reaction-derived volatile compounds (Cejudo-Bastante *et al.,* 2010; Sonni *et al.,* 2009).

As observed in Figure. II.3.6, the *untreated* and SO_2 red wine samples are located in PC1 positive being separated by PC2, explaining 10% of the total variability. The 10% of variability of the PC2 is lower when compared with the 55% of variability of the PC1, due to higher dispersion of data of the unpressurized wines along the PC2. These results show that, contrary to the white wines where the *untreated* white wine was well separated from the SO_2, due to the presence of volatile compounds possibly originating from microorganism contamination in the *untreated* white wines, the separation between the two unpressurized red wines was not so conclusive. Therefore the main separation in the red wine samples is due principally to the pressure treatments.

II.3.3.3 Evolution of ketones, aldehydes, furans, and acetals profile along wine storage

Since the pressurized wines with 9 months of storage were mainly characterized, by ketones acetals, furans, and aldehydes, it was necessary to understand the impact of the pressure treatments on these chemicals groups, after 2 months of storage, and their evolution during storage. For that, a heatmap (Figure II.3.7), a logarithmic normalization of the GC peak area, was performed for a direct and rapid interpretation of the relative abundance of each aldehydes, ketones, furans, and acetals compounds for the different white and red wines (with three independent assays) at 2 and 9 months of storage. White (Figure II.3.7A) and red wines (Figure II.3.7B) after 2 months of storage revealed a similar profile among the samples with different treatments, since the relative abundance of the chemical groups are homogenous for all the wines. These results are in accordance with the preview ones that show that after 2 months of storage, the impact of the two pressure treatments on the volatile composition of both white and red wine are minimal. However, after 9 months of storage is possible to observe that, for both pressurized white and red wines, the volatile profiles of each aldehyde, ketone, furan, and acetal compounds were very different when compared with the unpressurized wines. These results confirm that the impact of pressure treatments in both white and red wines, namely in these compounds, was only noticeable after several months of storage.

Acetals, ketones, and Maillard volatile compounds, such furfural and benzaldehyde, have a tendency to increase linearly during wine aging and are reported as potential age markers of Sherry wines (Fernández de Simón *et al.*, 2014; Sun *et al.*, 2013) and Madeira wines (Perestrelo *et al.*, 2011). Therefore, it seems that the pressurized wine samples present a volatile composition characteristic of faster aged/thermally treated

wines. These results indicate that the HHP treatment influences the white and red wine long term volatile composition and seems to accelerate their evolution during storage, being this particularly evident for lager storage periods.

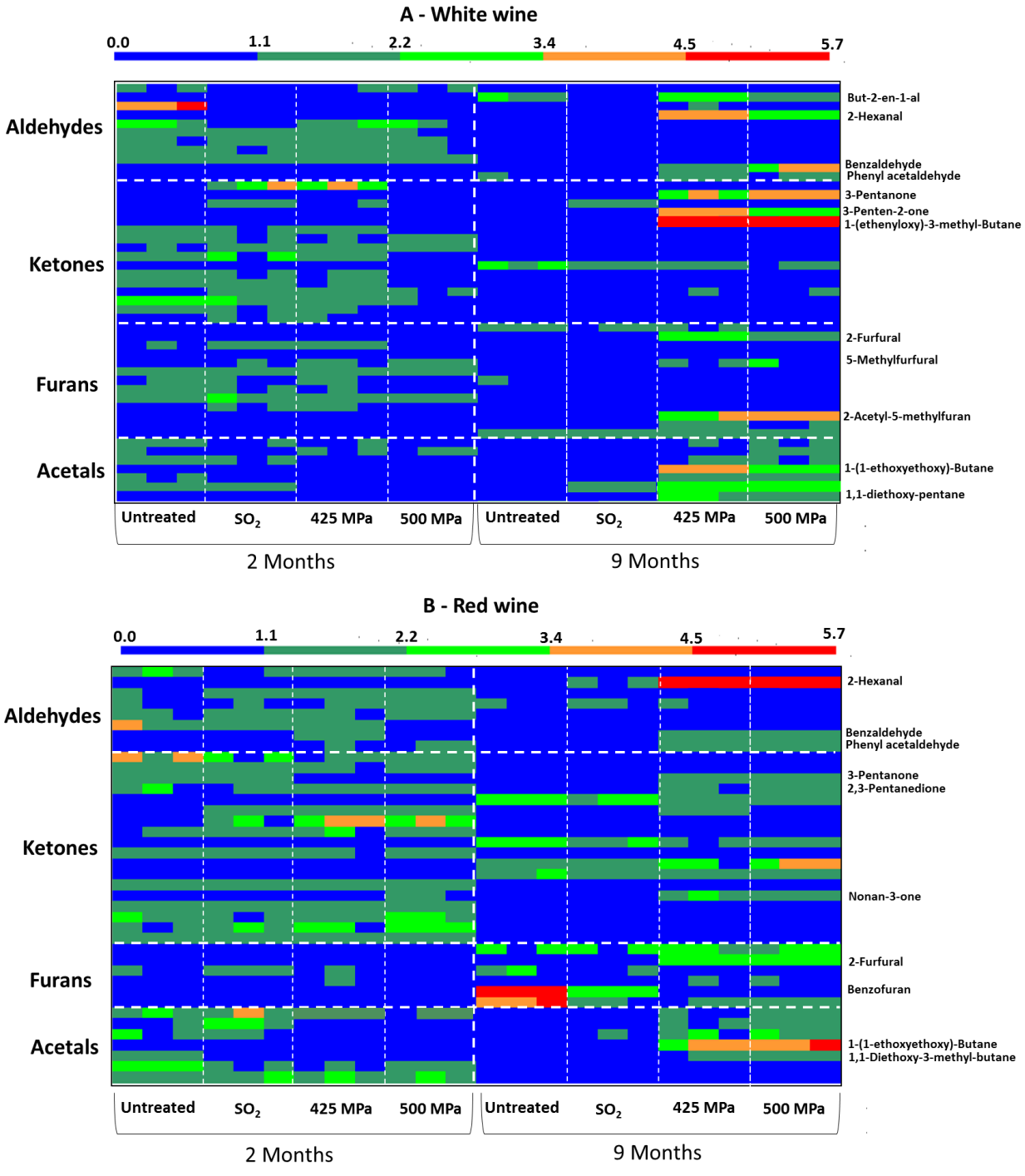

Figure II.3.7. Heatmaps (logarithmic normalization of the GC peak area) for white (A) and red (B) wines of the aldehyde, ketone, furan and acetal compounds. Different intensities correspond to the normalized GC peak areas of each compound (3 replicates).

II.3.4 Concluding remarks

The results obtained in this work demonstrate that high pressure treatments with processing time around 5 min and pressures between 400 and 500 MPa influence white and red wine volatile composition. However, the effect is only perceptible after some months of storage, changing the wine aroma characteristics. The two pressure treatments studied showed similar effects on both white and red wines. The changes on the volatile composition of the pressurized wines, namely the increase of furans, aldehydes, ketones, and acetals content, indicate that the HHP treatments accelerate the Maillard reactions, and the oxidation of alcohols and fatty acids, leading to wines with a volatile composition characteristic of faster aged and/or thermally treated wines.

CHAPTER III

Application of high hydrostatic pressure treatments in wine model solutions

CHAPTER III.1. Formation of Maillard volatile compounds in model wine solutions, during storage, promoted by high pressure treatments

CHAPTER III.2. Evaluation of the effect of high pressure treatments on anthocyanin condensation reactions in model wine solutions

CHAPTER III.1

Formation of Maillard volatile compounds in model wine solutions promoted, during storage, by high pressure treatments

Formation of Maillard volatile compounds in model wine solutions, during storage, promoted by high pressure treatments

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Keywords: High pressure; wine model solution; amino acids; carbohydrates; Maillard volatile compounds

III.1.1 Overview

As mentioned in Chapter I, the Maillard reaction is responsible for the development of colour, aroma and flavour precursors in food products. The large number of compounds that can be formed through the different pathways gives rise to a complex mixture of products characteristic of the different foods. Briefly, this reaction is initiated by a condensation between the carbonyl group, as for example a reducing sugar, and an amino group, as for example an amino acid, giving rise to different compounds, such as furfurals, aldehydes, acrylamides, and heterocyclic amines (Jaeger *et al.,* 2010). Brown polymers, so-called melanoidins, can be the final products of the reaction (Moreno *et al.*, 2003). Phenylacetaldehyde and benzaldehyde are also considered Maillard reaction-derived volatile compounds, since they can be formed by the Strecker degradation of amino acids as a result of the Maillard reaction (Pripis-Nicolau *et al.,* 2000). In the Strecker degradation, the amino acid in the presence of α-dicarbonyl compounds is decarboxylated and deaminated, forming an aldehyde with one carbon atom less than the amino acid, known as "Strecker aldehyde" (Keim *et al.,* 2002; Oliveira *et al.*, 2011). Nevertheless, some studies reported that the use of HHP treatments at moderate temperatures (30-60°C) may promote the Maillard reaction, changing the flavour, colour, and nutritional value of foods (Jaeger *et al.,* 2010; Schwarzenbolz and Henle, 2010; Tamaoka *et al.*, 1991).

The results of Chapters II.2 and II.3 showed that HHP treatments at room temperature accelerate the Maillard reaction in wine, leading to the decrease of amino acids content and the increase of Maillard derived volatile compounds (MVC) formation. Besides that, only few studies have evaluated the effects of pressure in model systems containing amino acids and sugars, but with no results on the influence of the

treatment during storage time (Bristow and Isaacs, 1999; Hill *et al.*, 1996; Moreno *et al.*, 2003; Schwarzenbolz *et al.*, 2000, 2002; van Boekel 2006). In an attempt to use HHP on wine, more studies concerning the chemical reactions caused by HHP are needed, namely the effects of HHP on the formation of MVC during storage. To achieve this objective, model wine solutions with equimolar (10 mM) mixtures of one sugar (arabinose or glucose) and one amino acid (lysine, serine or phenylalanine) were pressurized at 500 MPa for 5 min at 20 ºC. The content of MVC was determined along storage (up to 12 months) by gas chromatography coupled to mass spectrometry (GC-MS) combined with solid-phase microextractrion (SPME). As control, untreated model wine solutions and heated (at 60 ºC during 5 min) model wine solutions were also analysed.

III.1.2 Materials and methods

III.1.2.1 Chemicals

Milli-Q water (Millipore, Bedford, MA) was used in all this work. Absolute ethanol p.a, tartaric acid (99%), benzaldehyde (>99%), furfural (>99%) phenylacetaldehyde (>99%), L-amino acids standards (lysine, phenylalanine and serine), D-(-) arabinose (99%) and D-(-) glucose (99%) were purchased from Sigma (Seelze, Germany).

III.1.2.2 Model wine solutions and high pressure treatments

Model wine solutions (0.5% (w/v) tartaric acid containing 10% ethanol and adjusted to pH 3.5 with 0.1 M NaOH) with equimolar (10 mM) mixtures of one sugar (arabinose or glucose) and one amino acid (lysine, serine, or phenylalanine) were submitted to the pressure treatment (500 MPa for 5 min at 20 ºC) in polyethylene bottles (36.0 mL). The pressurisation was carried out using a hydrostatic press from Unipress Equipment, Model U33 (Warshaw, Poland), with a pressure vessel of 100 mL (35 mm diameter and 100 mm height), surrounded by an external jacket connected to a thermostatic bath in order to control the temperature. A mixture of propylene glycol and water (1:1) was used as pressurization fluid and the pressure build up was carried out at 450 MPa/min compression rate. Simultaneously, as controls, a non pressurized (*untreated*) model wine solution and a model solution heated at 60 ºC during 5 min

(*heated*) were also produced and stored at room temperature (20 ºC) in the absence of light. Figure III.1.1 shows a schematic representation of the model wine solution elaboration followed in this study.

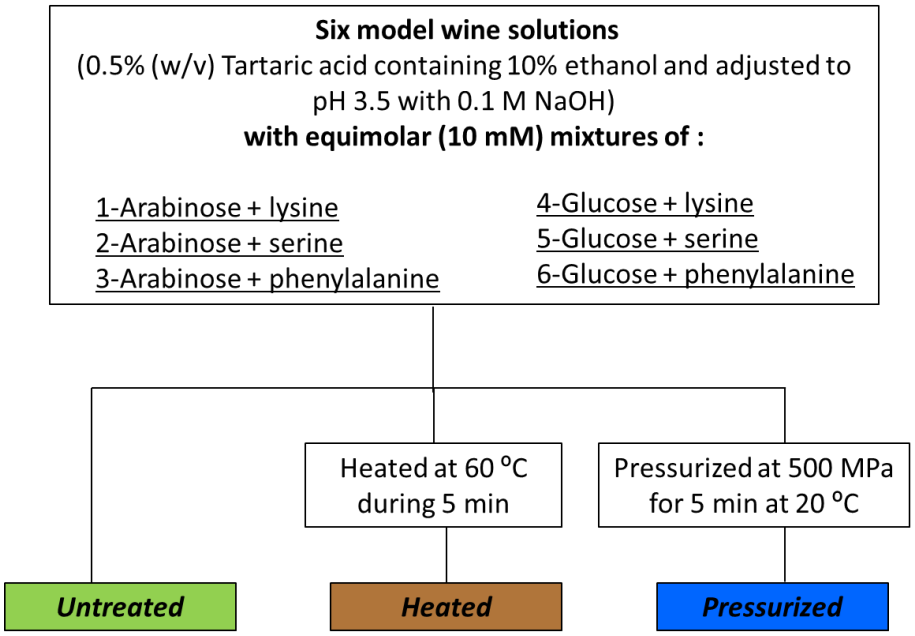

Figure III.1.1. Flow chart of the model wine solution elaboration followed in this study

III.1.2.3 Maillard derivated volatile compounds analysis

The content of MVC presented in the model wine samples was determined along storage by SPME-GC-MS. The SPME device used was a fused silica fiber coating partially cross-linked with 50/30 µm divinylbenzene/carboxen/ polydimethylsiloxane coating (DVB/CAR/PDMS). For the headspace-SPME assay, aliquots of 3 mL of the sample were placed into a 9 mL glass vial. After the addition of 0.6 g of NaCl and stirring at 400 rpm, the vial was capped with a screw cap with septum. The SPME fiber was exposed to the headspace for 20 min at 40 ºC to extract the volatile compounds.

The extracted compounds were analysed in a GC-qMS (Agilent Technologies 6890 N) equipped with DB-FFAP fused silica capillary column (J&W Scientific Inc., Folsom, CA, USA) with 30 m×0.25 mm (i.d.) and 0.25 µm film thickness. The following temperature programme was used: 3 min hold at 60 ºC, increase to 130 ºC at 10 ºC/min and then to 220 ºC (1 min hold) at 40 ºC/ min. The MS was operated in the electron impact mode with an electron impact energy of 70 eV and data collected at a rate of 3

scan s^{-1}. The ion source and the transfer line were kept at 230 ºC. Selected ion monitoring (SIM) was used for the detection of the compounds. The ions at *m/z 96*, 95, 67, 39; *m/z* 126, 97, 69, 41; *m/z* 106, 105, 77, 51; and *m/z* 120, 91, 61, were used, respectively for 2-furfural, 5-hydroxymethylfurfural, benzaldehyde, and phenylacetaldehyde detection. Three independent aliquots of each sample were analysed. For quantification of MVC, calibration curves of furfural (R^2=0.978), benzaldehyde (R^2=0.968) and phenylacetaldehyde (R^2=0.963) were performed. All analyses were performed in triplicate.

III.1.2.4 Statistical analysis

All statistical analyses were performed with the SPSS for Windows, 17.0. (SPSS Inc, Chicago, IL, USA). Data are expressed as means ± S.D. One-way analysis of variance (ANOVA) was carried out to determinate significant differences within and between groups. Tukey's test was applied to compare the mean values. Statistical significance was set at $p< 0.05$.

III.1.3 Results and discussion

Maillard derived volatile compounds (MVC), namely furfural, phenylacetaldehyde, and benzaldehyde, were not detected at the beginning of storage in the pressurized and untreated model wine solutions, whereas they were found in the heated model wine solution (Figures III.1.2, III.1.3, and III.4). Along storage, the MVC content increased in all model wine solutions. However, the pressurized model wine solutions presented a higher increase when compared to the controls (*untreated* and *heated* samples). These results show that pressure treatment (500 MPa for 5 min) did not prom ed immediately the Maillard reaction in model wine solutions, contrary to the temperature treatment (60 ºC for 5 min), but have an acceleration during storage.

III.1.3.1 Model solutions containing lysine or serine

In the wine model solutions containing arabinose with lysine or serine (Figure III.1.2), the formation of furfural in the *pressurized* samples presented, after 6 months of storage, no significant differences (p<0.05) when compared with the *heated* samples.

However, after 12 months, the amount of 2-furfural for the *pressurized* samples containing lysine and serine was 2-fold and 3-fold higher, respectively, when compared with the *heated* ones.

After 12 months, the content of 2-furfural was higher in the pressurized sample containing serine (448.95 µg/L) than in the pressurized sample containing lysine (301.68 µg/L). In fact, despite the formation rate of 2-furfural until 6 months of storage was slightly higher for the pressurized solution containing lysine (26.13 µg/L/month) when compared with the pressurized solution containing serine (21.01 µg/L/month), in the last 6 months of storage the formation of 2-furfural was 2.2-fold faster in pressurized samples containing serine than lysine.

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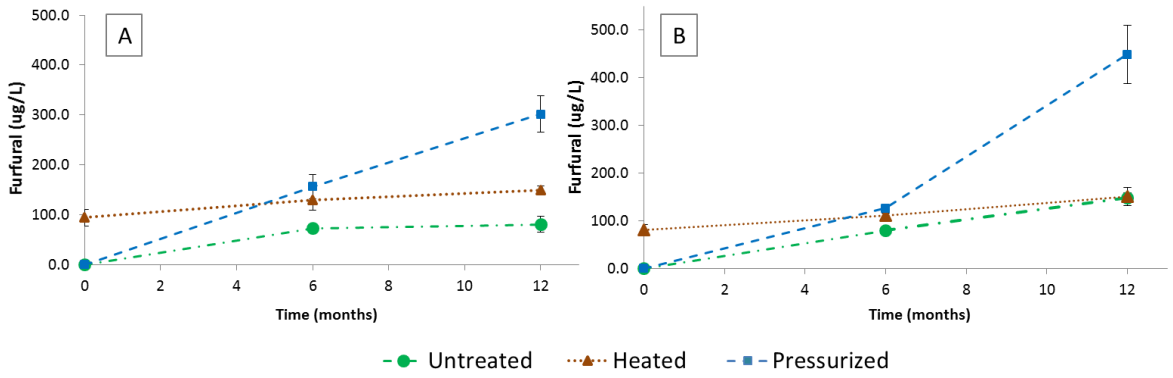

Figure III.1.2. 2-Furfural content in the model wine solutions containing the mixtures of arabinose/lysine (A) and arabinose/serine (B) during 12 months of storage

The possible formation of 5-hydroxymethylfurfural, that can arise from the dehydration of glucose through Maillard reaction, was also evaluated in the model wine solutions containing glucose with lysine or serine. Nevertheless, contrary to furfural that was detected in the model wine solutions containing arabinose, the 5-hydroxymethylfurfural was not detected in any of the model wine solutions. These results seem to indicate that after HHP treatments, the dehydration of pentoses through Maillard reaction might be more facilitated when compared to hexoses. These results are in agreement with the other reports that observed that pentoses, such arabinose (Biemel *et al.*, 2001) and xylose (Hofmann 1999), are more reactive than hexoses (Hwang *et al.*, 1994) during Maillard reactions promoted by high temperature treatments (60-100 ºC,

until 2 hours). Also the type of amino acid had a minor effect when compared to the type of sugar on the formation of both intermediates and final products of the Maillard reaction (Göğüş *et al.*, 1998; Lievonen *et al.*, 2002).

III.1.3.2 Model solutions containing phenylalanine

The content of MVC presented in the model wine solutions containing the mixtures arabinose/phenylalanine and glucose/phenylalanine are shown in Figures III.1.3 and III.1.4, respectively. After 9 months of storage, the *pressurized* model wine solutions containing arabinose and phenylalanine presented higher concentration of 2-furfural (1.6-fold higher), benzaldehyde (1.4-fold higher) and phenylacetaldehyde (2.3-fold higher) compared to the *heated* model wine solution (Figure III.1.3). Also, it can be observed that the formation rate of 2-furfural (Figure III.1.3A) and phenylacetaldehyde (Figure III.1.3C) of the pressurized samples containing the mixture arabinose/phenylalanine were almost constant along the 9 months of storage, contrary to the formation rate of benzaldehyde (Figure III.1.3B) that was 0.97 µg/L/month in the first 6 months of storage and 2.67 µg/L/month in the last 3 months.

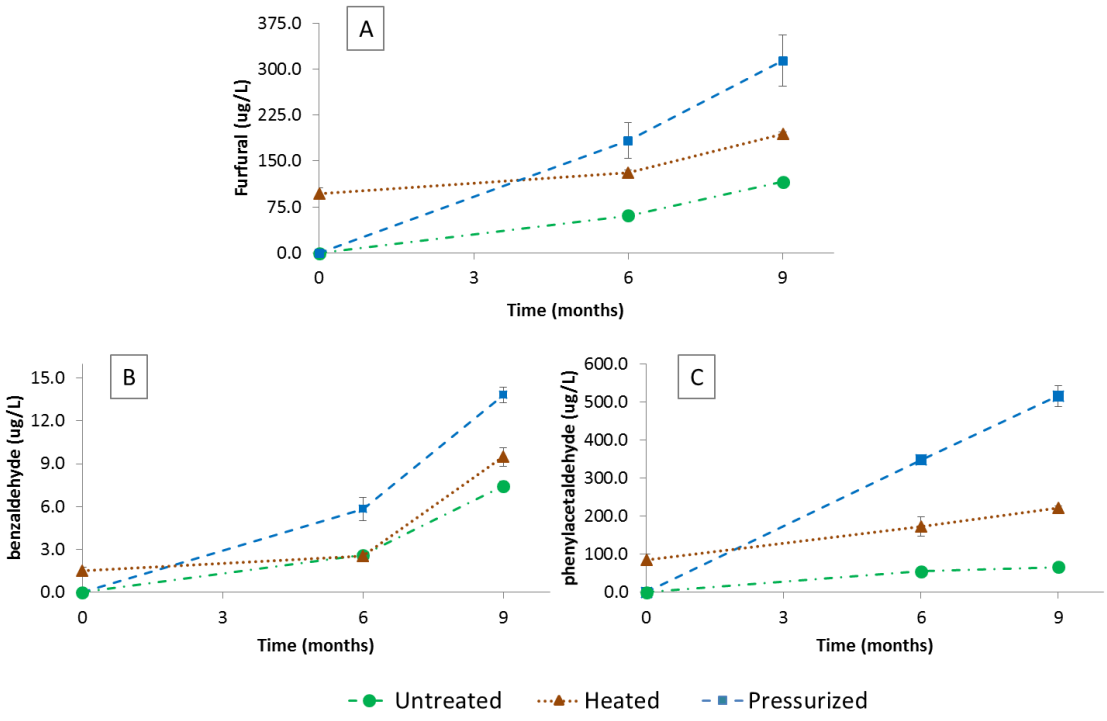

Figure III.1.3. 2-Furfural (A), benzaldehyde (B), and phenylacetaldehyde (C) content of the model wine solutions containing arabinose and phenylalanine during 9 months of storage

For the solutions containing glucose and phenylalanine (Figure III.1.4) the *pressurized* model wine solution presented, after 9 months, a benzaldehyde content 1.5-fold and 1.9-fold higher when compared with the *heated* and *untreated* samples, respectively. However, for the same storage period, the *pressurized* model wine solution presented no significant differences ($p<0.05$) and 4-fold higher phenylacetaldehyde content, when compared with the *heated heated* and *untreated* samples, respectively. For the *heated* and *pressurized* solutions, the formation rate of benzaldehyde (Figure III.1.4A) was slower in the first 6 months of storage (0.19 µg/L/months), than in the last 3 months (0.28 µg/L/months, respectively). However, for the same solutions, the formation rate of phenylacetaldehyde (Figure III.1.4B) was 4.1-fold faster in the first 6 months of storage, than in the last ones.

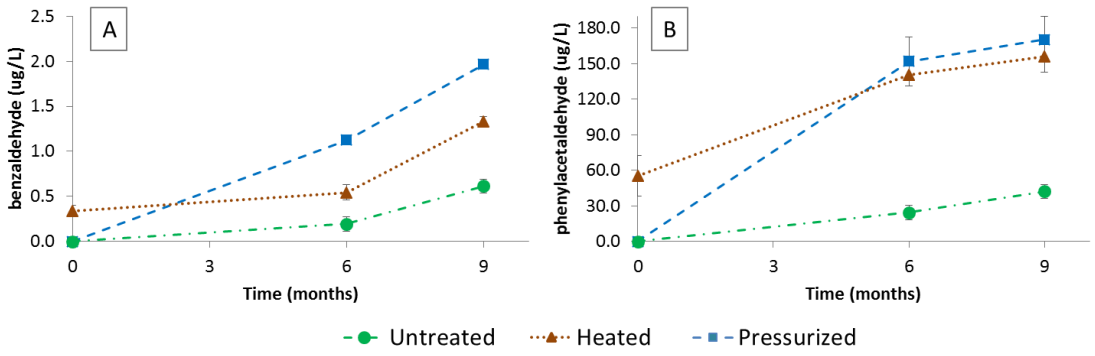

Figure III.1.4. Benzaldehyde (A) and phenylacetaldehyde (B) content of the model wine solutions containing glucose and phenylalanine during 9 months of storage

At the end of storage, the *pressurized* solution containing arabinose presented higher content of benzaldehyde (7-fold higher) and phenylacetaldehyde (3-fold higher) than the *pressurized* solution containing glucose. These results show that Strecker degradation of phenylalanine after HHP treatment and the corresponding formations of benzaldehyde and phenylacetaldehyde was more faster/facilitated in the mixture containing the pentose than the mixture containing the hexose.

Despite the fact that the results showed that high pressure treatment accelerated the Maillard reaction in the model wine solutions, the increase of 2-furfural and benzaldehyde contents in the pressurized model wine solution, after 9 months of storage, was lower that

their respective perception threshold (in wine and in water solutions), which are around 200 mg/L and 2 mg/L for 2-furfural (Cutzach *et al.*, 2000; Prida and Chatonnet, 2010) and benzaldehyde (Perestrelo *et al.*, 2006), respectively. However, the content of phenylacetaldehyde, at the end of storage, in all the model wine solutions containing arabinose/phenylalanine and glucose/phenylalanine was higher than the perception threshold in wine, which is around 15 µg/L (Bakker and Clark, 2012; Campo *et al.*, 2006; Mencarelli and Tonutti 2013), leading possibly to the perception, in these solutions, of "honey-like" and "sweet" odours (Schneider *et al.*, 1998).

Furthermore, the increase of MVC content in the pressurized model wine solutions, when compared with the untreated ones, was lower than that observed in Chapter II.3 for sulphur dioxide-free wines, being the content of 2-furfural and benzaldehyde 5-fold and 15-fold higher, respectively, in white wines pressurized at 500 MPa for 5 min, when compared with the untreated ones. These results are probably due to the higher range of compounds that are affected by the pressure treatment in wine, when compared with the model wine solutions, that indirectly participate (or catalyse) the formation of MVC during storage. In fact, the formation of *ortho*-quinones, that can be formed during oxidation of phenolic compounds in pressurized wines, can react with phenylalanine producing the Strecker aldehydes, benzaldehyde and phenylacetaldehyde (Oliveira *et al.*, 2011; Rizzi 2006). Therefore, it seems that, besides the direct effect of the pressure treatment in the acceleration of reactions between sugars and amino acids, the effect of the pressure treatments on others wine compounds, such phenolic compounds, cause complex chain reactions leading indirectly also to the formation of MVCs.

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III.1.4 Concluding remarks

The results show that high pressure treatment accelerates the Maillard reaction in model wine solutions (acidic media) and this effect is measurable, mainly, after 6 months of storage. Pressurized model wine solutions presented higher concentration of 2-furfural, phenylacetaldehyde, and benzaldehyde, compared to the controls.

5-hydroxymethylfurfural was not detected in any of the model wine solutions, contrary to 2-furfural that was detected in the model wine solutions containing arabinose. These results seem to indicate that the dehydration of pentoses (namely arabinose) through Maillard reaction might be more facilitated when compared to hexoses (namely glucose). Also, Strecker degradation of phenylalanine after HHP treatment and the

corresponding formations of benzaldehyde and phenylacetaldehyde were more facilitated in the mixture containing the pentose than the mixture containing hexose.

Despite the higher concentration of MVC in the pressurized samples, only the content of phenylacetaldehyde was higher than the respective perception threshold in wine, which allow inferring the perception of "honey-like" and "sweet" odours. Furthermore, the increase of MVC content in the pressurized model wine solutions was lower than that observed in wines (Chapter II.3), indicating that probably a high range of compounds are affected by the pressure treatment in wine, when compared with the model wine solutions, that indirectly participate in the formation of MVC during storage.

The implementation of HHP treatments to long term food products preservation, such wine, should be taken into consideration that Maillard reaction can occur influencing the sensorial characteristics of the foods along the storage.

CHAPTER III.2

Evaluation of the effect of high pressure treatments on anthocyanin condensation reactions in model wine solutions

Evaluation of the effect of high pressure treatments on anthocyanin condensation reactions in model wine solutions

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Keywords: High pressure; model wine solution; anthocyanin; polymerization reaction; Mass spectrometry; electrospray

III.2.1 Overview

As mentioned in Chapter I, the colour evolution of red wines is a complex process that is in part attributed to copigmentation phenomena and to the progressive displacement of the original anthocyanins by newly formed pigments (Marquez *et al.*, 2013; Mateus *et al.*, 2002; Pissarra *et al.*, 2004). These pigments usually arise from the interaction between anthocyanins and other phenolic compounds, especially flavanols such as catechins (He *et al.*, 2012). The direct reactions between anthocyanins and flavanols (Remy *et al.*, 2000), between anthocyanins and flavanols through ethyl bridges (Es-Safi *et al.*, 1999; Francia-Aricha *et al.*, 1997), or between anthocyanins and small compounds such as acetaldehyde or pyruvic acid (Benito *et al.*, 2011) have already been demonstrated in model solutions.

The results of Chapter II.1 showed that HHP treatments at room temperature accelerated the polymerization of the wine phenolic compounds, forming compounds with higher degree of polymerization that became insoluble in wine along the storage time. Nevertheless, it was already reported the formation of a vitisin A-type derivative, in model solution, by degradation of cyanidin 3-*O*-glucoside after HHP treatments at high temperatures (Corrales *et al.*, 2008). These results suggest that reactions involving covalent association of anthocyanins with other compounds can be accelerate by pressure treatments. However, the type of polymerization reactions of phenolic compounds promoted by HHP are not yet known. In order to understand this effect, model wine solutions with mixtures of malvidin-3-*O*-glucoside with (+)-catechin or/and acetaldehyde were pressurized at 500 MPa for 5 min at 20 ºC. The compounds formed were analysed immediately after the pressure treatment and after 8 months of storage by electrospray ionization mass spectrometry (ESI-MS) and tandem-mass spectrometry (ESI-MS^n) fragmentation analyses. As control, untreated model wine solutions were also analysed.

III.1.2 Materials and methods

III.1.2.1 Chemicals

Milli-Q water (Millipore, Bedford, MA) was used in all work. Absolute ethanol p.a, methanol p.a, acetaldehyde p.a, formic acid (for mass spectrometry, ~98%), and (+)-catechin (≥98%), were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Malvidin-3-*O*-glucoside (~95%) was extracted and purified in laboratory (Pissarra *et al.*, 2003) from grapes (*Vitis vinifera* L.) and kindly supplied by Professor Victor de Freitas of Faculty of Sciences, University of Porto.

III.1.2.2 Model wine solutions and high pressure treatments

Model wine solutions containing malvidin-3-*O*-glucoside (Mv3Glc) with (+)-catechin or/and acetaldehyde were prepared in 10% aqueous ethanol with a pH of 3.2 adjusted with formic acid using a molar ratio Mv3Glc (0.3 mM)/catechin/acetaldehyde of 1:2:20. The solutions were subjected to the pressure treatment (500 MPa for 5 min at 20 ºC) in polyethylene eppendorfs (500 µL). The pressurization was carried out using a hydrostatic press from Unipress Equipment (Model U33, Warshaw, Poland), with a pressure vessel of 100 mL (35 mm diameter and 100 mm height), surrounded by an external jacket connected to a thermostatic bath in order to control the temperature. A mixture of propylene glycol and water (1:1, v/v) was used as pressurization fluid and the pressure build up was carried out at 450 MPa/min compression rate. Simultaneously, as controls, non pressurized (untreated) model wine solutions were also produced. All model wine solutions were stored in the absence of light at room temperature (~20 ºC) for 8 months.

III.1.2.3 Electrospray Ionization Mass Spectrometry

ESI-MS and ESI-MS^2 spectra of the samples, at the beginning of storage, were carried out on a Q-TOF2 hybrid tandem mass spectrometer (Micromass, Manchester, U.K.). The cone voltage was set at 35 V and the capillary voltage was maintained at 3 kV. The source temperature was 80 ºC and the desolvation temperature was 150 ºC. MS^n spectra were obtained using argon as the collision gas, and the collision energy used was

set between 25 and 30 eV. The raw data were processed using a MassLynx software (version 4.0).

After 8 months of storage, ESI-MS and ESI-MS^n spectra of all the samples were carried out on an LXQ linear ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Typical operating conditions were as follows: electrospray voltage was 5 kV; capillary temperature was 275 ºC; capillary voltage was 1 V; and tube lens voltage was 40 V. Samples were introduced at a flow rate of 8 μL/min into the ESI source. Nitrogen was used as nebulizing and drying gas. In the MS^n experiments, the collision energy used was set between 18 and 31 (arbitrary units). Data acquisitions were carried out on an Xcalibur data system.

For all ESI analyses, samples were diluted (1:17, v/v) in methanol/formic acid (99:1, v/v), and the spectra were acquired in the positive mode, scanning the mass range from *m/z* 100 to 1500.

III.2.3 Results and discussion

III.2.3.1 Evaluation of anthocyanin condensation reaction after high pressure treatment

The model solutions containing the mixtures of 1) Mv3Glc with acetaldehyde; 2) Mv3Glc with catechin; and 3) Mv3Glc with acetaldehyde and catechin were analysed after the high pressure treatment, at the beginning of storage, by ESI-MS. The ESI-MS spectra of the pressurized model solutions are shown in Figure III.2.1. The compounds identified as $[M+H]^+$ and $[M]^+$ ions and the respective MS^2 fragments are shown in Table III.2.1.

Mv3Glc ($[M]^+$ at *m/z* 493) was detected in all model wine solutions as the ion with higher relative abundance, and the catechin ($[M+H]^+$ at *m/z* 291) was detected in the solutions containing this compound.

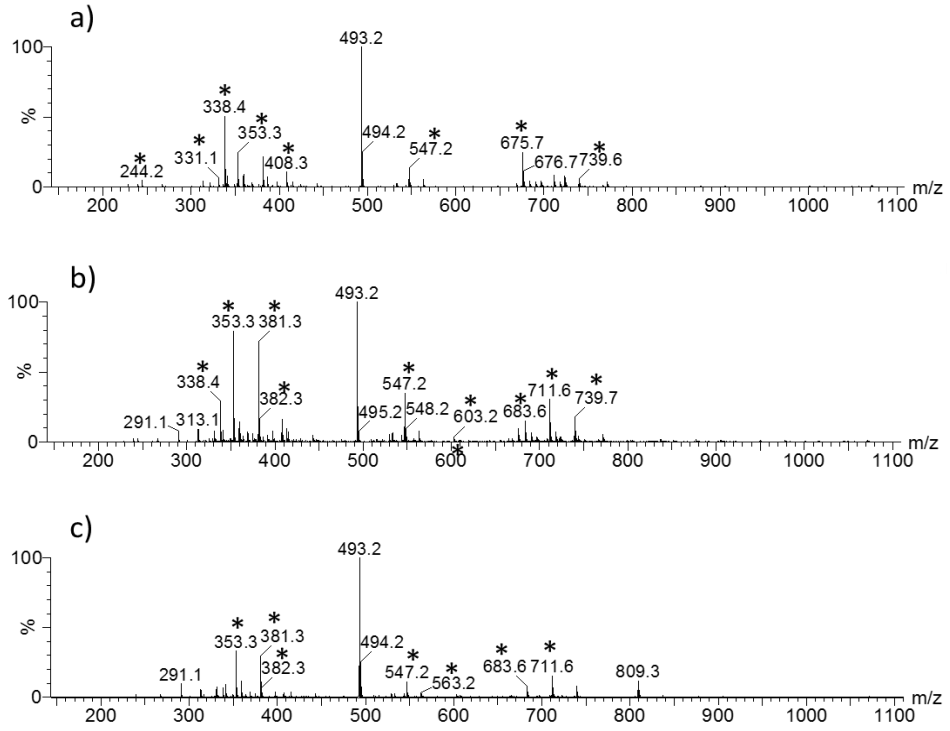

Figure III.2.1. ESI-MS spectra obtained for the pressurized solution containing (a) Mv3Glc and acetaldehyde, (b) Mv3Glc and catechin, and (c) Mv3Glc, acetaldehyde and catechin after high pressure treatment. Ions marked with an asterisk are attributed to impurities

Table III.2.1. Compounds identified in the model wine solutions after high pressure treatment, as $[M+H]^+$ and $[M]^+$ ions in ESI-MS spectra and the respective fragmentation.

Compounds	**m/z**			**Mv3Glc + acetaldehyde**		**Mv3Glc + catechin**		**Mv3Glc + acetaldehyde + catechin,**	
	$[M+H]^+$/ $[M]^+$	Main MS^2 fragments	Main MS^3 fragments	*Untr.*	*Press.*	*Untr.*	*Press.*	*Untr.*	*Press.*
(+)-Catechin	291	273 (-18 Da) 165 (-126 Da) 139 (-152 Da)	-	n.d	n.d	*	*	*	*
Malvidin-3-*O*-glucoside	493	331 (-162 Da)	-	*	*	*	*	*	*
Malvidin-3-*O*-glucoside-8-ethyl-catechin	809	647 (-162 Da) 519 (-290 Da) 357 (-452Da)	357 (-290 Da) 357 (-162 Da)	n.d	n.d	n.d	n.d	*	*

∗: Detected; n.d: not detected; Untr: untreated samples; Press: pressurized samples

The solutions containing Mv3Glc, acetaldehyde and catechin show the formation of a compound, since it was detected a molecular ion at *m/z* 809. Figure III.2.2 shows the schematic fragmentation pathways of the ion at *m/z* 809. The MS^2 of this ion gave the

major ions at *m/z* 647, 519, and 357, corresponding to the loss of one glucose moiety (162 Da), a loss of one flavanol molecule (290 Da), and the loss of both molecules (162 Da + 290 Da), respectively. MS^3 of the ion at *m/z* 647 produced a major ion at *m/z* 357, corresponding to the loss of one flavanol molecule (290 Da). The MS^3 of the ion at *m/z* 519 produced a major ion at *m/z* 357, corresponding to the loss of one glucose moiety (162 Da). These data permit to identify this compound as malvidin-3-*O*-glucoside-8-ethyl-catechin, formed by the reaction between Mv3Glc and catechin, mediated by acetaldehyde. This compound is usually formed during wine storage and is one of a group of pigments that are formed due to the condensation reactions of anthocyanins (de Freitas and Mateus, 2011; Flamini 2013). It plays a crucial role in colour evolution, namely the change of the initial purple-red colour to a more reddish brown hue (de Freitas and Mateus, 2011; Flamini 2013; He *et al.*, 2012). As stated in literature (He *et al.*, 2012; Pissarra *et al.*, 2003), the formation of malvidin-3-*O*-glucoside-8-ethyl-catechin starts with the protonation of acetaldehyde, followed by addition to a nucleophilic position of the flavanol unit; the dehydration of the resulting protonated adduct yields a new carbocation, which suffers a nucleophilic attack by the anthocyanin (Figure II.2.3).

In Table III.2.1, it can be observed that no new compound was formed in the pressurized samples when compared with the untreated ones. These results show that the pressure treatment (500 MPa for 5 min) did not promote, at the beginning of storage, new condensation reactions of the Mv3Glc with the catechin or acetaldehyde.

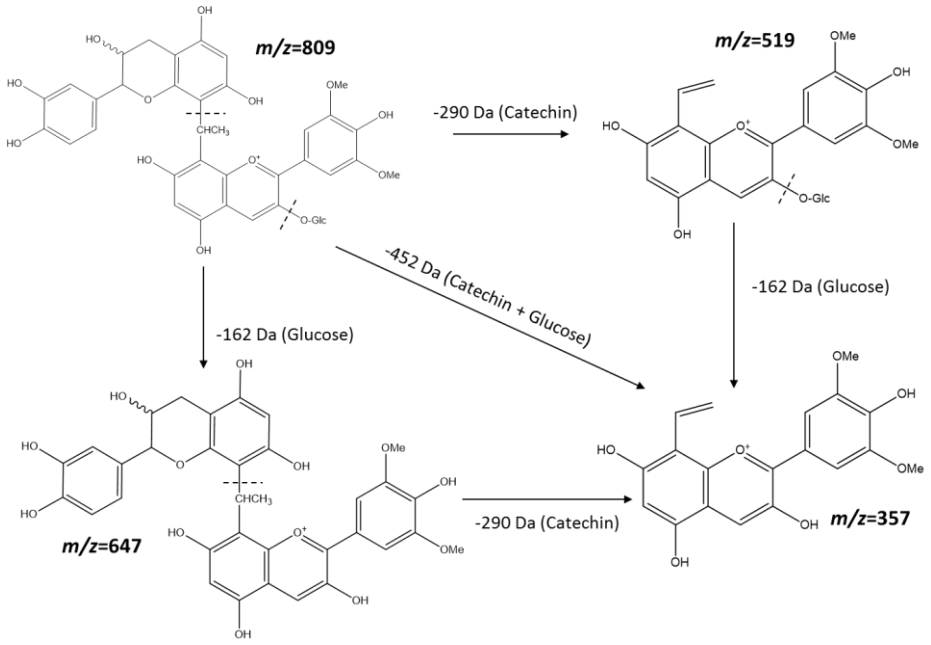

Figure III.2.2. Fragmentation patterns of the ion at *m/z* 809 studied in ESI-MS positive ion mode.

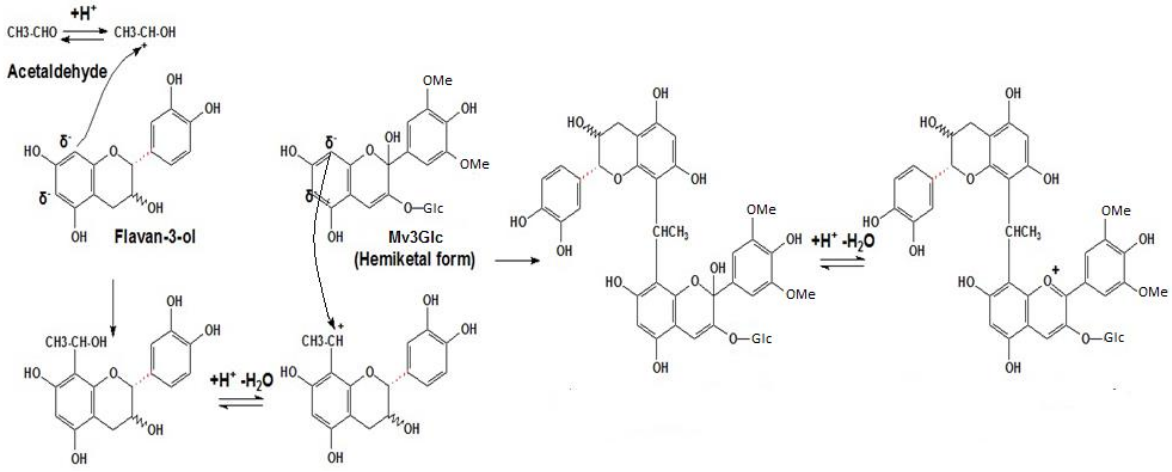

Figure III.2.3. Mechanism proposed for the formation of the malvidin-3-*O*-glucoside-8-ethyl-catechin (*m/z* 809) from the reaction between Mv3Glc and catechin, mediated by acetaldehyde (adapted from Pissarra *et al.*, 2003).

III.2.3.2 Evaluation of anthocyanin condensation reaction after 8 months of storage

After 8 months of storage, in the untreated and pressurized solutions containing the mixture of "Mv3Glc + catechin" and "Mv3Glc + acetaldehyde + catechin" a molecular ion at *m/z* 805 was detected, indicating the formation of a condensation compound (Table III.2.2).The possible formation of malvidin-3-*O*-glucoside-8-ethyl-catechin (*m/z* 809) was also detected in the untreated and pressurized solutions containing Mv3Glc, catechin and acetaldehyde, as observed in the beginning of storage, but also in the untreated and pressurized solutions containing only Mv3Glc and catechin (Table III.2.2). Despite the absence of acetaldehyde addition in the solution "Mv3Glc + catechin", the possible oxidation of ethanol to acetaldehyde during storage (Sun *et al.*, 2007) can explain the presence of this compound in the solution and, consequently, its participation in the catechin-vitisin B and malvidin-3-*O*-glucoside-8-ethyl-catechin formation. The untreated and pressurized solutions containing the mixture of Mv3Glc with acetaldehyde did not present the formation of any compound.

The fragmentation pattern of the molecular ion at *m/z* 805 (Figure III.2.4) was consistent with the structure of catechin-vitisin B. The fragments at *m/z* 397 and 517 confirm that catechin moiety is the upper unit (Figure III.2.4), as reported by other studies (Macz-Pop *et al.*, 2005; Nave *et al.*, 2010), since the presence of these ion fragments are characteristic, discriminating between the catechin-vitisin B and vitisin B-catechin dimers. In contrast to anthocyanins, pyranoanthocyanins are described to be much more

resistant to the attack of water (Oliveira *et al.*, 2009) and consequently could not become nucleophilic enough to react with the flavanol carbocation. Overall, the formation of catechin-vitisin B in the solutions probably arises from the attack of the nucleophilic C8 or C6 position of the anthocyanin molecule to the electrophilic C4 position of the flavan-3-ol, followed by a cycloaddition of the acetaldehyde in its enolic form forming a second pyranic ring between the C4 and the hydroxyl group on the C5 position of the anthocyanin molecule (Figure III.2.5).

Table III.2.2. Compounds identified in the model wine solutions after 8 months of storage, as $[M+H]^+$ and $[M]^+$ ions in ESI-MS spectra and the respective fragmentation.

Compounds	**m/z**			**Mv3Glc + acetaldehyde**		**Mv3Glc + catechin**		**Mv3Glc + acetaldehyde + catechin,**	
	$[M+H]^+$/ $[M]^+$	Main MS^2 fragments	Main MS^3 fragments	*Untr.*	*Press.*	*Untr.*	*Press.*	*Untr.*	*Press.*
(+)-Catechin	291	273 (-18 Da) 165 (-126 Da) 139 (-152 Da)	-	n.d	n.d	*	*	*	*
Malvidin-3-*O*-glucoside	493	331 (-162 Da)	-	*	*	*	*	*	*
Catechin-Vitisin B	805	653 (-152 Da) 643 (-162 Da)	491 (-162 Da) 517 (-126 Da) 491 (-156 Da) 397 (-246 Da)	n.d	n.d	*	*	*	*
Malvidin-3-*O*-glucoside-8-ethyl-catechin	809	647 (-162 Da) 519 (-290 Da) 357 (-452Da)	357 (-290 Da) 357 (-162 Da)	n.d	n.d	*	*	*	*
Unknown	851	699 (-152 Da) 689 (-162 Da)	537 (-162 Da) 473 (-226 Da)	n.d	n.d	n.d	*	n.d	n.d
Unknown	879	717 (-162 Da) 727 (-152 Da) 753 (-126 Da)	699 (-18 Da) 591 (-126 Da) 565 (-152 Da)	n.d	n.d	n.d	*	n.d	n.d

∗: detected; n.d: not detected; U: untreated samples; P: pressurized samples

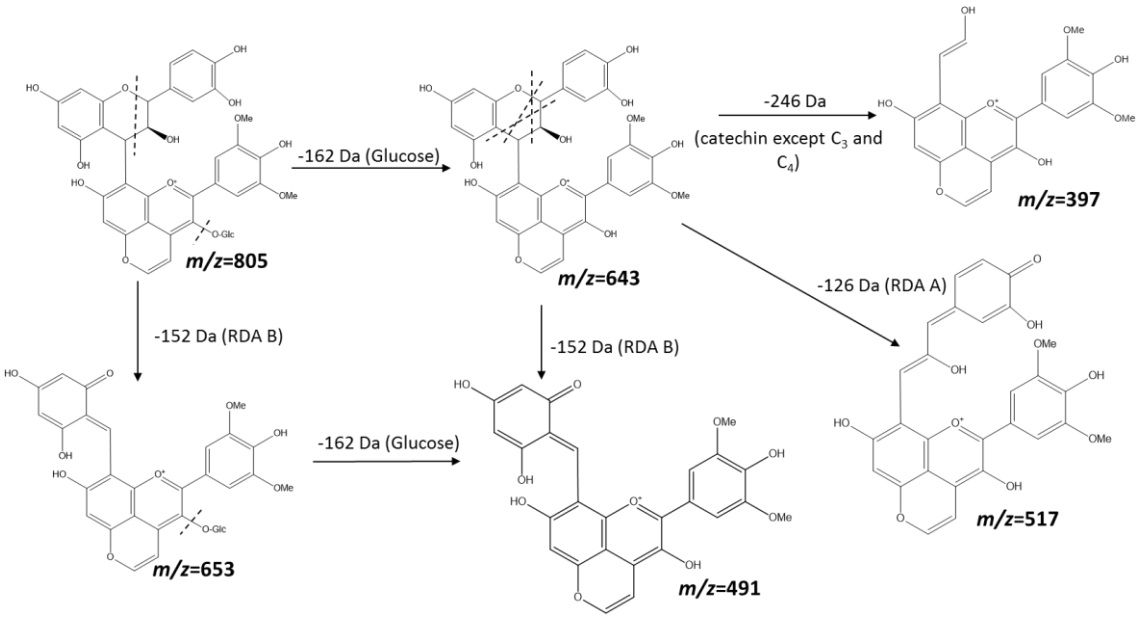

Figure III.2.4. Fragmentation patterns of the ion at *m/z* 805 studied in ESI-MS positive ion mode. RDA A: retro-Diels-Alder reaction with loss of ring A; RDA B: retro-Diels-Alder reaction with loss of ring A.

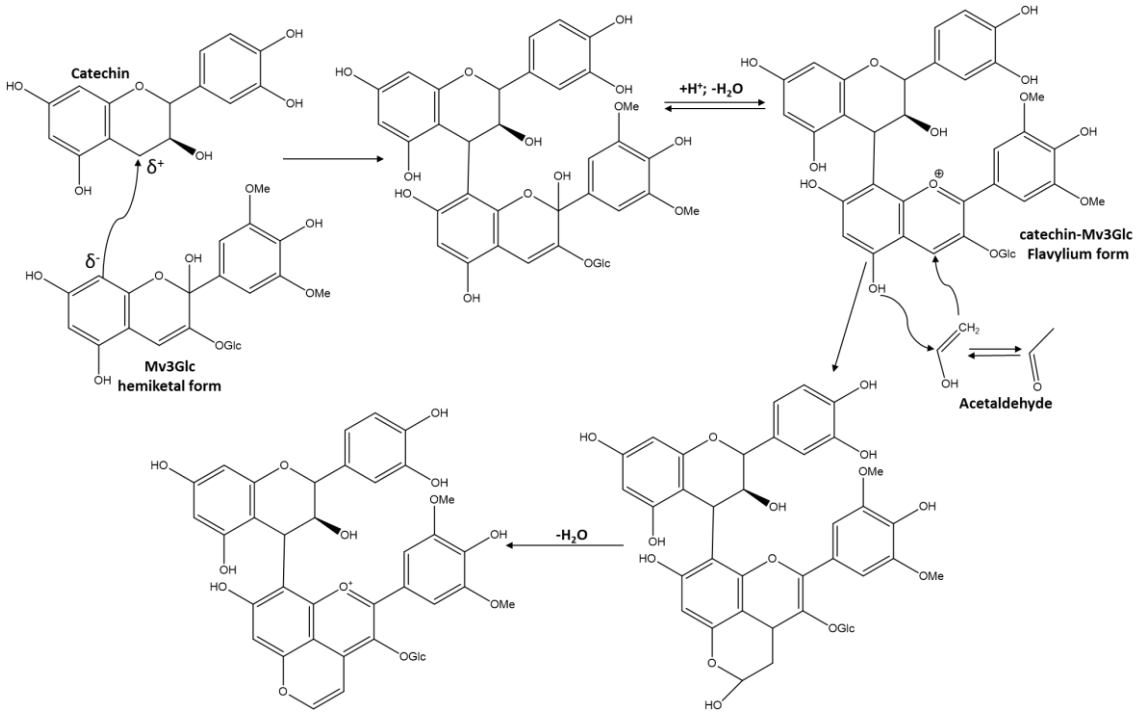

Figure III.2.5. Mechanism proposed for the formation of the catechin-vitisin B (*m/z* 805), adapted from He *et al.*, (2012).

Besides the molecular ions at *m/z* 805 and *m/z* 809, only the molecular ions at *m/z* 851 and *m/z* 879, detected in the pressurized model solution containing Mv3Glc with catechin (Figure III.2.6), indicated a possible effect of the pressure treatment on the formation of new compounds due to condensation reaction. In fact, the MS^2 of the ion at *m/z* 851 and 879 gave a major ion at *m/z* 689 and 717, respectively, corresponding to the loss of one glucose moiety (162 Da), and at *m/z* 699 and 727, respectively, corresponding to the loss of the fragment released by the retro-Diels-Alder (RDA B) decomposition (152 Da). These fragments indicate the presence of Mv3Glc and catechin in the structure of both compounds.

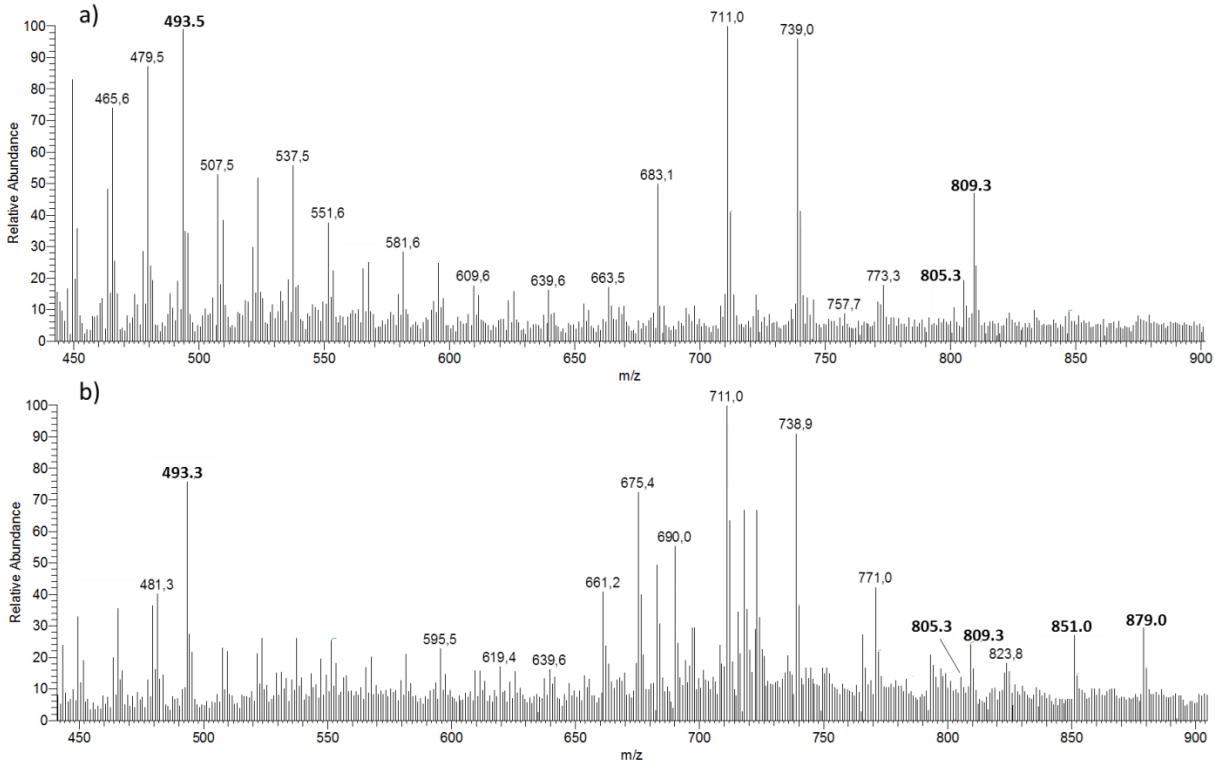

Figure III.2.6. ESI-MS spectra (*m/z* 450 to 900) obtained for the (a) untreated and (b) pressurized solution Mv3Glc and catechin after 8 months of storage.

A few number of structures were reported in the literature for the molecular ion at *m/z* 851, such as malvidin-3-*O*-glucoside-8-(3-methylbutyl)-catechin (Pissarra *et al.*, 2005), malvidin-3-*O*-glucoside-8-(2-methylbutyl)-catechin (Pissarra *et al.*, 2003), malvidin-3-*O*-(6-*O*-acetyl)-glucoside-8-ethyl-catechin (Flamini 2013), and pyruvic acid-catechin-malvidin-3-*O*-glucoside adduct resulting from the addition of the cationic form of pyruvic acid to the C6/C8 carbons of ring A of the catechin moiety or the C6 carbon

of the ring D of the malvidin moiety (Nave *et al.*, 2010). Nevertheless, the absence of malvidin-3-*O*-(6-*O*-acetyl)-glucoside, isovaleraldehyde, 2-methylbutyraldehyde, and pyruvic acid in the model wine solutions, suggest that the compound with the molecular ion at *m/z* 851 could not be one of those reported structures. The molecular ion at *m/z* 879 was not reported in the literature. Both molecular ions at *m/z* 851 and at *m/z* 879 can be derived from the polymerization of Mv3Glc and catechin with a formic acid adduct, since this acid was present in the model wine solution, used to adjust the pH.

III.2.4 Concluding remarks

This study show that pressure treatment (500 MPa for 5 min) did not promote, at the beginning of storage, condensation reactions between anthocyanins and flavonols in the model wine solutions.

After 8 months of storage, all the model solutions containing Mv3Glc with catechin and Mv3Glc with catechin and acetaldehyde presented the formation of condensation compounds, namely catechin-vitisin B and malvidin-3-*O*-glucoside-8-ethyl-catechin. Conversely, the pressure treatment promoted the formation of two unknown compounds in the mixture of Mv3Glc with catechin, which appear to result from the polymerization of Mv3Glc and catechin with other compounds presented in the model wine solution (potentially formic acid). Therefore, it is possible that the pressure treatment had a higher impact in terms of kineticks of reactions and in less extent in terms of different compounds formed. Also, it is feasible that, besides a possible direct effect of the pressure treatment in the polymerization of anthocyanins, the effect of the pressure treatments on other wine compounds can cause complex reactions leading indirectly to the acceleration of condensation reaction of anthocyanins in wine.

CHAPTER IV

Application of high hydrostatic pressure treatments in winemaking to accelerate wine aging

CHAPTER IV.1. Impact of high pressure treatments on the phenolic composition of a red wine after storage: occurrence of aged-like characteristics

CHAPTER IV.2. Impact of high pressure treatment on the phenolic composition of a red wine: comparison with different aging processes

CHAPTER IV.1

Impact of high pressure treatments on the phenolic composition of a red wine after storage: occurrence of aged-like characteristics

Impact of high pressure treatments on the phenolic composition of a red wine after storage: occurrence of aged-like characteristics

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KEYWORDS: High pressure; Red wine; Wine aging; Anthocyanins; Flavan-3-ols; Flavonols; Degree of polymerization; Tannin

IV.1.1 Overview

During the aging of wine, phenolic compounds participate in several reactions, namely polymerization and oxidations, and could be used to follow the aging process of wine (Chen *et al.*, 2012; Oliveira *et al.*, 2011; Ribéreau-Gayon *et al.*, 2006; Soto Vázquez *et al.*, 2010). Also, it was mentioned in Chapter II.1 that HHP treatments promote reactions that are similar to those observed during red wine aging, giving rise to sensorial characteristics of an aged-like wine. Given that, it seems possible to exploit this technology for production of young red wines with novel pleasant and distinct characteristics to address market and consumer demand. Therefore, the development of a novel aging-like HHP-based methodologies that can modify wine composition could benefit the wine industry, especially to improve wines with low aging potential (Tao *et al.*, 2014).

The aim of this work was to study the effect of high hydrostatic pressure treatments on the phenolic composition of a red wine after storage, since these compounds play an important role in wine colour and taste that are important wine quality parameters. For this purpose, a red wine was pressurized at two pressure conditions, 500 and 600 MPa, for 5 and 20 min at 20 ºC, respectively.

IV.1.2 Materials and methods

IV.1.2.1 Chemicals

Milli-Q water (Millipore, Bedford, MA) was used in all this work. HPLC-grade methanol, acetonitrile and formic acid (Merck, Darmstadt, Germany) were used. Delphinidin-3-*O*-glucoside (≥95%), cyanidin-3-*O*-glucoside (≥96%), petunidin-3-*O*-glucoside (≥95%), peonidin-3-*O*-glucoside (≥95%), malvidin-3-*O*-glucoside (≥95%), procyanidins B1 (≥80%), B2 (≥90%), B4 (≥80%) and C1 (≥80%), and phloroglucinol were purchased from Extrasynthese (Lyon, Genay-France). Gallic acid (≥99%), protocatechuic acid (≥90%), ferulic acid (≥90%), caftaric acid (≥95%), vanilic acid (≥95%), caffeic acid (≥95%), syringic acid (≥95%), *p*-hydroxybenzoic acid (≥98%), coutaric acid (≥90%) , *p*-coumaric acid (≥98%), sinapic acid (≥98%), chlorogenic acid (≥95%), myricetin (≥96%), quercetin (≥96%), kaempferol (≥90%), (+)-catechin (≥98%), and (−)-epicatechin (≥98%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

IV.1.2.2 Wine samples and high pressure treatments

Red wine samples were produced by Dão Sul SA (Carregal do Sal, Portugal) using Tinta Roriz (50%) and Touriga Nacional (50%) red grape varieties from Dão Appellation from 2013 harvest. An industrial batch fermenter of 16,000 L was used. After malolatic fermentation, the wine was transferred to 250 mL polyethylene bottles, stoppered, and pressurized at 600 MPa during 20 min or 500 MPa during 5 min, at 20 °C, in a hydrostatic press (Hiperbaric 55, Hiperbaric, Burgos, Spain), giving origin to samples *600 MPa* and *500 MPa*, respectively. The HHP equipment has a pressure vessel of 200 mm inner diameter and 2,000 mm length and a maximum operation pressure of 600 MPa. It was connected to a refrigeration unit (RMA KH 40 LT, Ferroli, San Bonifacio, Italy) that allowed to control the temperature of the input water used as a pressurizing fluid. Pressurizing water had a controlled temperature of 15 °C. Pressure build-up took place at a compression rate of about 600 MPa/min (adiabatic heating caused an increase in temperature of about 2.0 °C), while decompression was nearly instantaneous. A lot of the same wine was not submitted to high pressure treatment (*unpressurized*) and also bottled in the polyethylene bottles. All wines were stored at 80% relative humidity in the absence of light at room temperature ranging between 20 and 25 °C.

IV.1.2.3 Oenological parameters determination

The ethanol content, titratable acidity, volatile acidity, pH, reducing sugars, free and bound SO_2, HCl and gelatine indexes were determined for each wine according to the methods described by the Organisation International de la Vigne et du Vin (OIV 1990; Ough and Amerine, 1988; Ribéreau-Gayon *et al.*, 2006). All analyses were carried out in triplicate.

IV.1.2.4 Colour determination

The colour intensity (CI) was calculated as the sum of the absorbance values at 420 nm, 520 nm, and 620 nm and the colour tonality was determined by dividing the absorbance at 420 nm by the absorbance at 520 nm. Absorbance measurements were recorded on an Uvikon 922 spectrophotometer (Kontron Instruments, Saint Quentin en Yvelines, France). The contribution of each coloration (yellow, red, and blue) to the overall colour of wine was calculated by dividing the absorbance at 420 nm (Ye%), 520 nm (Rd%), and 620 nm (Bl%) by the colour intensity (CI). The proportion of red coloration produced by free and bound anthocyanins under their flavylium cations form (dA%) was calculated using the following formula, as describe by Kelebek *et al.,* (2010):

$$\mathrm{dA\%} = \left[1 - \frac{\mathrm{Abs420} + \mathrm{Abs620}}{2 \times \mathrm{Abs520}}\right] \times 100$$

IV.1.2.5 Phenolic Composition by spectrophotometric methods

The total phenolic (TP) content of the samples was determined by the Folin–Ciocalteu method (Singleton 1985). The samples were appropriately diluted in a solution of 10% ethanol. The calibration curve was performed using gallic acid as standard in a concentration range between 50 and 500 mg/L. The results were expressed as gallic acid equivalents.

Total proanthocyanidins were estimated according to Chira *et al.,* (2011a). This method is based on the Bate–Smith reaction, in which the proanthocyanidins in acid medium release anthocyanidins by heating. The wines were diluted to 1:50 in a 10% ethanol solution. One millilitre of the samples was added to 0.5 mL of water and 1.5 mL of 12 M HCl and the mixture was homogenised. Two tubes for each sample were

prepared: one was heated for 30 min in boiled water (sample A), while the other was maintained at room temperature (sample B). To each tube, 0.25 mL of 95% ethanol were added. The absorbance at 550 nm was then read through a 10 mm optical path. Total proanthocyanidins (g/L) were calculated as $19.33 \times (abs_{550nm}A - abs_{550nm}B)$.

Anthocyanins (Anth) were determined using the SO_2 bleaching method (Chira *et al.*, 2011b). A solution "A" was prepared as follows: 1 mL of wine, 1 mL of 0.1% HCl ethanol and 20 mL of 2% HCl. Blank (B) was prepared as follows: 2 mL of solution "A" and 0.8 mL of water. Sample (S) was prepared as follows: 2 mL of solution "A", 0.4 mL of water and 0.4 mL of $NaHSO_3$. After 20 min at room temperature, the absorbance at 520 nm was measured through a 10 mm optical path. Anth (mg/L) were calculated as $875 \times (Abs_{520nm}B - Abs_{520nm}S)$.

The proportion of polymerized pigments (%PP) was estimated according to Ribereau-Gayon and Stonestreet (1965). The assay consist to discolour the free fraction of anthocyanins with sodium metabisulphite ($Na_2S_2O_5$). For this, 1 mL of wine sample was placed in two test tubes with 9 mL of synthetic wine solution at pH 3.2. In one of the tubes (tube M), 40 µL of 20% sodium metabisulphite was added, and in the other tube (tube C) 40 µL of distilled water was added. The absorbance at 420 and 520 nm were measured on each of the tubes and the percentage of polymerized pigments calculation was performed using the formula:

$$\%PP = \left[\frac{(Abs420_M + Abs520_M)}{(Abs420_C + Abs520_C)}\right] \times 100$$

IV.1.2.6 Phenolic compounds composition by HPLC-MS

Samples were filtered through a 0.45 µm pore size membrane filter before injection. Analysis was performed on a Thermo-Finnigan Accela HPLC system consisting of an autosampler (Accela autosampler), a pump (Accela 600 Pump), a diode array detector (Accela PDA Detector) coupled to a Finnigan Xcalibur data system. Separation was performed on a reversed phase Agilent Nucleosil C18 (4.6 mm × 250 mm, 5 µm) column. Triplicate analyses were performed for each sample

For the monomeric anthocyanins analysis, the eluents used, water/formic acid (99:1, v/v) (solvent A) and acetonitrile/formic acid (99:1, v/v) (solvent B), were applied at a flow rate of 1 mL/min as follows: 10–35% B linear from 0–25 min, 35–100% B linear

from 25–26 min, 100% B isocratic from 26–28 min, 100–10% B linear from 28–29 min, with the re-equilibration of the column from 29–35 min under the initial gradient conditions. Detection was conducted at 520 nm. Quantification was performed by a comparison to malvidin-3-*O*-glucoside calibration curve.

For phenolic acids and flavonols analysis the mobile phase consisted of two solvents: Solvent A, water/formic acid (95:5; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v). Phenolic compounds were eluted under the following conditions: 1 mL/min flow rate and the temperature was set at 25 ºC, isocratic conditions from 0 to 15 min with 100% A, gradient conditions from 0% to 20% B in 30 min, from 20% to 50% B in 40 min, and from 50% to 100% B in 5 min, isocratic conditions with 100% B during 10 min, followed by washing and reconditioning the column. The ultra-violet-visible spectra (scanning from 200 nm to 600 nm) were recorded for all peaks. Identification of phenolic compounds were performed by comparison with their retention times and UV spectra of authentic standards and also confirmed by mass spectrometry analysis. Quantification was performed using external calibration curves using gallic acid, caffeic acid and kaempferol for benzoic acids, cinnamic acids and flavonols quantification, respectively, in the concentrations range normally present in wine (approximately 0.2–200 mg/L) and the obtained regression coefficients (r^2) were above 0.992 in all cases.

IV.1.2.7 HPLC-UV-Fluor/MS analysis of monomeric and oligomeric flavan-3-ols and mean degree of polymerisation (mDP)

The equipment used was a Thermo-Finnigan Surveyor HPLC system formed by UV–Vis detector (Surveyor PDA Plus), an autosampler (Surveyor autosampler Plus) and a quaternary pump (Surveyor LC pump Plus) controlled by Xcalibur data treatment system. This HPLC System was also coupled to a Thermo-Finnigan LCQ Advantage spectrometer equipped with an ion trap mass analyser.

The separation of monomeric and oligomeric flavan-3-ols was performed on a reversed phase Agilent Nucleosil C18 (250 mm × 4 mm, 5 μm). Water/formic acid (solvent A) (99:1, v/v) and acetonitrile/formic acid (99:1, v/v) (solvent B) were used at a flow rate of 1 mL/min. The gradient conditions were: 3% B isocratic from 0–3 min, 3–5% B linear from 3–14 min, 5–10% B linear from 14–22 min, 10–14% B linear from 22–26 min, 14–25% B linear from 26–40 min, 25– 100% B linear from 40–41 min, 100% B isocratic from 41–43 min, and 100–3% B linear from 43–44 min, with re-equilibration of

the column from 44–50 min under the initial gradient conditions. Detection was performed with a fluorescence detector set at 280 nm excitation wavelength and 320 nm emission wavelength with medium fluorescence intensity; as well as a diode array detector set at 280 nm. Identification of monomeric and oligomeric flavan-3-ols was carried out by comparison to the retention time of external standards ((+)-catechin, (-)-epicatechin, and procyanidins B1, B2, B4 and C1) and also confirmed by HPLC-MS analysis. Quantification was performed using external standard calibration curves.

For the determination of mean degree of polymerization (mDP), a solid-phase extraction (SPE) step was used to purify the wines. Each sample was diluted (3 times) and applied (10 mL) on a LC18 (octadecyl bonded, endcapped silica) cartridge (Supelco, St Quentin Fallavier, France) The column was washed with 50 mL of water and eluted with 50 mL of methanol. The methanol fraction was dried under reduced pressure, redissolved in 2 mL of methanol and used for mDP determination. The proanthocyanidin mDP concentrations were quantified by phloroglucinolysis (Drinkine *et al.*, 2007). Reversed-phase HPLC analysis of the products formed allowed the determination of the structural composition of proanthocyanidins, which was characterised by the nature of their constitutive extension units (released as flavan-3-ols phloroglucinol adducts) and terminal units (released as flavan-3-ols). These analyses were carried out in triplicate on a column Xterra RP18 (100 mm × 4.6 mm, 3.5 μm, Waters, France). The elution conditions were: solvent A, water/acetic acid (99:1, v/v); solvent B, methanol. The elution gradient for the analysis of the reaction mixture was as follows: 5% B for 25 min, a linear gradient from 5 to 32% B in 45 min, a linear gradient from 30 to 100% B in 2 min. The column was then washed with 100% B for 5 min and re-equilibrated with 5% B for 10 min. To calculate the apparent mDP, the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in molar basis) was divided by the sum of all flavan-3-ol monomers. To calculate the percentage of prodelphinidins (%P) the sum of (-)-epigallocatechin (EGC) subunits [(∑Pterminal units (EGC) concentrations) + (∑Pextension units (EGC-P) concentrations)] was divided by the sum of all flavan-3-ol.

IV.1.2.8 Sensorial analysis

A blind tasting test was done to the wines after 5 months of bottling by 25 expert panellists from the Oenology department of the University of Bordeaux. Wines (30 mL)

were presented in transparent glasses coded with a three-digit random code and distributed in a completely randomized order. In each session a descriptive analysis of each wine was conducted.

All tasters were informed that the wines had different treatments, but the panellists did not have any details of the experimental design. Three samples were given to each panellist: *unpressurized*, *500 MPa*, and *600 MPa*. Wines were evaluated on a predefined score sheet (intensity scale from 0 to 6) that included 23 descriptors in three categories: colour, aroma, and taste attributes. Also, a global evaluation was done on a scale from 0 to 10 in 4 categories: colour, aroma, taste, and global attributes. Averages of the scores for each descriptor were calculated.

IV.1.2.9 Statistical analysis

Statistical data analysis was performed using Analysis of Variance (ANOVA). Tukey's HSD Test was used for the data as comparison test when samples analyses showed significant differences after ANOVA ($p<0.05$).

IV.1.3 Results and discussion

IV.1.3.1 Effect of HHP treatments on the wine physicochemical characteristics

The physicochemical characteristics of the wine samples at the beginning of storage and after 5 months of bottle aging are summarized in Table IV.1.1. At the beginning of storage no significant difference among the different wine samples was observed for all the parameters analysed ($p<0.05$), indicating that both pressure treatments did not affect the physicochemical characteristics of the wine immediately after the pressure treatments. These results are in accordance with results obtained in Chapter II.1 and with previous studies that showed that pressure treatments around 300 to 600 MPa for few minutes have no impact in red wine properties at the beginning of storage (Mok *et al.,* 2006; Tao *et al.,* 2012).

After 5 months of storage all wine samples presented no significant differences among them in terms of density, ethanol content, titratable and volatile acidity, reducing sugars content, and lactic and tartaric acid content. The composition of wines were in

accordance with previous studies carried out on Touriga Nacional and Tinta Roriz wines (Jordão *et al.,* 2012; Rodrigues *et al.,* 2012, 2013). The wine samples presented values of HCl index around 20 and a gelatin index around 55. The HCl index represents the tannin polymerization level, and the gelatin index measures tannin reactivity toward proteins and, therefore, assesses wine astringency. The red wine HCl index usually ranges between 10 and 30 and the acceptable values of the gelatin index are between 40 and 60, in order to have a red wine with a satisfactory astringency for the consumer (Ribéreau-Gayon *et al.,* 2006). Therefore, overall the comparison of the three wines is in accordance with previously reported data on high quality red wine (Glories 1984; Ribéreau-Gayon *et al.,* 2006), showing that the red wine produced can be considered a high quality red wine regarding colour specificities, and HCl and gelatin indexes. After 5 months of bottle aging *unpressurized* wine presented less 41 and 46% content of free and total SO_2, respectively, when compared with the beginning of storage.

Table IV.1.1. Physicochemical analysis of the different wine samples at the beginning and after 5 months of bottle storage

Analysis / wine samples	Beginning of storage			5 months		
	Unpressurized	***500 MPa***	***600 MPa***	***Unpressurized***	***500 MPa***	***600 MPa***
Density (20 ºC/20 ºC)	0.9907 ±0.00	0.9906 ± 0.00	0.9906 ± 0.00	0.9915 ± 0.00	0.9915 ± 0.00	0.9915 ± 0.00
Ethanol (v/v; %)	13.67 ± 0.01	13.66 ± 0.00	13.63 ± 0.01	13.61 ± 0.01	13.61 ± 0.01	13.61 ± 0.01
Titratable acidity[a] (g/L)	2.80 ± 0.00	2.75 ± 0.00	2.72 ± 0.01	3.11 ± 0.01	3.13 ± 0.01	3.12 ± 0.00
pH	3.59 ± 0.00	3.58 ± 0.01	3.58 ± 0.01	3.62 ±0.00	3.61 ±0.00	3.62 ±0.00
Volatile acidity[b] (g/L)	0.40 ± 0.01	0.38 ± 0.02	0.38 ± 0.01	0.45 ± 0.01	0.42 ± 0.00	0.41 ± 0.00
Reducing sugar (g/L)	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.0	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
Free SO_2 (mg/L)	34.33 ± 0.58	33.33 ± 1.52	35.33 ± 1.15	20.33 ± 0.58	12.33 ± 0.58	11.67 ± 0.58
Total SO_2 (mg/L)	74.67 ± 0.58	74.33 ± 1.15	74.67 ± 1.5	40.33 ± 0.58	22.33 ± 1.53	25.33 ± 1.53
Lactic Acid (g/L)	1.06 ± 0.01	1.01 ± 0.02	0.99 ± 0.03	1.04 ± 0.00	1.03 ± 0.01	1.03 ± 0.01
Tartaric acid (g/L)	1.33 ± 0.01	1.19 ± 0.02	1.12 ± 0.01	2.22 ± 0.02	2.24 ± 0.01	2.21 ± 0.03
HCl index	28.8 ± 0.04	26.28 ± 0.93	29.33 ± 0.46	18.66 ± 0.25	21.49 ± 0.45	21.33 ± 0.19
Gelatin index	55.06 ± 0.65	53.83 ± 0.47	58.58 ± 1.71	55.97 ± 3.23	56.04 ± 5.74	60.81 ± 7.75
Tannin (g/L)	3.38 ± 0.12	3.31 ± 0.26	3.68 ± 0.27	3.46 ± 0.06	3.90 ± 0.07	3.77 ± 0.13
Phenolic compounds[c] (mg/L)	3454.33 ± 31.82	3681.82 ± 200.68	3644.42 ± 66.27	3624.50 ± 27.41	3550.08 ± 52.78	3493.91 ± 77.72
Anthocyanins[d] (mg/L)	519.98 ± 4.85	509.42 ± 10.72	522.81 ± 10.04	401.48 ± 3.99	387.33 ± 1.53	389.40 ± 2.81
Pigments polymerization (%)	58.15 ± 0.21	58.47 ± 0.08	61.78 ± 1.02	66.35 ± 0.29	71.62 ± 0.47	73.90 ± 0.27
Colour intensity	0.92 ± 0.01	1.11 ± 0.03	1.11 ± 0.01	0.87 ± 0.01	0.81 ± 0.02	0.83 ±0.01
Colour tonality	0.62 ± 0.01	0.67 ± 0.02	0.67 ± 0.01	0.75± 0.01	0.74 ± 0.02	0.75 ± 0.01
Ye%	34.10 ± 0.02	35.25± 0.90	36.03 ± 0.04	36.89 ± 0.20	37.79 ± 0.52	37.54 ± 0.37
Rd%	54.80 ± 0.02	52.77 ± 1.33	53.43 ± 0.05	49.18 ± 0.16	50.92 ± 0.67	49.85 ± 0.18
Bl%	11.09 ± 0.01	11.97 ± 2.22	10.53 ± 0.01	13.91 ±0.05	11.28 ± 0.20	12.60 ± 0.19
dA%	58.77 ± 0.03	55.22 ± 2.42	56.42 ± 0.09	48.34 ± 0.32	51.80 ± 1.29	49.71 ± 0.37

All data are expressed as mean value ± standard deviation (n = 3); [a] Expressed as tartaric acid equivalent; [b] expressed as acetic acid equivalent; [c] expressed as gallic acid equivalents; [d] expressed as malvidin-3-glucoside equivalents.

The pressurized wines presented a more pronounced decrease of SO_2 content during storage, since the samples *500 MPa* and *600 MPa* presented around 70% less of both free and total SO_2 after 5 months of storage. Sulphur dioxide plays an important role against oxidation in wine, since it acts in three different ways: direct oxygen scavenging; reacting with hydrogen peroxide; and reducing the quinones formed during the oxidation process back to their phenol form (Karbowiak *et al.,* 2010; Oliveira *et al.,* 2011). Once in wine, SO_2 may react with several constituents, namely acetaldehyde, pyruvic acid, 2-oxoglutaric acid, and, to a lesser extent, anthocyanins, cinnamic acids, and reducing sugars, contributing for the modulation of the wine properties (Karbowiak *et al.,* 2010; Ribéreau-Gayon *et al.,* 2006). The reaction with these compounds reduces the rate of phenolic polymerization and, consequently, the colour loss usually observed during wine aging. Nevertheless, it is already reported the generation of high-reactive radicals during pressurization (Bolumar *et al.,* 2012b; Tao *et al.,* 2012), and once radicals are generated, an increased oxidation level could take place (Bolumar *et al.,* 2012b). Therefore, HHP seems to alter the equilibrium of the SO_2 reaction in wine during storage, since the free SO_2 might react with the radicals formed in the pressure treatments, leading to a more pronounced decrease of SO_2 content in pressurised wine during storage. Despite the decrease of SO_2 content in pressurized wines, the pressurized wines did not present significant differences in the colour values (p<0.05) when compared with the *unpressurized* wine, indicating that the pressure treatments did not seem to promote the combination between SO_2 and anthocyanins during storage and the content of sulphur dioxide in these wines appears to remain enough to decrease the rate of the colour loss during wine aging.

After 5 months of storage the pressurized wines presented a slightly higher (p<0.05) tannin content and slightly lower content (p<0.05) of total anthocyanins than the *unpressurized* wine. In terms of percentage of polymerized pigments, the difference between the wine samples are more pronounced, since the samples *500 MPa* and *600 MPa* presented, respectively 5% and 7% more polymerized pigments when compared with the *unpressurized* wine. Therefore, the applied HHP treatments affected the rate of anthocyanins polymerization, leading to a higher percentage of polymerized pigment. These results are in agreement with the results obtained in Chapter II.1 for SO_2-free red wine that showed that HHP treatments (400-500 MPa for 5 min) increase of condensation reactions of phenolic compounds, resulting in compounds with higher polymerization degree, namely anthocyanins and proanthocyanidins, along the wine aging.

IV.1.3.2 Effect of HHP treatments on the wine phenolic compounds composition.

Monomeric anthocyanin composition

Nine different monomeric anthocyanins (MA), including five glucosides, two acetyl glucosides, and two coumaroyl glucosides were identified and quantified in the wine samples (Figure IV.1.1). All samples showed during storage a decrease in the individual anthocyanin content and, consequently, a decrease in total monomeric anthocyanins content (41% to 51% less), when compared with the begging of storage (Table IV.1.2). This decrease was mainly due to the high decrease of malvidin 3-glucoside content, the MA present in higher content in red wines. Therefore, the decrease observed in MA during aging in bottle should be mainly due to their participation in numerous condensation reactions, as well as in hydrolytic and other degradation reactions (Monagas *et al.*, 2006; Santos-Buelga *et al.*, 1999) in a minor extent.

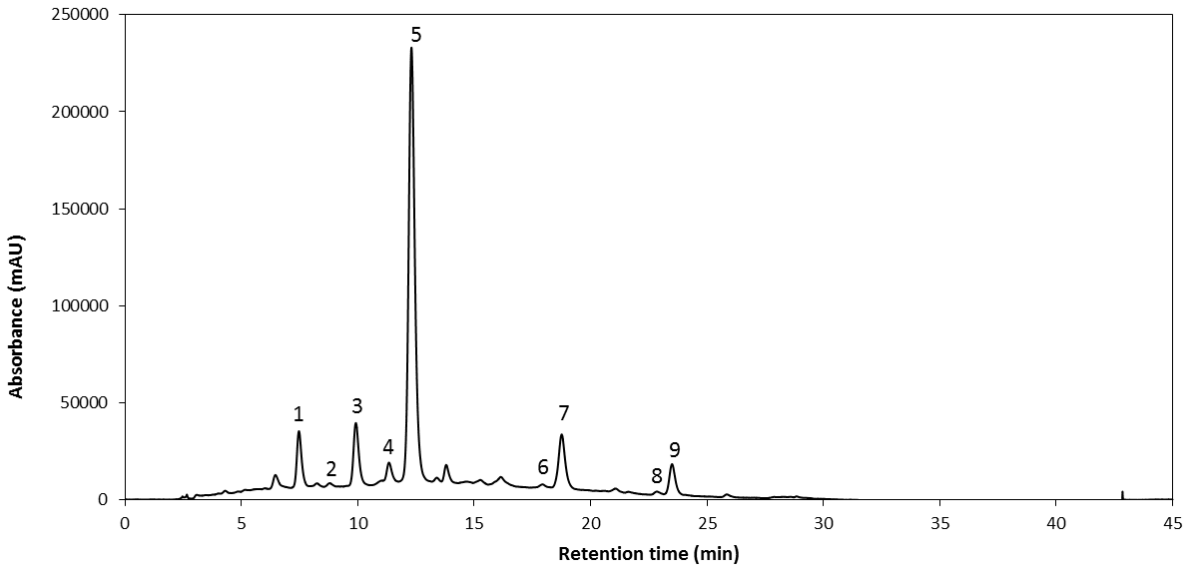

Figure IV.1.1. HPLC chromatogram of *unpressurized* wine at the beginner of storage recorded at 520 nm. Identification: 1. Delfinidin-3-*O*-glucoside, 2. Cyanidin-3-*O*-glucoside, 3. Petunidin-3-*O*-glucoside, 4. Peonidin-3-*O*-glucoside, 5. Malvidin-3-*O*-glucoside, 6. Peonidin-3-*O*-(6-*O*-acetyl)-glucoside, 7. Malvidin-3-*O*-(6-*O*-acetyl)-glucoside, 8: Peonidin-3-*O*-(6-*O*-*p*-coumaryl)-glucoside, 9. Malvidin-3-*O*-(6-*O*-*p*-coumaryl)-glucoside.

After 5 months of storage, the pressurized wines presented lower content of delphinidin-3-glucoside (10% less), petunidin-3-glucoside (13 to 15% less), malvidin-3-glucoside (13 to 15% less), malvidin-3-glucoside-acetaldehyde (15 to 16% less) and malvidin-3-(6-*p*-coumaroyl)-glucoside (14% less) when compared with the

unpressurized sample. Therefore, samples *500 MPa* and *600 MPa* presented, respectively, 13% and 14% less of total MA content than the *unpressurized* sample. These results are in agreement with previous works which showed that final anthocyanin content of red wine is affected by HHP treatment, due to the acceleration of condensation reactions during the wine storage period, involving monomeric anthocyanins and also other phenolic compounds (Tao *et al.*, 2012). However, the effect of the pressure treatments on the anthocyanins content did not alter significantly the colour of the wines (Table IV.1.1), contrary to a SO_2-free red wine pressurized with similar HHP treatments (Chapter II.1), probably because of the presence of sulphur dioxide in the wines that reduces the rate of phenolic polymerization and, consequently, the colour loss usually observed during wine aging (Bakker *et al.*, 1998).

Table IV.1.2. Monomeric anthocyanins content of the wine samples at the beginning and after 5 months of bottle storage

Peak number	Anthocyanin (mg/L)[1]	Beginning of storage			5 months		
		Unpressurized	*500 MPa*	*600 MPa*	*Unpressurized*	*500 MPa*	*600 MPa*
1	**Dp3Glc**	16.04 ± 0.17^{a}	16.62 ± 0.39^{a}	16.60 ± 0.12^{a}	10.29 ± 0.18^{b}	9.23 ± 0.10^{c}	9.22 ± 0.08^{c}
2	**Cy3Glc**	2.46 ± 0.03^{a}	2.48 ±0.09^{a}	2.57 ± 0.08^{a}	2.32 ± 0.02^{a}	2.45 ± 0.22^{a}	2.30 ± 0.02^{a}
3	**Pt3Glc**	21.01 ± 0.05^{a}	21.94 ± 0.19^{b}	22.22 ± 0.24^{b}	13.12 ± 0.26^{c}	11.44 ± 0.03^{d}	11.20 ± 0.02^{d}
4	**Pn3Glc**	7.88 ± 0.26^{a}	8.15 ± 0.30^{a}	8.14 ± 0.05^{a}	5.52 ± 0.20^{b}	4.93 ± 0.33^{b}	4.86 ± 0.42^{b}
5	**Mv3Glc**	160.49 ± 0.79^{a}	162.20 ± 0.67^{a}	162.48 ± 1.32^{a}	89.89 ± 0.36^{b}	77.91 ± 0.16^{c}	76.81 ± 0.09^{c}
6	**Pn3AcGl**	2.82 ± 0.10^{a}	2.80 ± 0.18^{a}	2.94 ± 0.10^{a}	2.39 ± 0.05^{b}	2.29 ± 0.06^{b}	2.23 ± 0.01^{b}
7	**Mv3AcGlc**	24.17 ± 0.35^{a}	24.57 ± 0.26^{a}	24.57 ± 0.28^{a}	14.08 ± 0.13^{b}	11.91 ± 0.05^{c}	11.77 ± 0.04^{c}
8	**Pn3CmGlc**	2.83 ± 0.06^{a}	2.84 ± 0.11^{a}	2.95 ± 0.03^{a}	2.31 ± 0.03^{b}	2.24 ± 0.02^{b}	2.23 ± 0.03^{b}
9	**Mv3CmGlc**	14.31 ± 0.15^{a}	14.78 ± 0.20^{b}	15.16 ± 0.22^{b}	7.91 ± 0.02^{c}	6.80 ± 0.04^{d}	6.77 ± 0.08^{d}
	Total	252.01 ± 1.30^{a}	256.38 ± 1.07^{b}	257.63 ± 1.84^{b}	147.82 ± 0.79^{c}	129.19 ± 0.68^{d}	127. 40 ± 0.47^{d}

All data are expressed as mean value ± standard deviation (n = 3). In the same line, different letters indicate significant differences according to a Tukey test ($p < 0.05$). [1]**Dp3Glc**-Delphinidin-3-*O*-glucoside, **Cy3Glc**-Cyanidin-3-*O*-glucoside**,** **Pt3Glc**-Petunidin-3-*O*-glucoside, **Pn3Glc**-Peonidin-3-*O*-glucoside, **Mv3Glc**-Malvidin-3-*O*-glucoside, **Pn3AcGlc**- Peonidin -3-*O*-(6-*O*-acetyl)-glucoside, **Mv3AcGlc**- Malvidin-3-*O*-(6-*O*-acetyl)-glucoside, **Pn3CmGlc**-Peonidin-3-*O*-(6-*O*-*p*-coumaryl)-glucoside, **Mv3CmGlc**- Malvidin-3-*O*-(6-*O*-*p*-coumaryl)-glucoside

Phenolic acids and flavonols composition

Twelve different phenolic acids and three flavonols were identified and quantified in the different wine samples (Figure IV.1.2).

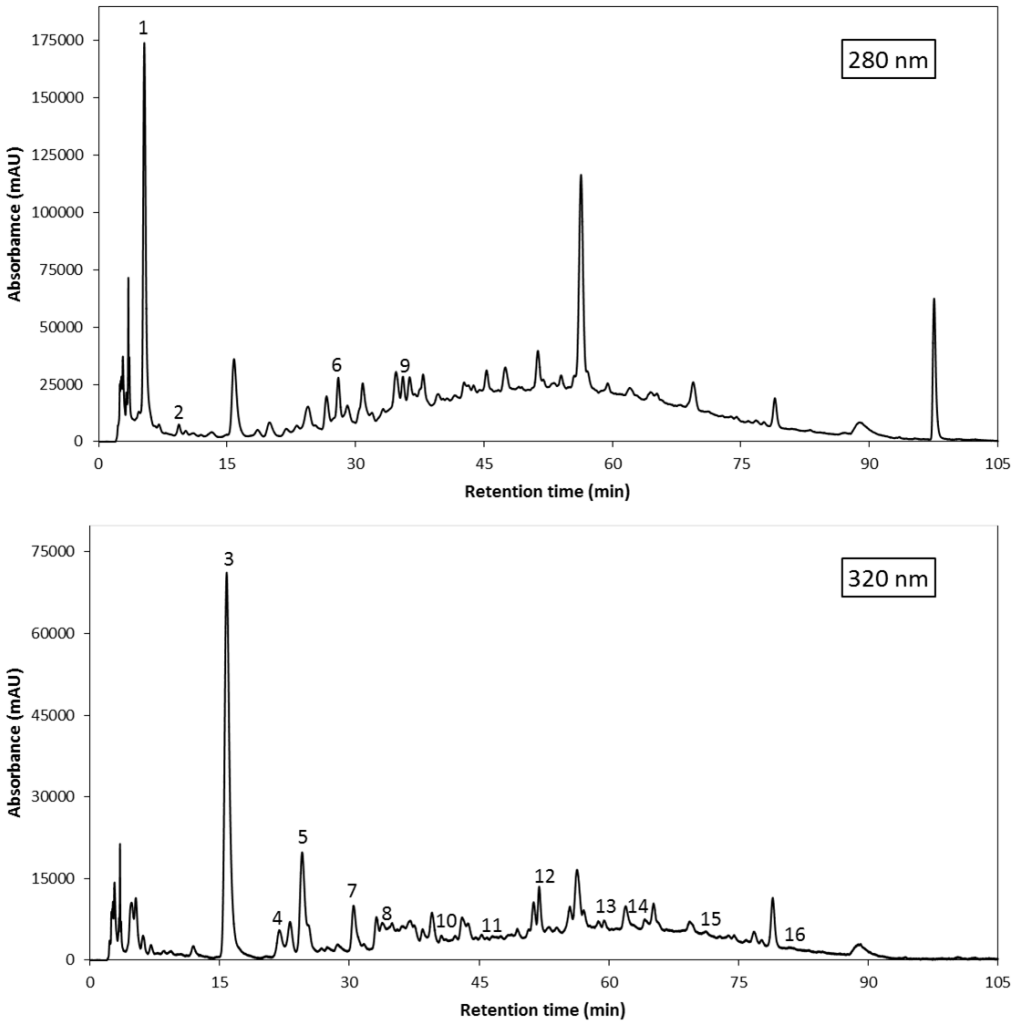

Figure IV.1.2. HPLC chromatogram of *Oak* wine after 5 months of storage recorded at 280 and 320 nm. Identification: 1. Gallic acid, 2. Protocatechuic acid, 3. Caftaric acid, 4. *p*-Hydroxybenzoic acid, 5. Coutaric acid, 6. Vanilic acid, 7. Caffeic acid, 8: Chlorogenic acid, 9. Syringic acid, 10. *p*-Coumaric acid, 11. Ferulic acid, 12. Sinapic acid, 13. Ellagic acid, 14. Myricetin, 15. Quercetin, 16. Kaempferol.

At the beginning of the storage, the wine samples did not show any statistical differences on phenolic acids and flavonols content (p<0.05) (Table IV.1.3). However, after 5 months of storage, the wines showed different evolution of phenolic acids and flavonols content.

Table IV.3. Phenolic acids and flavonols content of the wine samples at the beginning and after 5 months of bottle storage

Compounds (mg/L)	Beginning of storage			5 months		
	Unpressurized	***500 MPa***	***600 MPa***	***Unpressurized***	***500 MPa***	***600 MPa***
Phenolic acids						
Gallic acid	57.01 ± 0.29^{a}	56.07 ± 0.30^{a}	55.32 ± 0.28^{b}	54.60 ± 0.20^{b}	48.55 ± 2.04^{c}	42.66 ± 0.05^{d}
Protocatechuic acid	2.24 ± 0.05^{a}	2.19 ± 0.09^{ab}	2.14 ± 0.06^{ab}	2.02 ± 0.07^{bc}	1.99 ± 0.09^{bc}	1.79 ± 0.03^{c}
Caftaric acid	116.39 ± 1.39^{a}	117.65 ± 1.72^{a}	118.44 ± 0.47^{a}	102.13 ± 0.16^{b}	93.97 ± 1.30^{c}	95.85 ± 0.99^{c}
Vanilic acid	9.04 ± 0.21^{a}	8.89 ± 0.07^{a}	8.88 ± 0.10^{a}	7.43 ± 0.04^{b}	7.53 ± 0.41^{b}	7.92 ± 0.50^{b}
Syringic acid	5.13 ± 0.05^{a}	4.87 ± 0.05^{a}	4.92 ± 0.05^{a}	4.03 ± 0.08^{b}	3.85 ± 0.11^{bc}	3.48 ± 0.23^{c}
p-Hydroxybenzoic acid	3.73 ± 0.12^{a}	3.86 ± 0.05^{a}	3.89 ± 0.02^{a}	1.43 ± 0.06^{b}	1.16 ± 0.02^{c}	1.33 ± 0.01^{b}
Coutaric acid	13.37 ± 0.12^{a}	13.44 ± 0.05^{a}	13.34 ± 0.14^{a}	12.71 ± 0.18^{b}	11.61 ± 0.07^{c}	11.40 ± 0.21^{c}
Caffeic acid	2.47 ± 0.05^{a}	2.48 ± 0.03^{a}	2.47 ± 0.03^{a}	2.69 ± 0.07^{b}	3.13 ± 0.03^{c}	3.20 ± 0.05^{c}
Chlorogenic acid	0.48 ± 0.06^{a}	0.42 ± 0.02^{a}	0.42 ± 0.04^{a}	0.24 ± 0.04^{b}	0.20 ± 0.01^{b}	0.17 ± 0.02^{b}
p-Coumaric acid	0.48 ± 0.05^{a}	0.48 ± 0.02^{a}	0.53 ± 0.02^{ab}	0.53 ± 0.06^{ab}	0.61 ± 0.03^{b}	0.64 ± 0.06^{b}
Ferulic acid	0.16 ± 0.03^{a}	0.16 ± 0.02^{a}	0.16 ± 0.01^{a}	0.16 ± 0.02^{a}	0.16 ±0.02^{a}	0.19 ± 0.03^{a}
Sinapic acid	1.50 ± 0.05^{a}	1.43 ± 0.01^{a}	1.44 ± 0.12^{a}	1.60 ± 0.25^{a}	1.19 ± 0.01^{b}	1.17 ± 0.02^{b}
Total	211.99 ± 1.41^{a}	211.94 ± 1.84^{a}	211.96 ± 0.64^{a}	189.56 ± 0.64^{b}	173.95 ± 3.24^{c}	169.81 ± 1.78^{c}
Flavonols						
Myricetin	12.19 ± 0.28^{a}	11.00 ± 0.24^{b}	10.75 ± 0.04^{b}	9.73 ± 2.21^{b}	7.36 ± 0.15 ^{c}	7.42 ± 0.29^{c}
Quercetin	10.62 ± 0.10^{a}	10.41 ± 0.34^{ab}	11.16 ± 1.04^{ab}	9.72 ± 0.16^{b}	8.48 ± 0.43^{c}	8.73 ± 0.66^{c}
Kaempferol	10.68 ± 0.29^{a}	10.32 ± 0.22^{a}	10.20 ± 0.32^{a}	8.57 ± 0.58^{b}	6.95 ± 0.05^{c}	7.84 ± 0.77^{bc}
Total	33.50 ± 0.55^{a}	31.73 ± 0.49^{a}	32.11 ± 1.23^{a}	28.02 ± 1.73^{b}	22.79 ± 0.41^{c}	23.99 ± 0.93^{c}

All data are expressed as mean value ± standard deviation (n = 3). In the same line, different letters indicate significant differences according to a Tukey test (p < 0.05).

As seen previously for the anthocyanins, the total content of phenolic acids and flavonols in all wine samples decreased during the 5 months of bottle storage (up to 11%

and 19% less content for the case of phenolic acids and flavonols, respectively), although to a lower extent than the antocyanins. This behaviour should be due to the chemical oxidation and formation of copigments of this compounds with anthocyanins during storage (Burin *et al.*, 2011; Monagas *et al.*, 2006).

After 5 months of storage, the pressurised wines presented around 10% less phenolic acids content when compared with the unpressurised wine (Table IV.1.3). This behaviour was mainly due to the lower content of gallic and caftaric acids, the most dominant phenolic acids quantified, in the pressurized wines when compared with the *unpressurized* wine, since the *500 MPa* and *600 MPa* wines presented 11% and 22% less of gallic acid, and 8% and 6% less of caftaric acid, respectively. In relation to the flavonol content, the *500 MPa* and *600 MPa* wines presented 24% lower content of myricetin and 13% and 10% lower content of quercetin, respectively, when compared with the *unpressurized* wine. The kaempferol content was 19% lower in the wine pressurized at *500 MPa* when compared with the *unpressurized* wine, while for the *600 MPa* wine the content was not significantly different ($p<0.05$).

These results show that phenolic acids and flavonols were also affected by HHP treatments, leading to wines with lower content of these compounds and consequently to wines with possible lower bioactive activity. The decrease of these phenolic compounds in the pressurized wines may be related with the generation of high-reactive radicals during pressurization, enhancement of chemical oxidation, and polymerization of phenolic compounds during storage (Chen *et al.*, 2012; Clariana *et al.*, 2011).

Flavan-3-ols composition and mean degree of polymerisation

The flavan-3-ol monomers (+)-catechin and (-)-epicatechin, and oligomers (B1, B2, B4 dimers and C1 trimer) were identified and quantified in the wines at the beginning of storage and after 5 months of bottle aging (Table IV.1.4). Proanthocyanidins characteristics such as mean degree of polymerization (mDP) and percentage of prodelphinidins (%P) were also determined for all the wine samples (Table IV.1.4). At the beginning of storage no significant difference among the different wine samples was observed for the flavan-3-ols monomers and oligomers content. The mDP of the wines studied varied from 4.28 to 5.20, which are in agreement with values reported in the literature for Touriga Nacional, Trincadeira, Castelão, Syrah, and Cabernet Sauvignon wines with mDP varying from 2.1 to 9.6 (Cosme *et al.*, 2009). Therefore, the *600 MPa*

wine exhibited a slight higher mDP and %P values than the *500 MPa* and *unpressurized* wines (Table IV.1.4). These results allow inferring that pressure treatment of 600 MPa for 20 min increased the polymerization rate of higher molecular weight proanthocyanidins in the beginning of storage, leading to higher mDP and %P values without changing the flavan-3-ols monomers and oligomers content. In addition, pressure treatment of 500 MPa for 5 min did not affect the polymerization rate of high and low molecular weight proanthocyanidins, at the beginning of storage. These results are in line with others reported in the literature (Chen *et al.*, 2012; Tao *et al.*, 2012) that showed that more severe high pressure treatments (≥ 600 MPa for large minutes or hours) can promote the polymerisation reaction of flavan-3-ols and/or higher molecular weight proanthocyanidins, at the beginning of wine storage.

Table IV.1.4. Flavan-3-ol monomers and oligomers content, and tannin composition of the wine samples at the beginning and after 5 months of bottle storage

Compounds (mg/L)	Beginning of storage			5 months		
	Unpressurized	***500 MPa***	***600 MPa***	***Unpressurized***	***500 MPa***	***600 MPa***
Flavanols						
(+)-Catechin	71.82 ± 0.30^{a}	71.03 ± 0.66^{a}	72.41 ± 0.20^{a}	61.77 ± 0.66^{b}	59.35 ± 1.38^{c}	72.03 ± 1.22^{a}
(-)-Epicatechin	35.12 ± 0.39^{a}	35.14 ± 0.31^{a}	35.78 ± 0.04^{ac}	29.01 ± 0.23^{b}	26.34 ± 0.47^{d}	37.61 ± 2.10^{c}
Procyanidin B1	62.74 ± 0.46^{ab}	62.38 ± 0.29^{ab}	62.79 ± 0.91^{ab}	64.62 ± 1.58^{b}	61.26 ± 1.33^{a}	60.99 ± 0.65^{a}
Procyanidin B2	17.83 ± 0.11^{a}	17.56 ± 0.37^{a}	17.79 ± 0.16^{a}	17.23 ± 0.08^{a}	16.33 ± 0.30^{b}	15.92 ± 0.28^{b}
Procyanidin B4	3.84 ± 0.46^{a}	4.86 ± 0.79^{a}	4.77 ± 0.27^{a}	3.97 ± 0.43^{a}	3.95 ± 0.42^{a}	4.20 ± 0.38^{a}
Procyanidin C1	11.48 ± 0.11^{a}	11.69 ± 0.41^{a}	11.40 ± 0.30^{a}	9.79 ± 0.20^{b}	9.54 ± 0.43^{b}	9.05 ± 0.35^{b}
Total	202.83 ± 1.04^{a}	202.66 ± 0.62^{a}	204.95 ± 1.38^{a}	186.39 ± 2.56^{b}	176.77 ± 4.07^{c}	199.80 ± 2.04^{a}
Tannin composition						
mDP	4.72 ± 0.04^{a}	4.80 ± 0.03^{a}	5.20 ± 0.11^{b}	4.72 ± 0.11^{a}	4.99 ± 0.06^{b}	4.28 ± 0.14^{c}
%P	6.82 ± 0.42^{a}	6.32 ± 0.17^{a}	8.16 ± 0.73^{b}	7.72 ± 0.30^{b}	7.78 ± 0.18^{b}	5.82 ± 0.20^{c}

All data are expressed as mean value ± standard deviation (n = 3). In the same line, different letters indicate significant differences according to a Tukey test ($p < 0.05$). *mDP* mean degree of polymerization; *%P* percentage of prodelphinidins

Along the time of storage, the wines showed different evolution of flavan-3-ols content and degree of polymerization (Table IV.1.4). After 5 months of storage, the *500 MPa* wine presented the lower value (176.77 mg/L) of the total flavan-3-ols content, contrary to the *600 MPa* wine that present the higher value (199.80 mg/L) among the wine samples ($p<0.05$). These values are mainly due to the lower content of catechin and epicatechin for the *500 MPa* wine and the higher content of these compounds for the *600 MPa* wine ($p<0.05$). In relation to the mDP and %P the *500 MPa* wine showed a higher mDP (4.99) among the wines samples, ($p<0.05$). The *600 MPa* wine presented the lower mDP (4.28) and %P (5.82) values when compared with the *500 MPa* and *unpressurized* wines. These results, together with the higher content of flavan-3-ol monomers presented in the *600 MPa,* at 5 months of storage, lead to infer a possible effect of HHP treatments in the acceleration of flavan-3-ol/proanthocyanidins reactions that occur naturally during the wine storage period. In fact, during wine aging, different reactions can occur, giving rise to proanthocyanidins with different degree of polymerization. In the first months of wine storage, the mDP tends to increase due to condensation reactions of flavan-3-ols in the wine (Ribéreau-Gayon *et al.,* 2006). However, along aging, a decrease in mDP and prodelphinidins could occur due to the easier degradation of higher molecular weight proanthocyanidins and also due to their precipitation after condensation with other compounds, namely polysaccharides and proteins (Cheynier *et al.,* 1997; Chira *et al.,* 2011a; Cosme *et al.,* 2009). Vidal *et al.,* (2002) also attributed the decrease in mDP to a cleavage reaction that occurs in acidic media like wine. In this case, these reactions dominate in relation to the polymerisation reaction of proanthocyanidins that also occur. Therefore, with the pressure treatment of 600 MPa for 20 min, the condensation reactions between flavan-3-ol/proanthocyanidins increase immediately after the treatment, leading to higher mDP and %P values. However, during wine storage, the rate of cleavage reaction and the precipitation of proanthocyanidins in this pressurized wine are also higher, leading to a faster decrease in mDP and %P values and a higher content of flavan-3-ol monomers in this wine comparing with the *500 MPa* wine.

These results show that the pressure treatments studied increased the rate of flavan-3-ol/proanthocyanidins reactions during wine storage, being this effect more pronounced with the most severe pressure treatment.

IV.1.3.3 Effect of HHP treatments on the wine sensorial characteristics

The sensorial properties of the wines were analysed after 5 months of storage to assess the organoleptic characteristics of the wine in terms of colour, aroma, and taste. The results of the average scores of the panelists are displayed in Figure. IV1.3. Regarding the colour evaluation (Figure. IV1.3A), it can be observed that the pressurized wines did not present significant differences values for all the five colour descriptors (red, violet and brown colour, intensity and limpidity) when compared with the *unpressurized* wine (p>0.05). These results are in agreement with the chromatic parameters obtained for the pressurized wines (Table IV.1.1), since no significant difference (p>0.05) among the different wine samples was observed for all the parameters (colour intensity, Tint, Ye%, Rd%, Bl%, dA%).

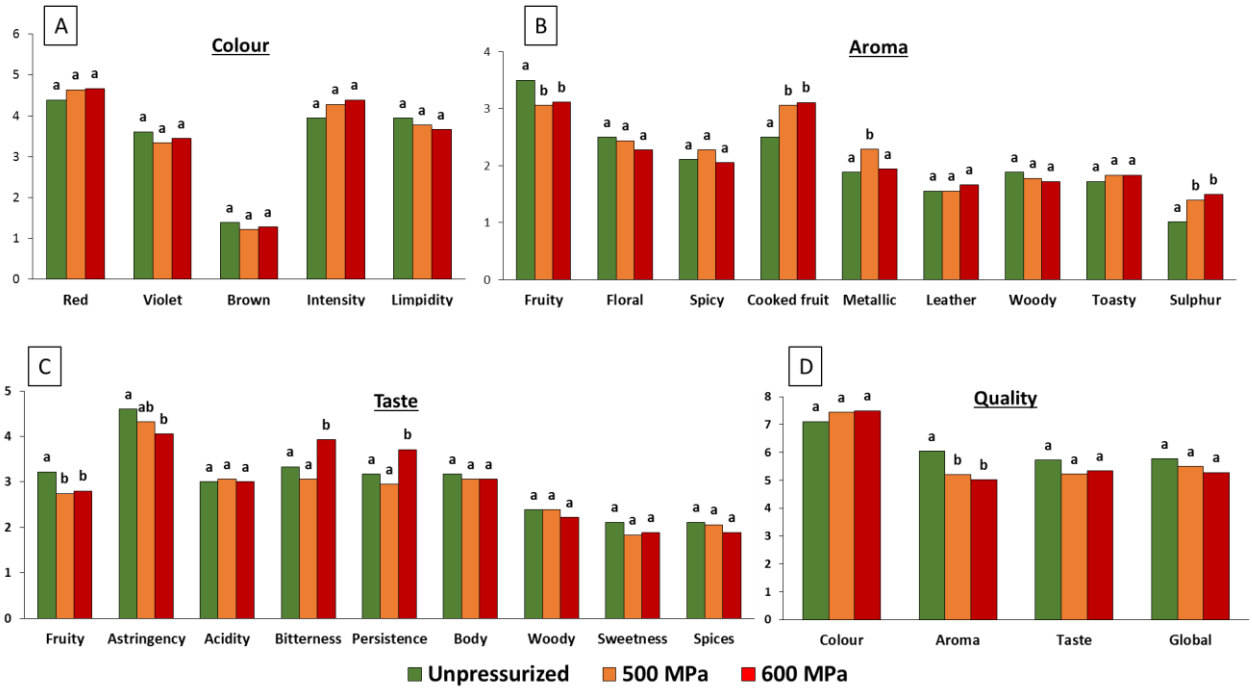

Figure. IV1.3. Descriptive sensory analysis of the colour (A), aroma (B), taste (C), and global attributes (D) of wines samples at 5 months of storage. All data are expressed as mean value. Different letters indicate significant differences according ANOVA followed by a Tukey test (p<0.05).

The aroma of pressurized wine samples (Figure. IV1.3B) presented a higher scents of cooked fruit, and sulphur aroma and lower scents of fruity aromas than the *unpressurized* wine (p<0.05). This result is in agreement with results reported for SO_2-

free red (Chapter II.1) and white wines (Chapter II.2) with pressure treatment that showed higher cooked fruit aroma perception, attributed to the increase of Maillard volatile compounds (Chapter II.3), namely 2-furfural, that have "roasty" fruit descriptors (Castro-Vázquez *et al.,* 2011; Jeleń *et al.,* 2011). Also, the *600 MPa* presented higher metallic notes than the *unpressurized* and *500 MPa* wines.

Comparing the taste assessment of the different wine samples (Figure. IV1.3C), the pressurized wines showed a slightly lower fruity level than the *unpressurized* wine ($p<0.05$). Furthermore, the wine pressurized at 600 MPa presented higher values of bitterness and persistence among the wine samples, and lower astringency when compared with the *unpressurized* wine ($p<0.05$). The lower astringency noticed in the *600 MPa* wine could be explained by the lower mDP and %P values in this wine, since it has been shown that the decrease of mean molecular mass of proanthocyanidins decreases their ability to precipitate proteins (Chira *et al.,* 2011a; McRae *et al.,* 2010; Obreque-Slíer *et al.,* 2010), as well the perception of astringency (Chira e*t al.,* 2011b; Kallithraka *et al.,* 2011; Vidal *et al.,* 2002). Therefore, higher values of bitterness and persistence detected in this wine could be explained by the higher content of flavan-3-ol monomers (Table IV.1.4), since flavan-3-ol monomers are reported to be more bitter than astringent, being these compounds the main responsible for wine bitterness (Chira *et al.,* 2009; Prieur *et al.,* 1994).

The pressurized wines showed at 5 months of storage a slight lower aroma assessment, (Figure. IV1.3D) probably due to the higher cooked fruit aroma and lower fruity notes (Figure. IV1.3B), when compared with the *unpressurized* wine. However, all the wines presented a similar global assessment among them.

In general, regarding sensorial analysis, it seems that the phenolic composition changes, namely in terms of mDP and proanthocyanidins content, due to the HHP treatments, modify significantly the taste of the wines, although without decreasing their final quality. In addition, it seems that the lower content of anthocyanins present in the pressurized wines (Table IV.1.2) did not alter the wine colour. According to the sensorial analysis, the pressurized wines are considered suitable for commercialization as table red wines.

IV.1.4 Concluding remarks

This work demonstrated that high pressure treatments with pressures of 500 MPa during 5 min and 600 MPa during 20 min influence red wine phenolic composition, and led to alterations in the wine sensorial characteristics. Most of these effects are only noticeable after storage for 5 months, being more pronounced for the pressure treatment of 600 MPa for 20 min. These data indicate that the conditions of pressure treatment, such as "pressure" and "pressure holding time", had significant influence in the phenolic composition of pressurized wines.

The main changes that occur on the pressurized red wine phenolic composition, such as the lower content of monomeric anthocyanins, phenolic acids, and flavonols, are probably due to an increase of condensation reactions and oxidation of these compounds. At the same time, HHP influenced the polymerisation and cleavage reactions in which proanthocyanidins are involved. Therefore, the HHP treatments seems to promote reactions that are similar to those observed during wine aging, leading to aged-like wine characteristics that are perceived in a sensorial analysis.

CHAPTER IV.2

Impact of high pressure treatment on the phenolic composition of a red wine: comparison with different aging processes

Impact of high pressure treatment on the phenolic composition of a red wine: comparison with different aging processes

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IV.2.1 Overview

Wine ageing is a common winemaking practice for improving wine quality and organoleptic characteristics (Gómez García-Carpintero *et al.,* 2012; Tao *et al.,* 2014). Aging in wood barrels leads to wine changes in colour, structure, and, especially, in aroma. This changes are mainly due to reactions favoured by oxygen that occur among phenolic compounds and extraction of several compounds from wood, increasing wine complexity and stability (Del Barrio-Galán *et al.,* 2011, 2012). However, several disadvantages are appointed for barrel aging, such as the high cost of oak barrel, the large space held by barrels in the winery, the wine loss due to evaporation, and mainly the long aging time required (Ruiz de Adana *et al.,* 2005; Tao *et al.,* 2014). For these reasons alternative techniques have been developed to simplify the ageing process, allow obtaining more economic wines with similar or novel characteristics (Gómez García-Carpintero *et al.,* 2012). The alternatives include oak chips (Arapitsas *et al.,* 2004; Chira and Teissedre, 2013a; del Alamo *et al.,* 2008; García-Carpintero *et al.,* 2011) and micro-oxygenation with and without the presence of oak chips (Cano-López *et al.,* 2010; Cejudo-Bastante *et al.,* 2011a, 2011b; del Alamo *et al.,* 2010; Pérez-Magariño *et al.,* 2009; Tao et *al.,* 2014), that have been an extended practice in the USA, Australia, and Chile for several years, but it was not a legal practice in EU countries until 2006 ("Commission Regulation (EC)." 2006). These two techniques were recognized as being cheaper than oak barrels aging techniques (Tao *et al.,* 2014), and also to accelerate the condensation reactions between flavonoids mediated by acetaldehyde and the

cycloaddition reactions between pyruvic acid and anthocyanins (Cano-López *et al.*, 2010; Cejudo-Bastante *et al.*, 2011a, 2011b; Del Barrio-Galán *et al.*, 2012). As result of these reactions, polymeric structures are formed that enhance wine sensorial characteristics such as colour stability and astringency (Cejudo-Bastante *et al.*, 2011a; Tao *et al.*, 2014).

In Chapter IV it was shown that HHP can be potentially used to produce wine with aged-like characteristics, since the pressure treatment accelerated the polymerisation and cleavage reactions of phenolic compounds that are similar to those observed during wine aging, leading, for example, to lower astringency and higher bitterness degree. Therefore, HHP showed great potential for accelerating the wine aging process, shortening the aging time and lowering the costs for the winemaking industry, but also in producing wines with different physicochemical and sensorial characteristics.

As the phenols compounds play an important role in wine colour and taste, that are important quality parameters of the wine, the aim of this work was to study the effect of a high hydrostatic pressure treatment in the phenolic composition of a red wine, comparing with the effect of different aging processes. For this purpose, a red wine was pressurized at 500 MPa for 5 min at 20 ºC and the impact of HHP treatment on the phenolic compounds composition was studied after 5 months of storage and compared with the effect of different aging processes, such oak barrels, oak chips and micro-oxygenation with oak chips. A wine without any treatment was also produced in order to be used as control.

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IV.2.2 Materials and methods

IV.2.2.1 Chemicals

Milli-Q water (Millipore, Bedford, MA) was used in all this work. HPLC-grade methanol, acetonitrile and formic acid (Merck, Darmstadt, Germany) were used. Delphinidin-3-*O*-glucoside (≥95%), cyanidin-3-*O*-glucoside (≥96%), petunidin-3-*O*-glucoside (≥95%), peonidin-3-*O*-glucoside (≥95%), malvidin-3-*O*-glucoside (≥95%), procyanidins B1 (≥80%), B2 (≥90%), B4 (≥80%) and C1 (≥80%), and phloroglucinol were purchased from Extrasynthese (Lyon, Genay-France). Gallic acid (≥99%), protocatechuic acid (≥90%), ferulic acid (≥90%), caftaric acid (≥95%), vanilic acid (≥95%), caffeic acid (≥95%), syringic acid (≥95%), *p*-hydroxybenzoic acid (≥98%), coutaric acid (≥90%) , *p*-coumaric acid (≥98%), sinapic acid (≥98%), chlorogenic acid (≥95%), myricetin (≥96%), quercetin (≥96%), kaempferol (≥90%), (+)-catechin (≥98%), and (−)-epicatechin (≥98%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

IV.2.2.2 Wine samples

Red wine samples were produced by Dão Sul SA (Carregal do Sal, Portugal) using Tinta-Roriz (50%) and Touriga Nacional (50%) red grape variety from Dão Appellation from 2013 harvest. An industrial batch fermenter of 16,000 L was used.

After malolactic fermentation, the wine was separated in 5 different batches. Figure IV.2.1 shows a schematic representation of the winemaking process followed in this study. In one batch, the wine was transferred to 250 mL polyethylene bottles, stoppered, and pressurized at 500 MPa during 5 min, at 20 °C, in a hydrostatic press (Hiperbaric 55, Hiperbaric, Burgos, Spain) of the department of Chemistry of the University of Aveiro, Portugal, giving origin to the sample *"pressurized"*. This equipment has a pressure vessel of 200 mm inner diameter and 2,000 mm length and a maximum operation pressure of 600 MPa. It is connected to a refrigeration unit (RMA KH 40 LT, Ferroli, San Bonifacio, Italy) that allows to control the temperature of the input water used as pressurizing fluid. Pressurizing water was used at a controlled temperature of 15 °C. Pressure build-up took place at a compression rate of about 600 MPa/min (adiabatic heating caused an increase in temperature of about 2.0 °C), while decompression was nearly instantaneous. In two other batches, the wine was transferred and stored during 3 months in two inox vats of 5,000 L with 5 g/L of a mixture of fresh

and toasted oak chips (Bioeno, Portugal) in both vats. One of these batches was also submitted to 1 month of micro-oxygenation treatment (5 mL/L/months of O_2) originating the sample *"Oak chips + mO_2"*, while the other batch, without micro-oxygenation treatment, gave origin to the sample *"Oak chips*". A lot of the original wine was stored for 3 months in four 225 L French oak barrels from the region of Allier (Vicard, France), giving origin to the sample *"Oak barrels".* As polyethylene bottles shown to have a little impact on the sensorial properties of the wine (Ghidossi *et al.*, 2012), all wine samples (pressurized and unpressurized) were bottled, after the different treatments, in polyethylene bottles. The wine with no treatment (*control*) was also bottled in the polyethylene bottles. All wines were stored for 5 months at 80% relative humidity in the absence of light at a temperature ranging between 10 and 15 °C.

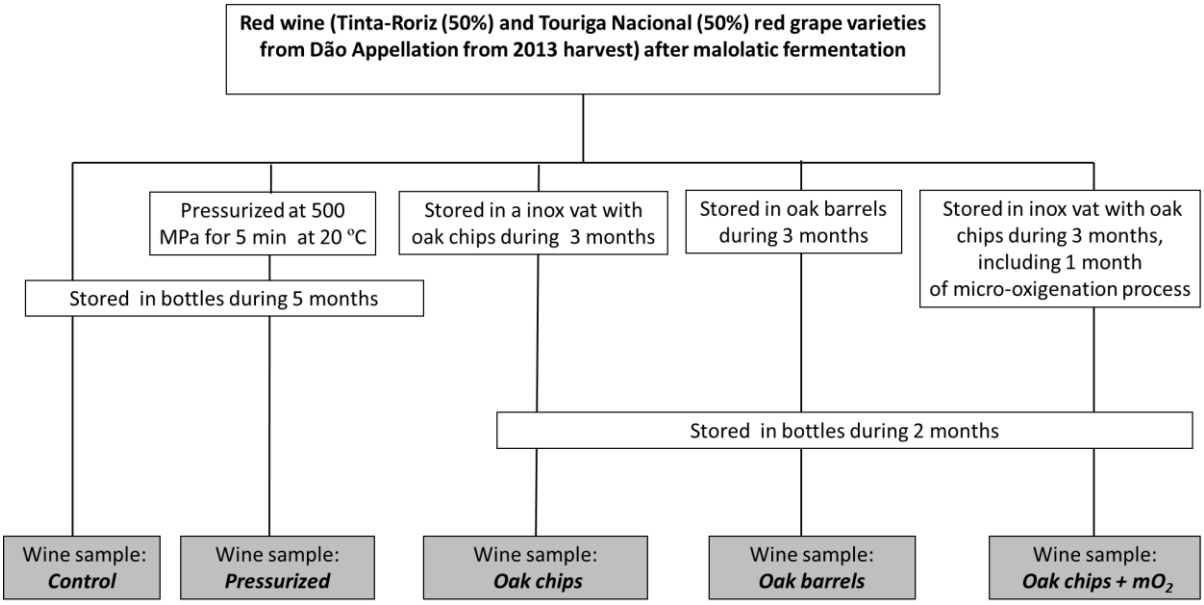


Figure IV.2.1. Flow chart of winemaking and storage process followed in this study for the production of the five wine samples.

IV.2.2.3 Oenological parameters determination

The ethanol content, titratable acidity, volatile acidity, pH, reducing sugars, free and bound SO_2, HCl, and gelatine indexes were determined for each wine samples according to the methods described by the Organization International de la Vigne et du Vin (OIV 1990; Ough and Amerine, 1988; Ribéreau-Gayon *et al.*, 2006). All analyses were carried out in triplicate.

IV.2.2.4 Colour determination

The colour intensity (CI) was calculated as the sum of the absorbance values at 420, 520, and 620 nm and the colour tonality was determined by the ratio between the absorbance at 420 and 520 nm. Absorbance measurements were recorded on an Uvikon 922 spectrophotometer (Kontron Instruments, Saint Quentin en Yvelines, France). The contribution of each coloration (yellow, red, and blue) to the overall colour of wine was calculated by dividing the absorbance at 420 nm (Ye%), 520 nm (Rd%) and 620 nm (Bl%) by the colour intensity (CI). The proportion of red coloration produced by free and bound anthocyanins under their flavylium cations form (dA%) was calculated using the following formula (Kelebek et *al.*, 2010):

$$\mathrm{dA\%} = \left[1 - \frac{\mathrm{Abs420} + \mathrm{Abs620}}{2 \times \mathrm{Abs520}}\right] \times 100$$

IV.2.2.5 Phenolic Composition by spectrophotometric methods

The total phenolic (TP) content of the samples was determined by the Folin–Ciocalteu method (Singleton 1985). The samples were appropriately diluted in a solution of 10% ethanol. The calibration curve was performed using gallic acid as standard in a concentration range between 50 and 500 mg/L. The results were expressed as gallic acid equivalents.

Total proanthocyanidins were estimated based on the Bate–Smith reaction, in which the proanthocyanidins in acid medium release anthocyanidins by heating (Chira *et al.*, 2011a). The wines were diluted to 1:50 in a 10% ethanol solution. One millilitre of the sample was added to 0.5 mL of water and 1.5 mL of 12 M HCl and the mixture was homogenised. Two tubes for each sample were prepared: one was heated for 30 min in boiled water (sample A), while the other was maintained at room temperature (sample B). To each tube, 0.25 mL of 95% ethanol were added. The absorbance at 550 nm was read through a 10 mm optical path. Total proanthocyanidins were calculated as 19.33 × ($Abs_{550nm}A$ - $Abs_{550nm}B$).

Anthocyanins (Anth) were determined using the SO_2 bleaching method (Chira *et al.*, 2011b). A solution ”A” was prepared as follows: 1 mL of wine, 1 mL of 0.1% HCl in ethanol and 20 mL of 2% HCl. Blank (B) was prepared as follows: 2 mL of solution ”A” and 0.8 mL of water. Sample (S) was prepared by adding 2 mL of solution ”A”, 0.4 mL of water and 0.4 mL of $HNaSO_3$ (15% w/v). After 20 min at room temperature, the

absorbance at 520 nm was measured through a 10 mm optical path. Anth were calculated as 875 × ($Abs_{520nm}B$ - $Abs_{520nm}S$).

The proportion of polymerized pigments (%PP) was estimated according to Ribereau-Gayon and Stonestreet (1965). The assay consists in discolouring the free fraction of anthocyanins with sodium metabisulphite ($Na_2S_2O_5$). For this, 1 mL of wine sample was placed in two test tubes with 9 mL of synthetic wine solution (10% ethanol and 5 g/L of tartaric acid at pH 3.2). In one of the tubes (tube M), 40 µL of 20% sodium metabisulphite was added, and in the other tube (tube C) 40 µL of distilled water was added. The absorbance at 420 and 520 nm were measured on each one of the tubes and calculation of the percentage of polymerized pigments was performed using the formula:

$$\%PP = \left[\frac{(Abs420_M + Abs520_M)}{(Abs420_C + Abs520_C)}\right] \times 100$$

IV.2.2.6 Phenolic compounds composition by HPLC-MS

Wine samples were filtered through a 0.45 µm pore size membrane filter before injection. Analysis was performed on a Thermo-Finnigan Accela HPLC system consisting of an autosampler (Accela autosampler), a pump (Accela 600 Pump), and a diode array detector (Accela PDA Detector) coupled to a Finnigan Xcalibur data system. Separation was performed on a reversed phase Agilent Nucleosil C18 (4.6 mm × 250 mm, 5 µm) column. Triplicate analyses were performed for each sample.

For the monomeric anthocyanins analysis, the eluents used, water/formic acid (99:1, v/v) (solvent A) and acetonitrile/formic acid (99:1, v/v) (solvent B), were applied at a flow rate of 1 mL/min as follows: 10–35% B linear from 0–25 min, 35–100% B linear from 25–26 min, 100% B isocratic from 26–28 min, 100–10% B linear from 28–29 min, with the re-equilibration of the column from 29–35 min under the initial gradient conditions. Detection was conducted at 520 nm. Quantification was performed by a comparison to malvidin-3-*O*-glucoside calibration curve.

For phenolic acids and flavonols analysis the mobile phase consisted of two solvents: Solvent A, water/formic acid (95:5; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v). Phenolic compounds were eluted under the following conditions: 1 mL/min flow rate and the temperature was set at 25 °C, isocratic conditions from 0 to 15 min with 100% A, gradient conditions from 0% to 20% B in 30 min, from 20% to 50%

B in 40 min, and from 50% to 100% B in 5 min, isocratic conditions with 100% B during 10 min, followed by washing and reconditioning the column. The ultraviolet-visible spectra (200 to 600 nm) were recorded for all peaks. The identification of phenolic compounds was performed by comparison with their retention times and UV-Vis spectra of authentic standards and also it was confirmed by mass spectrometry analysis. Quantification was performed using external calibration curves using gallic acid, caffeic acid, and kaempferol for benzoic acids, cinnamic acids, and flavonols quantification, respectively. The standards concentration ranges were those normally present in wines (approximately 0.2–200 mg/L) and the obtained regression coefficients (r^2) were above 0.992 in all cases.

IV.2.2.7 HPLC-UV-MS/MS analysis of pyranoanthocyanins

The pyranoanthocyanins present in the wine samples were analysed by HPLC-UV-MS/MS using the method described by Pechamat et al. (2014). These analyses were performed on a Thermo-Finnigan Surveyor HPLC-UV system composed of an UV−Vis detector (Surveyor PDA Plus), an autosampler (Surveyor autosampler Plus), and a quaternary pump system (Surveyor LC pump Plus), controlled by a Xcalibur data treatment system. These analyses were carried out on a 250 × 4.6 mm, 5 μm Lichrospher 100 RP 18 column. The solvents used for the gradient were solvent A, water with 0.5% of formic acid, and solvent B, acetonitrile with 0.5% formic acid. The gradient used was as follows: from 100 to 85% of A in 5 min, from 85 to 70% of A in 30 min, from 70 to 0% of A in 1 min, then 0% of A during 4 min. The flow rate was set at 1 mL/min, and the temperature was fixed at 12 °C. The wavelengths used were 280 and 520 nm. This HPLC-UV system was also coupled to a Thermo-Finnigan LCQ Advantage spectrometer equipped with an electrospray ionization source and an ion trap mass analyzer. The electrospray ionization mass spectrometry detection was performed in positive mode with the following optimized parameters: capillary temperature 300 °C, capillary voltage 5 V, nebulizer gas flow 1.75 L/min, desolvation gas flow 1 L/min, and spray voltage 5 kV. The pyranoanthocyanins were identified according to their mass spectra and fragmentation (Flamini 2013; Pechamat et al. 2014; Sánchez-Ilárduya et al. 2014). The pigments were quantified using pure malvidin-3-*O*-glucoside as standard and chlorogenic acid as internal standard. The calibration curve was accomplished by injecting solutions

of malvidin-3-*O*-glucoside ranging from 1 to 256 mg/L with 50 mg/L of chlorogenic acid. The response factor was established by plotting the concentrations ratios versus the peak area ratios of malvidin-3-*O*-glucoside ion (i.e., *m/z* 493) to the internal standard ion (i.e., *m/z* 355). The R^2 obtained was 0.9998. The quantification of the pyranoanthocyanins-procyanidin dimers was performed using their molecular ion: vitisin A $[M]^+ = 561$, vitisin B $[M]^+ = 517$, Pyranomalvidin-3-*O*-glucoside-phenol $[M]^+ = 609$, Pyranomalvidin-3-*O*-glucoside-catechin $[M]^+ = 805$, and Pyranomalvidin-3-*O*-glucoside-catechin dimer $[M]^+ = 1093$ (Flamini 2013; Pechamat et al. 2014; Sánchez-Ilárduya et al. 2014).

IV.2.2.8 HPLC-UV-Fluor/MS analysis of monomeric and oligomeric flavan-3-ols and mean degree of polymerisation (mDP)

The equipment used was a Thermo-Finnigan Surveyor HPLC system formed by UV–Vis detector (Surveyor PDA Plus), an autosampler (Surveyor autosampler Plus) and a quaternary pump (Surveyor LC pump Plus) controlled by Xcalibur data treatment system. This HPLC System was also coupled to a Thermo-Finnigan LCQ Advantage spectrometer equipped with an ion trap mass analyser.

The separation of monomeric and oligomeric flavan-3-ols was performed on a reversed phase Agilent Nucleosil C18 (250 mm × 4 mm, 5 μm). Water/formic acid (solvent A) (99:1, v/v) and acetonitrile/formic acid (99:1, v/v) (solvent B) were used at a flow rate of 1 mL/min. The gradient conditions were: 3% B isocratic from 0–3 min, 3–5% B linear from 3–14 min, 5–10% B linear from 14–22 min, 10–14% B linear from 22–26 min, 14–25% B linear from 26–40 min, 25– 100% B linear from 40–41 min, 100% B isocratic from 41–43 min, and 100–3% B linear from 43–44 min, with re-equilibration of the column from 44–50 min under the initial gradient conditions. Detection was performed with a fluorescence detector set at 280 nm excitation wavelength and 320 nm emission wavelength with medium fluorescence intensity; as well as a diode array detector set at 280 nm. Identification of monomeric and oligomeric flavan-3-ols was carried out by comparison to the retention time of external standards ((+)-catechin, (-)-epicatechin, and procyanidins B1, B2, B4 and C1) and also confirmed by HPLC-MS analysis. Quantification was performed using external standard calibration curves.

For the determination of mean degree of polymerization (mDP), a solid-phase extraction (SPE) step was used to purify the wines. Each sample was diluted 3 times and applied (10 mL) on a LC18 (octadecyl bonded, endcapped silica) cartridge (Supelco, St

Quentin Fallavier, France). The column was washed with 50 mL of water and eluted with 50 mL of methanol. The methanol fraction was dried under reduced pressure, redissolved in 2 mL of methanol and used for mDP determination. The proanthocyanidin mDP concentrations were quantified by phloroglucinolysis (Drinkine *et al.,* 2007). Reversed-phase HPLC analysis of the products formed allows determination of the structural composition of proanthocyanidins, which are characterised by the nature of their constitutive extension units (released as flavan-3-ols phloroglucinol adducts) and terminal units (released as flavan-3-ols). These analyses were carried out in triplicate on a column Xterra RP18 (100 mm × 4.6 mm, 3.5 μm, Waters, France). The elution conditions were: solvent A, water/acetic acid (99:1, v/v); solvent B, methanol. The elution gradient for the analysis of the reaction mixture was as follows: 5% B for 25 min, a linear gradient from 5 to 32% B in 45 min, a linear gradient from 30 to 100% B in 2 min. The column was then washed with 100% B for 5 min and re-equilibrated with 5% B for 10 min. To calculate the apparent mDP, the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in molar basis) was divided by the sum of all flavan-3-ol monomers. To calculate the percentage of prodelphinidins (%P) the sum of (-)-epigallocatechin (EGC) subunits [(∑Pterminal units (EGC) concentrations) + (∑Pextension units (EGC-P) concentrations)] was divided by the sum of all flavan-3-ol.

IV.2.2.9 Sensorial analysis

A blind tasting test was done to the wines after 5 months of storage by 25 expert panellists from the Oenology Department of the University of Bordeaux. Wines (30 mL) were presented in transparent glasses coded with a three-digit random code and distributed in a completely randomized order. In each session a descriptive analysis of each wine was conducted.

All tasters were informed that the wines had different treatments, but the panellists did not have any details of the experimental design. Each panellist was presented with the five samples: *control*, *pressurized*, *oak chips*, *oak barrels* and *oak chips + mO_2*. Wines were evaluated on a predefined score sheet (intensity scale from 0 to 6) that included 23 descriptors in three categories: colour, aroma, and taste attributes. Also, a global evaluation was performed on a scale from 0 to 10 in 4 categories: colour, aroma, taste, and global attributes. Averages of the scores for each descriptor were calculated.

IV.2.2.10 Statistical analysis

Statistical data analysis was performed using Analysis of Variance (ANOVA). Tukey's HSD Test was used for the data as comparison test when samples analyses showed significant differences after ANOVA (p<0.05).

IV.2.3 Results and discussion

IV.2.3.1 Wines physicochemical characteristics

The physicochemical characteristics of the wine samples at the beginning of storage and after 5 months of bottle aging are summarized in Table IV.2.1. At the beginning of storage no significant differences among the wine samples were observed for all the parameters analysed (p<0.05), indicating that the pressure treatment did not immediately affect the physicochemical characteristics of the wine. These results are in accordance with previous studies that showed that pressure treatments around 300 to 600 MPa for few minutes have no impact in red wine properties at the beginning of storage (Mok *et al.*, 2006, Tao *et al.*, 2012, Chapter II.1, II2).

After 5 months of storage, the wine samples presented no significant differences (p>0.05) among them in terms of density, ethanol content, titratable and volatile acidity, reducing sugar content, and lactic and tartaric acid content (Table IV.2.1). Their composition were in accordance with previous studies carried out on Touriga Nacional and Tinta Roriz wines (Jordão *et al.*, 2012; Rodrigues *et al.*, 2012; 2013). In terms of SO_2 content, the unpressurized wines presented around 40 and 45% less free and total SO_2, respectively, when compared with the beginning of storage. The pressurized wine presented a more pronounced decrease of SO_2 content during storage (54 and 51% less content of both free and total SO_2, respectively). These results are in accordance with previous study that show the decrease of sulphur dioxide content with pressure treatments in wine during storage *(unpublished results*). Therefore, HHP seems to alter the equilibrium of the SO_2 reactions in wine during storage, by promotion of the reaction of free SO_2 with the radicals formed in the pressure treatments (Bolumar *et al.*, 2012b; Tao *et al.*, 2012), leading to a more pronounced decrease of sulphur dioxide content in pressurised wine during storage. The wine samples treated with wood (*Oak chips*, *Oak barrels*, and *Oak chips + mO₂)* presented a free and total SO_2 content similar to the *control*

sample, showing that, contrary to the high pressure, these treatments did not alter significantly the content of SO_2 in wine.

A red wine with a satisfactory astringency for the consumer should have a HCl index (tannin polymerization level) ranging between 10 and 30 and a gelatin index (tannin reactivity toward proteins) between 40 and 60 (Ribéreau-Gayon *et al.*, 2006). All the wine samples presented HCl and gelatin indexes within the appropriate ranges at the beginning and after 5 months of storage. Nevertheless, after 5 months of storage, the four treated wines presented slightly higher values ($p<0.05$), more pronounced for the *Oak chips + mO_2* wines, when compared with the *control*, as well as a higher tannin content (Table IV.2.1). These results showed that pressurized wines, like the wines treated with wood, presented higher tannin polymerization level than the *control*. In terms of percentage of pigments polymerization, all the wines treated with aging processes presented 5 to 7% more polymerization when compared with the untreated wine. This higher percentage is in accordance with the lower values of colour intensity observed (Table IV.2.1), since it was reported that formation of polymeric pigments contributes to the loss in colour intensity (Monagas *et al.*, 2006). These results showed that the pressure treatment, addition of oak chips with and without microoxygenation process, and storage in oak barrels had a similar impact on the physicochemical properties of the wine. All the aging treatments seem to increase the condensation reactions of phenolic compounds, resulting in compounds with higher polymerization degree along the wine aging.

Table IV.2.1 Physicochemical analysis of the different wine samples at the beginning and after 5 months of storage

Analysis	Beginning of storage		5 months				
	Control	*Pressurized*	*Control*	*Pressurized*	*Oak chips*	*Oak barrels*	*Oak chips + mO_2*
Density (20 ◦C/20 ◦C)	0.9907 ±0.00	0.9906 ± 0.00	0.9915 ± 0.00	0.9915 ± 0.00	0.9914 ± 0.00	0.9912 ± 0.00	0.9914 ± 0.00
Ethanol (v/v; %)	13.67 ± 0.01	13.66 ± 0.00	13.55 ± 0.00	13.60 ± 0.00	13.66 ± 0.01	13.80 ± 0.01	13.66 ± 0.00
Titratable acidity[a] (g L^{-1})	2.80 ± 0.00	2.75 ± 0.00	3.03 ± 0.00	3.11 ± 0.00	3.04 ± 0.01	3.05 ±0.00	3.04 ± 0.01
pH	3.59 ± 0.00	3.58 ± 0.01	3.64 ± 0.01	3.62 ± 0.00	3.63 ± 0.00	3.63 ± 0.01	3.64 ± 0.00
Volatile acidity[b] (g L^{-1})	0.40 ± 0.01	0.38 ± 0.02	0.43 ±0.01	0.41 ±0.01	0.39 ±0.01	0.45±0.01	0.39 ±0.01
Reducing sugar (g L^{-1})	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.0
Free SO_2 (mg L^{-1})	34.33 ± 0.58	33.33 ± 1.52	21.33 ± 0.58	15.33 ± 0.58	22.67 ± 0.58	21.00 ± 1.00	21.33 ± 0.58
Total SO_2 (mg L^{-1})	74.67 ± 0.58	74.33 ± 1.15	45.67 ± 0.58	36.67 ± 1.52	56.33 ± 0.58	43.33 ± 0.58	45.33 ± 0.58
Lactic acid (g L^{-1})	1.06 ± 0.01	1.01 ± 0.02	1.01 ± 0.00	1.04 ± 0.01	1.02 ± 0.01	1.01 ± 0.02	1.01 ± 0.00
Tartaric acid *(g L^{-1})*	1.33 ± 0.01	1.19 ± 0.02	2.08 ± 0.00	2.22 ± 0.03	2.10 ± 0.00	1.94 ± 0.00	2.10 ± 0.02
HCl index	28.8 ± 0.04	26.28 ± 0.93	16.66 ± 0.51	19.54 ± 0.32	21.13 ± 0.29	18.91 ± 0.40	23.68 ± 0.26
Gelatin index	55.06 ± 0.65	53.83 ± 0.47	44.90 ± 0.21	54.00 ± 0.15	56.85 ± 0.19	54.56 ± 0.56	58.47 ± 1.36
Tannin (g L^{-1})	3.38 ± 0.12	3.31 ± 0.26	3.69 ± 0.01	3.95 ± 0.02	3.86 ± 0.02	3.98 ± 0.3	3.96 ± 0.14
Phenolic compounds[c] (mg/L)	3454.33 ± 31.82	3681.82 ± 200.68	3423.70 ± 42.67	3416.68 ± 89.00	3418.08 ± 145.10	3468.63 ± 147.76	3373.14 ± 240.57
Anthocyanins[d] (mg/L)	519.98 ± 4.85	509.42 ± 10.72	443.53 ± 6.03	423.50 ± 7.88	458.76 ± 7.28	448.23 ± 9.68	448.12 ± 4.59
Pigments polymerization (%)	58.15 ± 0.21	58.47 ± 0.08	61.68 ± 0.59	68.44 ± 0.74	66.32 ± 0.75	67.34 ± 0.99	69.13 ± 0.94
Colour intensity	0.92 ± 0.01	1.11 ± 0.03	1.11 ± 0.01	0.93 ± 0.00	0.95 ± 0.00	0.95 ± 0.00	0.88 ±0.0
Colour tonality	0.62 ± 0.01	0.67 ± 0.02	0.64 ± 0.00	0.64 ± 0.00	0.66 ± 0.01	0.66 ± 0.01	0.68 ± 0.01
Ye%	34.10 ± 0.02	35.25± 0.90	34.84 ± 0.02	34.74 ± 0.01	35.28 ± 0.02	35.47 ± 0.02	35.83 ± 0.02
Rd%	54.80 ± 0.02	52.77 ± 1.33	54.49 ± 0.02	54.42 ± 0.01	53.62 ± 0.03	53.77 ± 0.02	53.04 ± 0.03
Bl%	11.09 ± 0.01	11.97 ± 2.22	10.66 ± 0.01	10.84 ± 0.01	11.10 ± 0.01	10.76 ± 0.01	11.12 ± 0.01
dA%	58.77 ± 0.03	55.22 ± 2.42	58.25 ± 0.04	58.13 ± 0.02	56.75 ± 0.05	57.02 ± 0.04	55.74 ± 0.05

All data are expressed as mean value ± standard deviation (n = 3); [a] Expressed as tartaric acid equivalent; [b] expressed as acetic acid equivalent; [c] expressed as mg of gallic acid equivalents; [d] expressed as mg of malvidin 3-glucoside equivalents.

IV.2.3.2 Effect of HHP treatments on the wine phenolic compounds composition

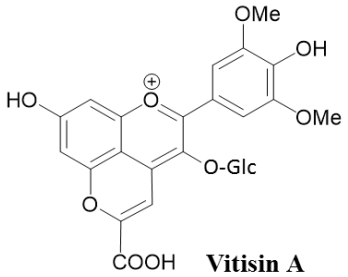
Monomeric anthocyanin and pyranoanthocyanins composition

Nine monomeric anthocyanins (MA), including five glucosides, two acetyl glucosides, and two coumaroyl glucosides, and five pyranoanthocyanins, were identified and quantified in the wine samples. Molecular ion and fragmentation information, together with λ_{max} values for the identified pyranoanthocyanins are present in Table IV.2.2. Pyranoanthocyanins are formed by a cycloaddition reaction of anthocyanins in the flavylium form with different compounds, giving rise to the formation of a new pyranic ring (de Freitas and Mateus, 2011; Oliveira *et al.*, 2010; Sánchez-Ilárduya *et al.*, 2014). The formation of these anthocyanin derivatives could be the result of a nucleophilic cycloaddition reaction involving the C4 and the hydroxyl group at C5 of the anthocyanin and an ethylene of the other compound (Sánchez-Ilárduya *et al.*, 2014). Pyranoanthocyanins were shown to arise mainly from the association of anthocyanins, such as malvidin 3-*O*-glucoside, with pyruvic acid, acetaldehyde, hydroxycinnamic acids or their decarboxylation products, and vinylflavanols during wine storage (Carvalho *et al.*, 2010; Marquez *et al.* 2013; Monagas *et al.*, 2006; Oliveira *et al.*, 2014; Rentzsch *et al.*, 2010; Sánchez-Ilárduya et al., 2014), resulting in the structures showed in Figure IV.2.2.

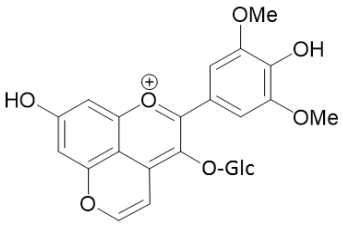
Table IV.2.2. Mass spectral details and UV data of pyranoanthocyanins identified in the wines

Pyranoanthocyanins	$[M]^+$	MS^2 fragments (*m/z*)	MS^3 fragments (*m/z*)	λ_{max} (nm)	Ref [a]
Vitisin A	561	399	-	513	a, b
Vitisin B	517	355	-	490	a, b
Pyranomalvidin-3-*O*-glucoside-phenol	609	447	-	503	a, b
Pyranomalvidin-3-*O*-glucoside-catechin	805	643	491	506	a, b
Pyranomalvidin-3-*O*-glucoside-catechin dimer	1093	931,803	641	513	a, b, c

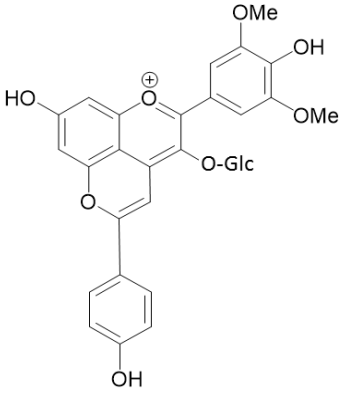
[a](a) Flamini 2013; (b) Sánchez-Ilárduya *et al.*, 2014; (c) Pechamat *et al.*, 2014,



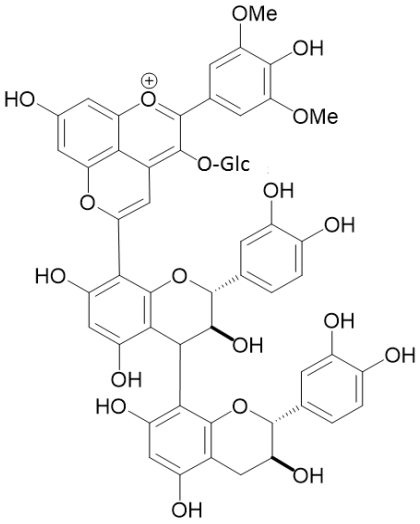
Vitisin A
(derivative of malvidin-3-*O*-glucoside and pyruvic acid)



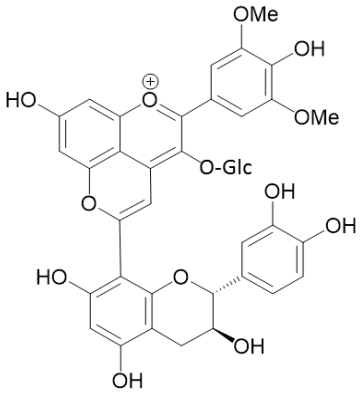
Vitisin B
(derivative of malvidin-3-*O*-glucoside and acetaldehyde)



Pyranomalvidin-3-*O*-glucoside-phenol
(derivative of malvidin-3-*O*-glucoside and 4-vinylfenol acid)



Pyranomalvidin-3-*O*-glucoside-catechin dimer
(derivative of malvidin-3-*O*-glucoside and vinylflavanol)



Pyranomalvidin-3-*O*-glucoside-catechin
(derivative of malvidin-3-*O*-glucoside and vinylflavanol)

Figure IV.2.2. Structures of pyranoanthocyanins identified and quantified in all wine samples.

All samples showed during storage a decrease in the individual anthocyanin content and, consequently, a decrease in total MA content (26% to 34% less), when compared with the beginning of storage (Table IV.2.3). This decrease was mainly due to the high decrease of malvidin 3-*O*-glucoside content, the MA present in higher content in red wines. Therefore, this decrease should be mainly due to MA participation in numerous condensation reactions, forming pyranoanthocyanins, as well as in hydrolytic and other degradation reactions (Monagas *et al.*, 2006; Santos-Buelga *et al.*, 1999) in a minor extent. In fact, the content of pyranoanthocyanins of the *pressurized*, *Oak chips*, *Oak barrels*, and *Oak chips* + mO_2 wines increased significantly (p>0.05) during wine storage

Table IV.2.3 Monomeric anthocyanins and pyranoanthocyanins content of the wine samples at the beginning and after 5 months of storage

Compounds (mg/L)	Beginning of storage		5 months				
	Control	***Pressurized***	***Control***	***Pressurized***	***Oak chips***	***Oak barrels***	***Oak chips + mO_2***
Monomeric anthocyanins*							
Delphinidin-3-*O*-glucoside	16.04 ± 0.17^{a}	16.62 ± 0.39^{a}	12.00 ± 0.14^{bc}	11.63 ± 0.16^{c}	12.44 ± 0.15^{b}	11.87 ± 0.08^{c}	12.04 ± 0.07^{bc}
Cyanidin-3-*O*-glucoside	2.46 ± 0.03^{a}	2.48 ±0.09^{a}	2.39 ± 0.07^{a}	2.50 ± 0.26^{a}	2.44 ± 0.05^{a}	2.39 ± 0.04^{a}	2.37 ± 0.05^{a}
Petunidin-3-*O*-glucoside	21.01 ± 0.05^{a}	21.94 ± 0.19^{b}	15.76 ± 0.07^{c}	15.06 ± 0.52^{c}	16.26 ± 0.02^{d}	15.43 ± 0.07^{c}	15.58 ± 0.17^{c}
Peonidin-3-*O*-glucoside	7.88 ± 0.26^{a}	8.15 ± 0.30^{a}	6.57 ± 0.16^{b}	6.35 ± 0.31^{b}	6.74 ± 0.25^{b}	6.23 ± 0.08^{b}	6.26 ± 0.20^{b}
Malvidin-3-*O*-glucoside	160.49 ± 0.79^{a}	162.20 ± 0.67^{a}	111.81 ± 0.20^{b}	100.82 ± 1.92^{c}	118.41 ± 0.16^{d}	110.37 ± 0.26^{b}	111.26 ± 0.35^{b}
Peonidin-3-*O*-(6-acetyl)-glucoside	2.82 ± 0.10^{ad}	2.80 ± 0.18^{ab}	2.50 ± 0.08^{c}	2.52 ± 0.04^{bc}	2.56 ± 0.06^{bcd}	2.47 ± 0.06^{c}	2.48 ± 0.11^{c}
Malvidin- 3-*O*-(6-acetyl)-glucoside	24.17 ± 0.35^{a}	24.57 ± 0.26^{a}	17.16 ± 0.23^{b}	16.20 ± 0.23^{c}	18.39 ± 0.05^{d}	16.97 ±0.02^{b}	17.24 ± 0.03^{b}
Peonidin-3-*O*-(6-*p*-coumaroyl)-glucoside	2.83 ± 0.06^{a}	2.84 ± 0.11^{a}	2.49 ± 0.01^{b}	2.43 ± 0.02^{b}	2.50 ± 0.02^{b}	2.39 ± 0.03^{b}	2.39 ± 0.01^{b}
Malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside	14.31 ± 0.15^{a}	14.78 ± 0.20^{b}	9.62 ± 0.05^{c}	8.97 ± 0.13^{d}	10.05 ± 0.03^{e}	9.44 ± 0.06^{cf}	9.19 ± 0.07^{df}
Total	252.01 ± 1.30^{a}	256.38 ± 1.07^{b}	180.30 ± 0.42^{c}	166.49 ± 1.90^{d}	189.79 ± 0.27^{e}	177.55 ± 0.26^{c}	178.82 ± 0.82^{c}
Pyranoanthocyanins**							
Vitisin A	16.74 ± 1.28^{a}	22.98 ± 0.90^{b}	17.05 ± 0.75^{a}	21.41 ± 0.28^{b}	18.17 ± 0.08^{a}	18.06 ± 0.41^{a}	21.96 ± 0.18^{b}
Vitisin B	6.68 ± 0.64^{a}	6.39 ± 0.73^{a}	3.55 ± 0.05^{b}	5.02 ± 0.14^{c}	5.04 ± 0.09^{c}	5.18 ± 0.37^{c}	3.69 ± 0.07^{b}
Pyranomalvidin-3-*O*-glucoside-phenol	10.26 ± 1.44^{ab}	8.97 ± 0.42^{a}	9.53 ± 0.74^{a}	8.06 ±0.91^{ab}	7.83 ± 0.25^{b}	7.47 ± 0.58^{b}	7.99 ± 0.51^{ab}
Pyranomalvidin-3-*O*-glucoside-catechin	0.82 ± 0.06^{a}	0.76 ± 0.02^{a}	1.15 ± 0.29^{b}	3.08 ± 0.81^{c}	2.69 ± 0.80^{c}	3.51 ± 0.88^{cd}	4.68 ± 0.38^{d}
Pyranomalvidin-3-*O*-glucoside-catechin dimer	1.55 ± 0.32^{a}	1.93 ± 0.58^{a}	5.10 ± 0.99^{b}	8.97 ± 0.74^{c}	6.09 ± 0.69^{b}	7.37 ± 1.41^{bc}	9.36 ± 0.77^{c}
Total	36.04 ± 2.55^{a}	41.03 ± 0.33^{b}	36.38 ± 1.25^{a}	46.53 ± 0.83^{c}	39.82 ± 0.70^{b}	41.58 ± 0.45^{b}	47.69 ± 0.95^{c}

* Data expressed as mean value ± standard deviation (n = 3); ** Data expressed as mean value ± standard deviation (n = 4). In the same line, different letters indicate significant differences according to Tukey test ($p < 0.05$).

(up to 24 % more in the case of *Oak chips + mO_2*), indicating a higher polymerization rate of monomeric anthocyanins. These results are in agreement with the pigments polymerization analyses (Table IV.2.1), since the wines treated by aging processes presented higher proportion of polymerized pigments (%PP) when compared with the *control* wine.

After 5 months of storage, the *pressurized* wine presented a lower content of malvidin-3-*O*-glucoside (8% less), malvidin-3-*O*-(6-acetyl)-glucoside (7% less) and malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside (6% less), and consequently 8% less of total MA content when compared with the *control* sample. In terms of pyranoanthocyanins content, the *pressurized* wine presented a higher content of vitisins A and B (20% and 29% more, respectively), and pyranomalvidin-3-*O*-glucoside-catechin and pyranomalvidin-3-*O*-glucoside-catechin dimer (43% and 62% more, respectively) than the *control* sample. These results are in line with the results reported in Chapter IV.1, which showed a lower anthocyanin content of red wine treated with HHP, due to the acceleration of condensation reactions during the wine storage period involving monomeric anthocyanins with other phenolic compounds.

Comparing the different aging processes, as can be observed in Table IV.2.2, the *pressurized* sample presented the lower content of monomeric anthocyanins ($p<0.05$). In addition, the *pressurized* sample presented a higher content of pyranoanthocyanins, than the *Oak chips* and *Oak barrels* samples (14% and 11% higher, respectively), and no significant different value when compared with *Oak chips + mO_2* sample. These results indicate that the HHP treatment and the addition of oak chips to the wine with microoxygenation process were the treatments that accelerated more the polymerization of the monomeric anthocyanins during storage. These results are in line with other results reported in the literature that showed that HHP treatments (Tao *et al.*, 2012) and microoxygenation process (Cano-López *et al.* 2010; Cejudo-Bastante *et al.* 2011a, 2011b; Gómez-Plaza and Cano-López, 2011; Pechamat *et al.*, 2014) can be effective procedures for wine aging acceleration.

Phenolic acids and flavonols composition

Thirteen different phenolic acids (Table IV.2.4) and three flavonols (Table IV.2.5) were identified and quantified in the different wine samples. At the beginning of the storage, the wine samples did not show any statistical difference on phenolic acids and flavonols content ($p<0.05$). However, after 5 months of storage, different evolution of

these compounds were noticed (18% and 25% less content for the case of phenolic acids and flavonols, respectively), as seen previously for the anthocyanins, although to a lower extent.

Table IV.2.4. Phenolic acids content of the wine samples at the beginning and after 5 months of storage

Phenolic acids **(mg/L)**	**Beginning of storage**		**5 months**				
	Control	***Pressurized***	***Control***	***Pressurized***	***Oak chips***	***Oak barrels***	***Oak chips + mO_2***
Gallic acid	57.01 ± 0.29^{a}	56.07 ± 0.30^{a}	57.02 ± 2.39^{a}	47.87 ± 3.02^{b}	76.85 ± 0.07^{c}	78.90 ± 0.56^{d}	78.95 ± 0.13^{d}
Protocatechuic acid	2.24 ± 0.05^{a}	2.19 ± 0.09^{ab}	1.78 ± 0.09^{bc}	1.91 ± 0.06^{bd}	1.56 ± 0.06^{c}	2.06 ± 0.07^{d}	1.62 ± 0.18^{c}
Caftaric acid	116.39 ± 1.39^{a}	117.65 ± 1.72^{a}	102.48 ± 4.76^{b}	93.27 ± 1.68^{c}	94.58 ± 1.00^{c}	99.09 ± 1.65^{b}	93.66 ± 2.00^{c}
Vanilic acid	9.04 ± 0.21^{a}	8.89 ± 0.07^{a}	8.31 ± 0.50^{ac}	7.52 ± 0.10^{bc}	7.63 ± 0.08^{bc}	7.62 ± 0.08^{bc}	7.35 ± 0.11^{b}
Syringic acid	5.13 ± 0.05^{a}	4.87 ± 0.05^{a}	3.87 ± 0.03^{b}	4.35 ± 0.10^{c}	4.21 ± 0.12^{c}	3.91 ± 0.12^{bc}	3.63 ± 0.36^{b}
p-Hydroxybenzoic acid	3.73 ± 0.12^{a}	3.86 ± 0.05^{a}	1.62 ± 0.17^{b}	1.32 ± 0.08^{b}	1.51 ± 0.04^{b}	1.58 ± 0.07^{b}	1.57 ± 0.02^{b}
Coutaric acid	13.37 ± 0.12^{a}	13.44 ± 0.05^{a}	12.23 ± 0.12^{b}	11.73 ± 0.33^{bc}	11.84 ± 0.06^{bc}	11.50 ± 0.18^{c}	11.26 ± 0.40^{c}
Caffeic acid	2.47 ± 0.05^{a}	2.48 ± 0.03^{a}	2.54 ± 0.03^{ac}	3.18 ± 0.04^{b}	2.80 ± 0.07^{c}	3.04 ± 0.02^{b}	2.87 ± 0.15^{c}
Chlorogenic acid	0.48 ± 0.06^{a}	0.42 ± 0.02^{a}	0.37 ± 0.01^{a}	0.23 ± 0.04^{b}	0.39 ± 0.03^{a}	0.40 ± 0.02^{a}	0.38 ± 0.02^{a}
p-Coumaric acid	0.48 ± 0.05^{a}	0.48 ± 0.02^{a}	0.46 ± 0.10^{b}	0.27 ± 0.01^{a}	0.46 ± 0.04^{a}	0.32 ± 0.02^{b}	0.41 ± 0.03^{a}
Ferulic acid	0.16 ± 0.03^{a}	0.16 ± 0.02^{a}	0.25^{b} ± 0.04	0.15 ± 0.03^{ab}	0.14 ± 0.02^{a}	0.15 ± 0.02^{a}	0.17 ± 0.04^{ab}
Sinapic acid	1.50 ± 0.05^{a}	1.43 ± 0.01^{a}	2.10 ± 0.20^{b}	1.61 ± 0.01^{c}	1.83 ± 0.05^{c}	1.66 ± 0.02^{c}	1.61 ± 0.02^{c}
Ellagic acid	-	-	-	-	1.09 ± 0.04^{a}	0.33 ± 0.03^{b}	1.41± 0.02^{c}
Total	211.99 ± 1.41^{a}	211.94 ± 1.84^{a}	193.24± 4.73^{b}	173.42 ± 0.95^{c}	204.88 ± 1.21^{d}	210.54 ± 2.19^{a}	204.90 ± 2.52^{d}

All data are expressed as mean value ± standard deviation (n = 3). In the same line, different letters indicate significant differences according to a Tukey test ($p < 0.05$).

After 5 months of storage, the pressurised wines presented around 10% less of phenolic acids content when compared with the *control* (Table IV.2.4). This behaviour was mainly due to the lower content of gallic (16%) and caftaric acids (19%), the most

dominant phenolic acids quantified in the *pressurized* wine. The *Oak chips*, *Oak barrels* and *Oak chips + mO_2* samples showed, after 5 months of storage, higher content of phenolic acids when compared with the *pressurized* and *control* wines, mainly due to the higher content of gallic acid and the presence of ellagic acid. In fact, it is known that hydrolysable tannins, formed by gallic and/or ellagic acids esterified to glucose are extracted from the wood to the wine due to the contact with the wood and by action of esterases, releasing gallic and ellagic acids into the wine (Chira and Teissedre, 2013b; Jordão *et al.*, 2005, 2008; Vázquez *et al.*, 2010). The content of gallic acid was higher than that of ellagic acid since the wood utilized in this work was French oak, that is generally richer in gallic acids than the American oak (Cabrita *et al.*, 2011).

Table IV.2.5. Flavonols content of the wine samples at the beginning and after 5 months of storage

Flavonols **(mg/L)**	**Beginning of storage**		**5 months**				
	Control	**Pressurized**	**Control**	**Pressurized**	**Oak chips**	**Oak barrels**	**Oak chips + mO_2**
Myricetin	12.19 ± 0.28^{a}	11.00 ± 0.24^{b}	9.90 ± 2.54^{b}	7.62 ± 0.29^{c}	11.92 ± 0.58^{ab}	12.29 ± 1.00^{ab}	11.70 ± 0.61^{ab}
Quercetin	10.62 ± 0.10^{a}	10.41 ± 0.34^{a}	10.13 ± 0.56^{ab}	9.13 ± 0.57^{b}	11.27 ± 0.87^{a}	10.32 ± 0.62^{a}	11.57 ± 2.69^{ab}
Kaempferol	10.68 ± 0.29^{a}	10.32 ± 0.22^{a}	10 .48 ± 1.20^{a}	7.18 ± 1.46^{b}	8.27 ± 0.48^{b}	8.26 ± 0.17^{b}	7.87 ± 0.67^{b}
Total	33.50 ± 0.55^{a}	31.73 ± 0.49^{a}	30.51 ± 1.99^{a}	23.92 ± 1.02^{b}	31.46 ± 1.24^{a}	30.87 ± 1.77^{a}	31.14 ± 3.02^{a}

All data are expressed as mean value ± standard deviation (n = 3). In the same line, different letters indicate significant differences according to a Tukey test ($p < 0.05$).

In relation to the flavonol content (Table IV.2.5), the *pressurized* wine presented, after 5 months of storage, up to 34% and 19% lower content of myricetin and quercetin, respectively, when compared with the other four wines. The kaempferol content was 31% lower in the *pressurized* wine when compared with the *control*, while compared with the other wines treated with aging processes the content was not significantly different ($p<0.05$). These results show that the phenolic acids and flavonols were also affected by HHP treatments, leading to wines with lower content of these compounds and, consequently, to wines with possible lower bioactive activity, when compared with wines treated with wood. The decrease of these phenolic compounds in the pressurized wine may be related with the generation of high-reactive radicals during pressurization,

enhancement of chemical oxidation, and polymerization of phenolic compounds during storage (Chen *et al.*, 2012; Clariana *et al.*, 2011).

Flavan-3-ols composition and mean degree of polymerisation

The flavan-3-ol monomers ((+)-catechin and (-)-epicatechin) and oligomers (B1, B2, B4 dimers and C1 trimer) were identified and quantified in the wines at the beginning of storage and after 5 months of bottle aging (Table IV.2.6). Proanthocyanidins characteristics such as mean degree of polymerization (mDP) and percentage of prodelphinidins (%P) were also determined for all the wine samples (Table IV.2.6).

Table IV.2.6. Flavan-3-ol monomers and oligomers content, and tannin composition of the wine samples at the beginning and after 5 months of storage

Compounds (mg/L)	Beginning of storage		5 months				
	Control	***Pressurized***	***Control***	***Pressurized***	***Oak chips***	***Oak barrels***	***Oak chips + mO_2***
Flavanols							
Catechin	71.82 ± 0.30^{a}	71.03 ± 0.66^{a}	65.70 ± 0.55^{b}	63.63 ± 0.05^{c}	62.34 ± 0.88^{c}	62.02 ± 1.88^{c}	61.74 ± 0.22^{c}
Epicatechin	35.12 ± 0.39^{a}	35.14 ± 0.31^{a}	30.26 ± 0.29^{b}	27.19 ± 0.34^{c}	29.29 ± 0.57^{b}	29.82 ± 1.56^{bc}	29.71 ± 0.24^{b}
Procyanidin B1	62.74 ± 0.46^{ab}	62.38 ± 0.29^{a}	65.55 ± 0.76^{b}	62.04 ± 1.64^{a}	64.18 ± 1.51^{ab}	63.80 ± 1.15^{ab}	63.24 ± 1.03^{ab}
Procyanidin B2	17.83 ± 0.11^{a}	17.56 ± 0.37^{ab}	17.71 ± 0.32^{a}	16.98 ± 0.01^{b}	17.46 ± 0.38^{ab}	17.68 ± 0.07^{ab}	17.40 ± 0.26^{ab}
Procyanidin B4	3.84 ± 0.46^{a}	4.86 ± 0.79^{a}	4.41 ± 0.47^{a}	3.70 ± 0.31^{a}	3.77 ± 0.33^{a}	3.93 ± 0.30^{a}	4.01 ± 0.59^{a}
Procyanidin C1	11.48 ± 0.11^{a}	11.69 ± 0.41^{a}	10.82 ± 0.29^{ab}	9.80 ± 0.14^{b}	10.04 ±0.57^{bc}	10.38 ± 0.89^{abc}	10.15 ± 0.38^{bc}
Total	202.83 ± 1.04^{a}	202.66 ± 0.62^{a}	194.86 ± 0.39^{b}	183.33 ± 2.10^{c}	187.09 ± 2.05^{c}	187. 63 ± 2.44^{c}	186.26 ± 2.16^{c}
Tannin composition							
mDP	4.72 ± 0.04^{a}	4.80 ± 0.03^{a}	4.77 ± 0.07^{a}	5.12 ± 0.12^{b}	5.45 ± 0.02^{c}	5.14 ± 0.05^{b}	5.28 ± 0.04^{b}
%P	6.82 ± 0.42^{a}	6.32 ± 0.17^{a}	5.42 ± 0.01^{b}	7.78 ± 0.23^{c}	8.97 ± 0.19^{d}	8.13 ± 0.21^{c}	8.19 ± 0.36^{cd}

All data are expressed as mean value ± standard deviation (n = 3). In the same line, different letters indicate significant differences according to a Tukey test ($p < 0.05$). *mDP* mean degree of polymerization; *%P* percentage of prodelphinidins

At the beginning of storage no significant difference among the different wine samples was observed for the flavan-3-ols monomers and oligomers content, and for mDP

and %P values. The flavan-3-ols content of the wines studied varied from 183.33 to 202.83 mg/L and the mDP varied from 4.72 to 5.45, which are in agreement with values reported in the literature for Touriga Nacional, Trincadeira, Castelão, Syrah, and Cabernet Sauvignon wines, with flavan-3-ols content and mDP varying from 62.3 to 228.3 mg/L and 2.1 to 9.6, respectively (Cosme *et al.*, 2009).

Along the time of storage, the wines showed different evolution of flavan-3-ols content and degree of polymerization (Table IV.2.6). After 5 months of storage, the *pressurized* wine presented 6% less total flavan-3-ols content than the *control* ($p<0.05$). This value was mainly due to the lower content of (+)-catechin, (-)-epicatechin, and procyanidins B1 and B2 in the *pressurized* wine ($p<0.05$)., whereas the *pressurized* wine presented a higher mDP (4.8) and %P (5.4) than the *control* ($p<0.05$).

The *pressurized* wine showed no significant difference in terms of flavan-3-ol monomers and oligomers ($p>0.05$) when compared with *Oak chips*, *Oak barrels*, and *Oak chips + mO_2* wine samples, having all the samples a content of flavan-3-ol monomers and oligomers lower than the *control*. In terms of mDP and %P, the *pressurized*, *Oak barrels* and *Oak chips + mO_2* wine samples presented similar values among them, but slight lower values when compared with the *Oak chips sample* ($p<0.05$). These results together with the lower content of flavan-3-ol monomers and oligomers presented in the four wines treated with aging processes (*pressurized*, *Oak chips*, *Oak barrels*, and *Oak chips + mO_2* wine) at 5 months of storage, lead to infer similar effect among the four aging processes in the acceleration of flavan-3-ol/proanthocyanidins condensation reactions that occur naturally during the wine storage period. The acceleration of these condensation reactions in wines treated with wood aging processes are probably due to the presence of compounds extracted from the wood during aging, such as phenolic acids, aldehydes, and ellagitannins that participate in chain reactions that can promote flavan-3-ol/proanthocyanidins condensation reactions (Cano-López *et al.*, 2010; Gómez García-Carpintero *et al.*, 2012; González-Sáiz *et al.*, 2014; Sartini et al., 2007; Soto Vázquez *et al.*, 2010; Tao *et al.*, 2014). Dissolution of some quantities of oxygen into the wine by the diffusion of the oxygen through the barrels semipermeable walls during barrels storage (Garde-Cerdán and Ancín-Azpilicueta, 2006; Martínez-Gil *et al.*, 2011; Tao *et al.*, 2014) or by microoxygenation process (Gambuti *et al.*, 2013; Gómez-Plaza and Cano-López, 2011; Parpinello and Versari, 2012) are also reported to influence the acceleration of some phenolic condensation reactions. The pressure treatment, as referred previously, can generate high-reactive radicals during pressurization that enhances chemical oxidation

(Chen *et al.*, 2012; Clariana *et al.*, 2011), and probably lead also to flavan-3-ol/proanthocyanidins condensation reactions acceleration. In addition, as a thermodynamic factor, it is expected that HHP treatments can influence these reactions, since pressure, by "Le Chatelier principle", influences equilibrium of chemical reactions according to changes in volume (Corrales *et al.*, 2008).

IV.2.3.3 Effect of HHP treatments on the wine sensorial characteristics

The sensorial properties of the wines were analysed after 5 months of storage, to assess the organoleptic characteristics of the wine in terms of colour, aroma, and taste. Regarding the colour evaluation (Figure IV.2.3), it can be observed that the wine samples did not present significant differences among them for all the five colour descriptors (red, violet, and brown colour, intensity and limpidity) (p>0.05). These results are in agreement with the chromatic parameters obtained for the wines (Table IV.2.1), since no significant differences (p>0.05) among the different wine samples were observed for the colour tonality, Ye%, Rd%, Bl%, and dA% parameters. Therefore, the slight lower colour intensity observed in the wines treated with aging processes (Table IV.2.1) was not enough to be detected in the sensorial analysis.

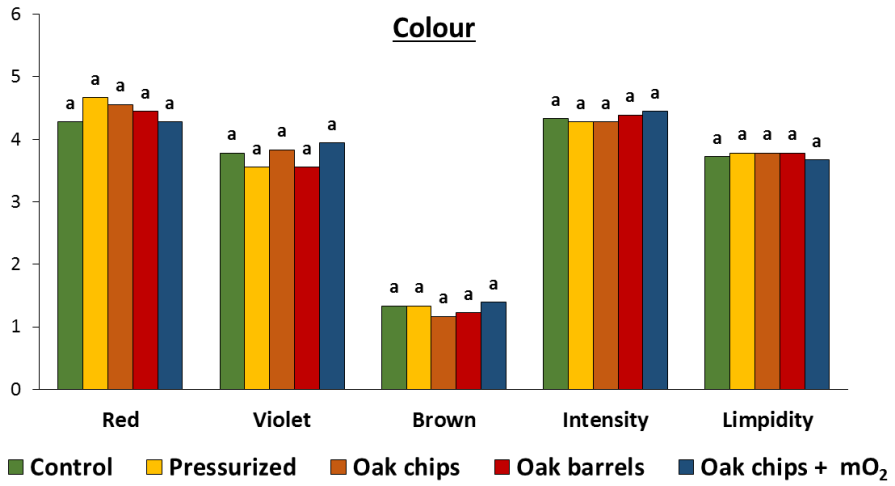


Figure IV.2.3. Descriptive sensory analysis of the colour of wine samples at 5 months of storage. All data are expressed as mean value. Different letters indicate significant differences according ANOVA followed by a Tukey test (p<0.05).

In terms of aroma (Figure IV.2.4), the *pressurized* wine presented the higher scents of cooked fruit and lower scents of fruity aromas (p<0.05). This result is in agreement with results obtained for sulphur dioxide-free red (Chapter II.1) and white

wines (Chapter II.2) that showed that pressure treatments increase the cooked fruit aroma perception due to the increase of Maillard volatile compounds, namely 2-furfural and benzaldehyde (Chapter II.3), since these compounds are described to have "roasty" fruit notes (Castro-Vázquez *et al.*, 2011; Jeleń *et al.*, 2011). The *Oak chips*, *Oak barrels*, and *Oak chips* + mO_2 samples presented higher scents of fruity, spicy, woody, and toasty aromas, when compared with the *control* and *pressurized* wines. Also, the *Oak barrels* presented the higher scents of leather aroma. These results could be explained by the migration of volatile compounds from the wood to the wines. The volatile compounds extracted from wood are mainly 2-furfural, oak lactone, eugenol, vanillin, syringaldehyde, and guaiacol (Arapitsas *et al.*, 2004; Tao *et al.*, 2014), most of them described to have "spicy", "woody" and "toasty" notes (Castro-Vázquez *et al.*, 2011; Jeleń *et al.*, 2011).

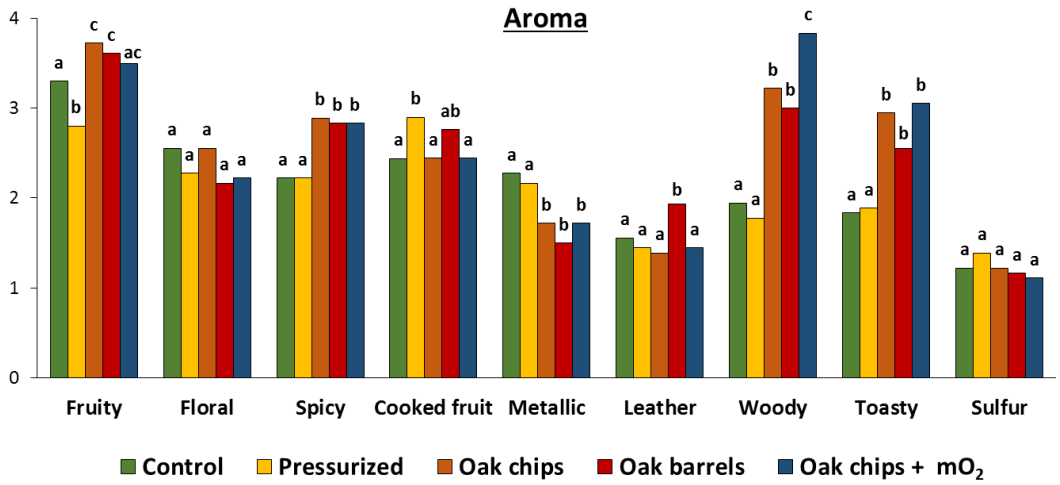


Figure IV.2.4. Descriptive sensory analysis of the aroma of wine samples at 5 months of storage. All data are expressed as mean value. Different letters indicate significant differences according ANOVA followed by a Tukey test (p<0.05).

Comparing the taste assessment of the different wine samples (Figure IV.2.5), no significant differences values were observed for eight of the nine taste descriptors (fruity, astringency, acidity, bitterness, persistence, body, sweetness, and spices). The only significant difference noticed was a higher woody taste for the wines treated with wood when compared with the *pressurized* and *control*.

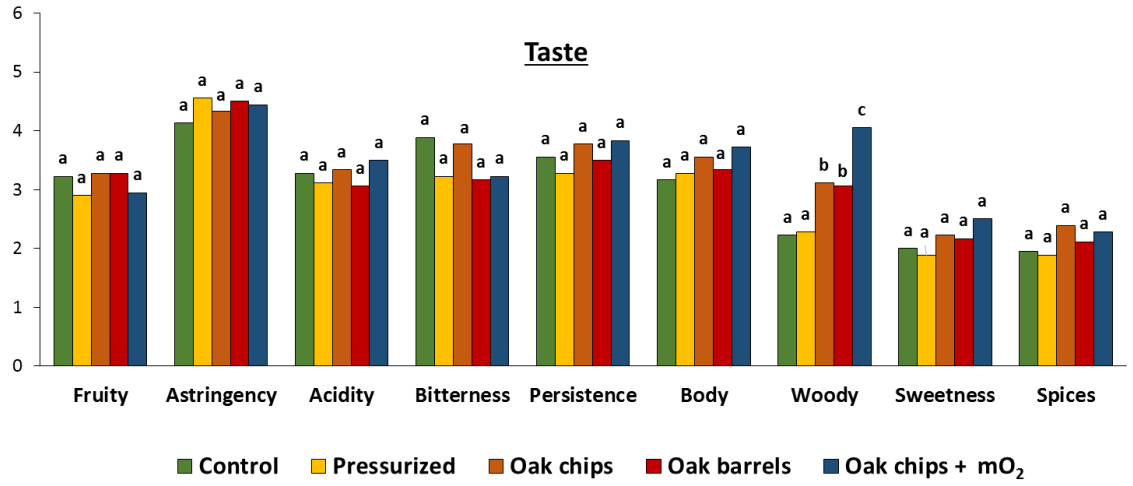


Figure IV.2.5. Descriptive sensory analysis of the taste of wine samples at 5 months of storage. All data are expressed as mean value. Different letters indicate significant differences according ANOVA followed by a Tukey test (p<0.05).

The *Oak chips*, *Oak barrels*, and *Oak chips + mO_2* wines showed a better aroma assessment (Figure IV.2.6) due probably to the higher fruity, spicy, woody, and toasty notes in these wines (Figure IV.2.4), when compared with the *control* and *pressurized* wines. However, all the wines presented a good and similar global assessment.

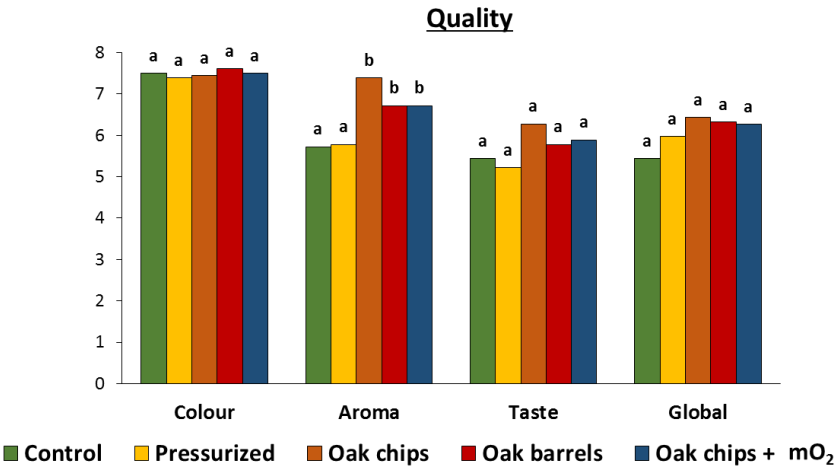


Figure IV.2.6. Descriptive sensory analysis of the global attributes of wine samples at 5 months of storage. All data are expressed as mean value. Different letters indicate significant differences according ANOVA followed by a Tukey test (p<0.05).

In general, regarding sensorial analysis, it seems that the phenolic composition changes, namely in terms of anthocyanins polymerization, mDP, and proanthocyanidins content in HHP treated wines did not alter significantly their taste and colour assessment. This behavior can also be observed for the wine treated by wood, since these wines only showed differences in taste and aroma due to compounds extracted from wood during the

wine storage. According to the sensorial analysis, the pressurized wines could be considered suitable for commercialization as table red wines.

IV.2.4 Concluding remarks

This study shown that a high pressure treatment of 500 MPa during 5 min and the wood aging treatments influenced red wine phenolic composition after 5 months of storage. The main changes that occured in the pressurized wine phenolic composition was the decrease of monomeric (anthocyanins, phenolic acids, and flavonols) and an increase of polymeric compounds, due to the promotion of condensation reactions. These changes are similar to those observed in wines treated with wood, such as storage in oak barrels, and addition of oak chips with or without microoxygenation process, leading to aged wine-like characteristics. Despite the chemical composition changes verified, all the wines presented a similar global assessment, with the aroma and taste characteristics of the pressurized wine resembling more to the wine without any aging process (*control*) than the wines treated with wood.

HHP can be potentially used to accelerate the wine aging process of wines with low aging potential, producing young red wines with pleasant and distinct characteristics. In spite of the promising results, the ideal wine aging process is quite extensive. The challenge for the academic community and wine industry is the combination of these or other new methodologies in a concerted strategic approach in order to produce novel wines meeting the modern wine consumer demands, keeping tradition along with innovation.

CHAPTER V

Conclusions and Future Perspectives

In this PhD thesis, the influence of HHP treatments on wine chemical and sensorial characteristics was studed in order to increase the fundamental knowledge about the effect of HHP on wine, contributing for the evaluation of the feasibility of using this technology in winemaking.

The application of high hydrostatic pressure treatments in winemaking for wine preservation, as an alternative to sulphur dioxide, was evaluated studying the effect of HHP in the physicochemical and sensorial properties of sulphur dioxide-free in both red and white wines, during bottle storage. This is the first report where physicochemical and sensorial characteristics of pressurized red and white wines were evaluated during one year of bottle aging.

High pressure treatments with 5 min of processing time and pressures of 425 and 500 MPa were shown to have influence on both red and white wine physicochemical and sensorial characteristics. However, the effects are only perceptible after, at least, 6 months of storage. The alterations that occurred on the pressurized red wine characteristics, such as the more orange-red colour and the lower antioxidant activity (15- 27% less), total phenolic content (9% less), and anthocyanins content (45–61% less), were due to an increase of condensation reactions of phenolic compounds. The increase of these condensation reactions lead to the formation of compounds with higher degree of polymerisation that became insoluble in wine along storage, increasing consequently the amount of wine deposits in the pressurized wines. In terms of white wines, pressurized wines showed, after one year of storage, a more brownish colour and a lower antioxidant activity (15% less) and total content of phenolic compounds (10% less) compared to the unpressurized wines. These results, together with the lower content of free amino acids (*e.g* 87% less of serine) and higher content of furans (*e.g* 10 fold higher of 2-furfural), present in the pressurized wines after nine months of storage, lead to propose an effect of HHP treatments in the acceleration of Maillard reactions occurring during the wine storage period. Sensorial analysis showed that the pressurized wines presented a higher cooked fruit aroma and lower fruity and floral aromas, and also slightly higher bitterness level and a lower body and balance level than the unpressurized wines. Therefore, contrary to the pressurized sulphur dioxide-free red wine, the pressurized white wines are not considered suitable for commercialization as table white wines due to the higher brownish colour and cooked fruit aroma characteristic of an aged or thermally treated wine.

As the aroma is one of the most important quality parameters of the wine for consumers, the impact of the pressure treatments on the volatile composition of the sulphur dioxide free-red and white wines, during bottle storage, was also evaluated. Despite some differences observed in the volatile composition of the pressurized wines after 2 months of storage, namely lower content of esters in pressurized white wines and lower content of carboxylic acids and higher content of norisoprenoids in pressurized red wines, when compared with the unpressurized wines, the impact of the pressure treatments was minimal in the first months of storage. After 9 months, the pressurized wines presented a higher content of Maillard derived volatile compounds (*e.g* up to 10- and 15-fold higher of furfural and benzaldehyde, respectively), acetals and ketones when compared to the unpressurized wines. The two pressure treatments studied showed similar effects on both white and red wine volatiles. The changes on the volatile composition of the pressurized wines, namely the increase of furans, aldehydes, ketones, and acetals content, indicated that, in addition to the acceleration of the Maillard reactions mentioned above, the HHP treatments accelerate also the oxidation of alcohols and fatty acids. This effect lead to wines with a volatile composition network approaching the characteristic of faster aged and/or thermally treated wines.

The acceleration of the Maillard reactions by high pressure treatments was also studied in model wine solutions to better understand the effects of HHP on the formation of Maillard derived volatile compounds (MVC) during storage. The results showed that high pressure treatment accelerates the Maillard reaction in model wine solutions (acidic media) and this effect was quantifiable, mainly, after 6 months of storage. Pressurized model solutions presented higher concentration of 2-furfural, phenylacetaldehyde and benzaldehyde, when compared to the controls. Despite the higher concentration of MVC in the pressurized samples, only the content of phenylacetaldehyde was higher than the respective perception threshold in wine, which allow inferring the perception of "honey-like" and "sweet" odours. Furthermore, the increase of MVC content in the pressurized model wine solutions was lower than that observed in wines, indicating that probably a high range of compounds are affected by the pressure treatment in wine, when compared with the model wine solutions, that indirectly participate in the formation of MVC during storage.

Also, in model wine solutions, it was observed that pressure treatment (500 MPa for 5 min) did not promote, at the beginning of storage, condensation reactions between anthocyanins and flavonols. Conversely, after 8 months of storage it was observed the

formation of two unknown compounds in pressurized model wine solution, which appear to result from the polymerization of malvidin-3-*O*-glucoside and catechin with other compounds presented in the model wine solution (potentially formic acid), being an artifact. Therefore, it is possible that the pressure treatment had a higher impact in terms of kineticks of reactions and in less extent in terms of different compounds formed.

The application of high hydrostatic pressure treatments in winemaking to treat young wines was evaluated. For this propose, the effect of a high hydrostatic pressure treatment in the phenolic composition of a red wine was studied. High pressure treatments with pressures of 500 MPa during 5 min and 600 MPa during 20 min showed to influence red wine phenolic composition, and led to alterations in the wine sensorial characteristics. Most of these effects are only noticeable after storage for 5 months, being more pronounced for the pressure treatment of 600 MPa for 20 min. These data indicate that the conditions of pressure treatment, such as "pressure" and "pressure holding time", had significant influence in the phenolic composition of pressurized wines. The main changes that occur on the pressurized red wine phenolic composition, such as the lower content of monomeric anthocyanins (up to 14% less), phenolic acids (up to 11% less), and flavonols (up to 19% less), are due to an increase of condensation reactions and oxidation of these compounds. At the same time, HHP influenced the polymerisation and cleavage reactions in which proanthocyanidins are involved. The sensorial analysis of pressurized wines showed a slight lower evaluation regarding the aroma, due to the higher intensity of cooked fruit aroma and lower intensity of fruity notes, when compared with the unpressurized wine. The wine pressurized at 600 MPa presented the higher values of bitterness and persistence, and lower astringency among all the wine samples that could be explained by the lower mDP value (4.28) and higher content of flavan-3-ol monomers (199.80 mg/L).

The effect of the pressure treatment of 500 MPa for 5 min on the wine phenolic composition was also compared with the effect of different aging processes, such oak barrels, oak chips and micro-oxygenation with oak chips. The main changes that occurred in the pressurized wine phenolic composition such the decrease of monomeric anthocyanins, phenolic acids, and flavonols and an increase of polymeric compounds, due to the promotion of condensation reactions, were similar to those observed in wines treated with wood aging processes. Also, the pressurized wines presented a higher content of pyranoanthocyanins than the wines aged with oak chips and oak barrels (14% and 11% higher, respectively), and no significant different value was observed when compared

with the wine aged with addition of oak chips and microoxygenation process. These results indicated that HHP treatment and the addition of oak chips to the wine with microoxygenation process were the treatments that accelerated more the polymerization of the monomeric anthocyanins during storage. Despite the chemical composition changes verified, all the wines presented a similar global assessment, where the aroma and taste characteristics of the pressurized wine resembled more to the wine without any aging process than the wines treated with wood, due to the absence of woody taste and aromas. Therefore, the HHP treatments seem to promote reactions that are similar to those observed in wine wines treated with wood aging processes.

In conclusion, the results presented in this thesis showed that HHP treatments accelerate the Maillard reaction and the polymerization reactions between phenolic compounds present in the wine, influencing the chemical and sensorial properties of wine. However, it seems that HHP can be potentially used to preserve red wine, as an alternative to sulphur dioxide, producing wines with pleasant and distinct characteristics. The use of this technology proved to be most promising for preservation of red wines than white wines due its effect on sensorial properties that was evaluated positively for red wines and negatively for white wines. These aspects should be taken into consideration in the implementation of HHP treatments to wine conservation as an alternative to SO_2.

From the wine aging point of view, it was shown that HHP can be potentially used to modify the organoleptic properties of wine. As the traditional barrel aging technology has several disadvantages, the application of HHP processing during wine aging process can benefit the winemaking industry in overcoming these disadvantages. On the other hand, the utilization of HHP processing is recommended to commence with the aging of wines with low aging potential. For these wines, HHP processing can be potentially used to accelerate their aging process, modify their sensorial properties and stop the quality decrease. However, the cost of HHP equipment also needs to be taken into account when using it for wine aging in the winemaking industry. In addition, a market research and consumer preference tastings are essential to know the acceptance of wine consumers towards high-pressure treated wines.

From the results obtained herein future studies can be proposed:

i) Evaluate the application of HHP treatments in the production of different sherry wines, in substitution of thermal treatments.

ii) Evaluate the application of HHP treatments in wine with low aging potential in order to accelerate their aging process and conferring different sensorial properties.

iii) Evaluate the application of HHP treatments on wine with oak chips in order to accelerate the aging process and extract some compounds of the wood to confer some wood sensorial properties to the pressurized wine.

iv) Study the effect of HHP treatments on the structure, antioxidant capacity, and interactions of phenolic compounds in model wine solutions.

v) Study the effect of HHP treatments on the volatility and perception of wine aroma compounds in model wine solutions.

vi) Study the formation of Maillard derived volatile compounds by HHP treatments in model wine solutions containing also phenolic compounds.

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Supplementary data

Table S1. Volatile compounds identified by HS-SPME/ GC×GC -ToFMS in the white wine samples (Chapter II.3)

Peak number	^1t_R [a] (s)	^2t_R [a] (s)	Compound	$RI_{lit.}$ [b]	RI_{cal} [c]	After 2 months of storage				After 9months of storage			
						Untreated	SO_2	425 MPa	500 MPa	Untreated	SO_2	425 MPa	500 MPa
						Peak Area[d] ($x10^5$) and RSD [f] (%)							
			Carboxylic Acids										
1	95	3.176	Acetic acid	600	619	533.36 (6)	751. 31 (6)	41.03 (24)	48.36 86)	674.02 (10)	607.97 (27)	341.55 (6)	331.51 (11)
2	155	3.856	2-Methylpropanoic acid (Isobutyric acid)	767	762	5.02 (35)	8.57 (13)	7.34 (9)	6.97 (6)	-	-	-	-
3	175	4.632	Butanoic acid (Butyric acid)	808	806	13.34 (18)	16.59 (29)	15.44 (30)	10.17 (28)	14.46 (1)	11.26 (2)	-	-
4	225	3.896	2-Methyl butanoic acid	867	878	6.85 (22)	9.77 (24)	7.84 (18)	3.70 (24)	-	-	-	-
5	235	3.680	3-Methylbutanoic acid (Isovaleric acid)	876	880	10.79 (19)	17.05 (18)	10.14 (38)	13.02 (27)	19.59 (34)	13.65 (3)	-	-
6	360	3.864	Hexanoic acid (Caproic acid)	1017	1015	225.02 (13)	533.28 (14)	370.42 (11)	237.24 (12)	286.59 (7)	306.94 (39)	360.79 (21)	286.23 (8)
7	525	2.704	Octanoic acid (Caprylic acid)	1179	1203	596.36 (28)	859.19 (10)	595.55 (22)	537.90 (5)	397.22 (20)	424.85 (40)	504.07 (21)	365.63 (13)
8	690	2.056	*n*-Decanoic acid (*n*-Caprinic acid)	1380	1372	47.55 (14)	94.55 (8)	126.41 (10)	65.56 (9)	32.88 (44)	23.78 (33)	13.93 (30)	19.11 (33)
9	1070	1.488	*n*-Hexadecanoic acid (Palmitic acid)	1985	1984	-	21.48 (1)	28.16 (26)	17.60 (24)	-	-	-	-
			Subtotal (GC Peak Area)			1438.28 (17)	2311.79 (10)	1202.32 (18)	940.51 (8)	1424.77 (13)	1388.45 (33)	1220.33 (17)	1002.48 (11)
			Subtotal (Number of Compounds)			8	9	9	9	6	6	4	4
			Subtotal (%)			13.6	19.2	14.6	11.9	17.9	14.2	14.6	11.4
			Esters										
			Aliphatics Ethyl esters										
10	95	0.456	Ethyl ethanoate (Ethyl acetate)	613	613	585.77 (4)	453.84 (20)	334.97 (26)	357.59 (2)	510.28 (9)	541.90 (11)	278.12 (18)	313.52 (23)
11	125	0.496	Ethyl propanoate (Ethyl proprionate)	714	684	51.26 (12)	54.33 (19)	38.39 (23)	41.45 (30)	48.45 (2)	42.85 (3)	46.37 (5)	45.76 (7)
12	150	0.488	Ethyl 2-methylpropanoate (Ethyl isobutyrate)	762	742	15.48 (26)	11.52 (14)	13.07 (18)	13.14 (8)	33.71 (3)	39.87 (2)	35.66 (12)	37.96 (14)
13	180	0.536	Ethyl butanoate (Ethyl butyrate)	800	807	230.97 (10)	201.75 (13)	192.13 (8)	187.32 (6)	171.28 (11)	167.61 (2)	172.98 (2)	167.42 (6)
14	190	1.184	Ethyl 2-hydroxypropanoate (Ethyl lactate)	815	820	22.80 (13)	29.74 (4)	25.92 (7)	21.94 (3)	274.84 (12)	262.08 (11)	53.06 (5)	53.46 (5)
15	210	0.656	Ethyl but-2-enoate	844	845	27.20 (4)	23.53 (24)	22.37 (13)	19.55 (3)	16.09 (5)	16.49 (1)	17.30 (1)	17.38 (1)
16	215	0.512	Ethyl 2-methylbutanoate (Ethyl 2-methylbutyrate)	849	851	-	-	-	-	15.12 (7)	15.83 (2)	18.77 (3)	20.06 (3)
17	220	0.528	Ethyl 3-methylbutanoate (Ethyl isovalerate)	856	857	33.03 (4)	25.92 (34)	26.75 (19)	21.33 (4)	38.91[e]	41.12 (1)	48.09 (3)	48.78 (2)
18	260	0.552	Ethyl pentanoate (Ethyl valerate)	898	906	4.13 (17)	3.44 (17)	3.30 (18)	2.37 (19)	3.75 (26)	2.79 (5)	3.92 (32)	3.92 (32)
19	300	1.352	Ethyl 3-hydroxy-butanoate	949	949	1.95 (20)	4.60 (10)	3.24 (17)	2.75 (13)	-	-	-	-
20	350	0.576	Ethyl hexanoate (Ethyl caproate)	1001	1001	489.23 (7)	422.62 816)	181.08 (35)	394.23 (39)	415.41 (11)	460.34 (11)	474.81 (26)	497.69 (28)
21	360	0.632	Ethyl hex-3-enoate	1006	1012	5.91 (12)	4.63 (27)	3.55 (13)	3.84 (8)	2.51 (9)	3.35 (61)	2.41 (14)	2.69 (19)

22	390	0.64	Ethyl hex-2-enoate	1040	1045	17.72 (3)	16.88 (36)	14.06 (17)	10.78 (4)	12.04 (4)	11.56 (5)	12.26 (3)	12.79 (6)
23	405	0.952	Ethyl 2-hydroxy-4-methyl-pentanoate	1060	1062	1.25 (30)	2.28 (14)	1.51 (26)	1.45 (10)	8.65 (12)	9.17 (28)	6.87 (4)	5.88 (19)
24	420	2.040	Ethyl 4-hydroxybutanoate	1039	1080	79.80 (21)	89.16 (34)	74.19 (47)	62.43 (8)	-	15.73 (22)	9.86 (7)	9.37 (18)
25	440	0.658	Ethyl heptanoate	1104	1100	28.30 (17)	21.04 (29)	22.16 (14)	12.84 (26)	6.72 (5)	9.23 (4)	10.15 (11)	9.61 (6)
26	515	0.936	Diethyl butanedioate (Diethyl succinate)	1182	1189	20.95 (8)	44.85 (15)	33.46 (4)	19.43 (5)	248.57 (31)	403.92 (1)	216.85 (8)	221.49 (23)
27	530	0.576	Ethyl octanoate (Ethyl caprylate)	1199	1207	646.16 (30)	488.72 (19)	260.90 (7)	272.72 (22)	740.75 (20)	1090.71 (17)	901.46 (25)	1061.91 (21)
28	570	0.64	Ethyl oct-2-enoate	1246	1254	1.89 (27)	1.75 (23)	2.12 (6)	-	1.69[e]	1.19 (10)	0.67 (5)	1.03 (3)
29	595	0.880	Ethyl-*n*-propyl butanedioate (*m/z* = 129,101,73,43)	-	1283	-	-	-	-	0.90 (38)	0.59 (45)	0.44 (52)	0.56 (11)
30	610	0.576	Ethyl nonanoate (Ethyl pelargonate)	1294	1301	42.99 (11)	43.82 (27)	38.10 (26)	20.35 813)	5.17 (1)	5.38 (45)	1.88 (11)	1.77 (11)
31	640	0.816	Ethyl butyl butanedioate (*m/z* = 147,129,101,56)	-	1339	-	-	-	-	1.79 (27)	1.92 (34)	0.69 (16)	0.56 (17)
32	690	0.584	Ethyl decanoate (Ethyl caprinate)	1394	1401	668.03 (5)	703.06 (9)	75.34 (19)	114.51 (20)	202.45 (35)	248.32 (25)	244.67 (45)	393.92 (34)
33	715	0.816	Ethyl 3-methylbutyl succinate	1429	1435	0.91 (15)	1.77 (21)	1.25 (12)	0.65 (13)	20.06 (36)	22.08 (36)	9.26 (14)	7.92 (19)
34	795	0.920	Ethyl 3-hydroxytridecanoate	1539	1553	0.97 (32)	2.25 (26)	1.95 (5)	1.26 (13)	1.71 (40)	1.64 (18)	1.89 (23)	1.59 (5)
35	835	0.592	Ethyl dodecanoate (Ethyl laurate)	1593	1601	121.28 (6)	117.53 (1)	129.32 814)	125.72 (5)	88.02 (1)	53.34 (8)	24.81 (7)	119.80 (13)
36	965	0.600	Ethyl tetradecanoate (Ethyl myristate)	1793	1801	8.97 (22)	12.55 (15)	27.67 (31)	11.70 (15)	5.13 (56)	4.92 (10)	6.38 (32)	9.54 (21)
37	1085	0.616	Ethyl hexadecanoate (ethyl palmitate)	1994	2010	19.28 (2)	22.99 (34)	16.83 (24)	14.34 (19)	15.19 (35)	11.89 (3)	23.31 (42)	23.06 (54)
			Aromatics Ethyl esters										
38	510	0.96	Ethyl benzoate	1179	1184	1.00 (12)	1.11 (22)	1.00 (11)	0.86 (7)	1.52 (27)	0.98 (18)	1.06 (8)	0.98 (15)
39	570	1.016	Ethyl 2-phenylethanoate (Ethyl 2-phenylacetate)	1251	1254	3.92 (24)	5.98 (15)	5.86 (8)	3.07 (18)	3.01 (28)	2.82 (22)	4.01 (11)	3.54 (16)
			Subtotal (GC Peak Area)			3131.17 (12)	2811.64 (16)	1550.51 (20)	1737.62 (17)	2893.75 (16)	3489.64 (12)	2628.01 (21)	3093.95 (22)
			Subtotal (Number of Compounds)			27	27	27	26	28	29	29	29
			Subtotal (%)			29.7	23.4	18.8	21.9	36.3	35.5	31.5	35.2
			Aliphatic acetate esters										
40	75	0.456	Methyl ethanoate (Methyl acetate)	559	566	5.79 (27)	6.49 (12)	13.07 (15)	7.44 (24)	-	3.18 (66)	2.88 (28)	4.85 (33)
41	125	0.52	*n*-Propyl ethanoate (*n*-Propyl acetate)	712	684	58.91 (5)	46.85 (31)	49.76 (9)	43.06 (8)	11.31 (41)	14.06 (4)	12.14 (2)	14.00 (23)
42	160	0.52	2-Methylpropyl ethanoate (Isobutyl acetate)	767	766	149.62 (20)	143.19 (23)	118.75 (9)	111.06 (4)	26.35 (10)	42.86 (1)	38.82 (3)	40.76 (2)
43	190	0.56	Butyl ethanoate (Butyl acetate)	812	819	25.36 (5)	22.72 (28)	21.70 (8)	19.03 (9)	6.67 (16)	3.92 (16)	3.33 (32)	3.24 (2)
44	235	3.208	2-Methylbutyl ethanoate (2-Methylbutyl acetate)	885	879	30.05 (14)	32.36 (17)	10.07 (42)	10.14 (47)	8.46 (19)	16.24 (3)	4.87 (27)	5.01 (4)
45	240	0.544	3-Methylbutyl ethanoate (Isoamyl acetate)	876	882	393.62 (1)	681.65 (11)	307.91 (15)	335.99 (26)	310.89 (7)	280.80 (14)	363.87 (6)	337.09 (16)
46	270	0.576	Pentyl ethanoate (Amyl acetate)	915	916	19.63 (18)	21.16 (20)	19.76 (17)	11.47 (29)	2.57 (50)	2.42 (24)	1.16 (4)	1.29 (18)
47	270	0.680	Pent-2-enyl ethanoate (Pent-2-enyl acetate)	909	916	3.17 (19)	1.79 (26)	2.55 (29)	15.48 (14)	-	-	-	-
48	355	0.672	Hex-3-enyl ethanoate (Hex-3-enyl acetate)	1007	1006	83.37 (11)	88.71 (13)	79.20 (2)	75.30 (15)	14.25 (52)	24.41 (7)	16.00 (28)	12.75 (15)

49	360	0.64	Hexyl ethanoate (Hexyl acetate)	1014	1006	370.82 (13)	457.23 (26)	338.09 (34)	332.11 (28)	86.79 (7)	137.91 (14)	113.21 (3)	109.55 (2)
50	455	0.592	Heptyl ethanoate	1118	1118	3.44 (2)	3.95 (4)	2.88 823)	1.44 (45)	-	-	-	-
51	535	0.624	Octyl ethanoate	1213	1211	6.74 (5)	5.45 (25)	5.38 822)	3.82 (6)	-	-	-	-
			Aromatics acetates										
52	580	1.064	2-Phenylethyl ethanoate (2-Phenylethyl acetate)	1256	1260	307.61 (2)	273.32 (14)	263.64 (23)	235.91 (7)	86.95 (10)	98.50 (11)	79.86 (4)	78.95 (8)
			Subtotal (GC Peak Area)			1458.13 (8)	1784.88 (17)	1232.75 (20)	1202.23 (19)	554.22 (10)	624.30 (12)	636.15 (6)	607.48 (12)
			Subtotal (Number of Compounds)			13	13	13	13	9	10	10	10
			Subtotal (%)			13.8	14.9	14.9	15.18	6.9	6.4	7.6	6.9
			Aliphatic esters										
53	170	0.568	3-Methylbutyl methanoate (Isoamyl formate)	792	780	-	-	-	-	-	-	1.52 (17)	1.46 (5)
54	280	0.600	Methyl hexanoate (Methyl caproate)	934	927	17.39 (1)	14.60 (28)	12.94 (30)	11.67 (3)	11.76 (25)	6.74 (6)	4.73 (16)	6.03 (9)
55	300	1.352	2-Methylpropyl butanoate (Isobutyl butyrate)	961	949	-	-	-	-	1.19 (11)	-	-	-
56	320	0.560	Pentyl propanoate (Amyl propionate)	969	972	10.64 (16)	11.07 (11)	8.02 (14)	6.77 (5)	-	-	-	-
57	365	0.520	3-Methylpropyl butanoate (Isoamyl butyrate)	1013	1017	7.22 (6)	1.36 (31)	3.51 833)	1.94 (29)	-	-	-	-
58	405	0.544	Butyl 3-methylbutanoate (*n*-Butyl isovalerate)	1048	1062	-	-	-	-	6.84 (11)	7.27 (8)	7.39 (2)	7.28 (7)
59	410	1.080	3-Methylbutyl 2-hydroxypropanoate (Isoamyl lactate)	1047	1068	-	-	2.04 (15)	1.46 (18)	26.14[e]	25.94 (30)	3.58 (18)	3.60 (21)
60	420	0.936	Octy methanoate (Octyl formate)	1104	1079	-	-	-	-	36.27 (13)	31.87 (12)	16.25 (27)	14.94 (31)
61	440	0.560	Propyl hexanoate (Propyl caproate)	1093	1101	3.96 (10)	4.46 (26)	3.86 (28)	2.51 (3)	4.89 (25)	-	-	-
62	465	0.600	Methyl octanoate (Methyl caprylate)	1126	1130	35.40 (5)	34.64 (18)	26.00 (19)	17.00 (5)	21.25 (8)	22.73 (8)	24.12 (4)	23.32 (4)
63	490	0.536	2-Methylpropyl hexanoate (Isobutyl caproate)	1148	1160	3.22 (13)	3.35 (21)	2.35 (9)	1.70 (2)	3.91 (13)	4.58 (5)	4.97 (3)	5.26 (6)
64	515	0.592	Butyl hexanoate (Butyl caproate)	1189	1188	-	1.73 (19)	-	-	-	-	-	-
65	560	0.544	Hexyl 2-methyl-butanoate (Hexyl 2-methyl-butyrate)	1234	1242	6.99 (20)	7.24 (20)	4.70 (23)	3.18 (7)	2.76 (58)	-	-	-
66	575	0.552	3-Methylbutyl hexanoate (Isoamyl caproate)	1250	1259	41.32 (7)	28.31 (28)	31.57 823)	20.58 (4)	30.46 (12)	30.03 (27)	47.40 (48)	86.66 (32)
67	610	0.560	Propyl octanoate (Propyl caprylate)	1296	1301	8.30 (5)	8.11 (7)	5.80 (12)	4.05 (4)	7.75 (32)	7.79 (34)	7.16 (24)	6.13 (35)
68	635	0.600	Methyl decanoate (Methyl caprinate)	1326	1332	3.46 (10)	3.03 (33)	4.39 (12)	2.24 (4)	2.18 (43)	1.13 (3)	0.80 (5)	1.54 (5)
69	650	0.552	2-Methylpropyl octanoate (Isobutyl caprylate)	1348	1351	6.42 (7)	5.16 (22)	5.51 815)	4.20 85)	3.84 (1)	4.48 (5)	3.98 (6)	4.69 (3)
70	730	0.552	3-Methylbutyl octanoate (Isoamyl caprylate)	1444	1456	79.33 (5)	63.38 (12)	79.85 (10)	63.74 (4)	36.75 (19)	36.64 (5)	26.12 (26)	27.90 (16)
71	840	0.608	1-[2-(Isobutyryloxy)-1-methylethyl]-2,2-dimethylpropyl 2-methylpropanoate (*m/z* = 243,159,111,83,71,43)	-	1607	8.09 (4)	4.56 (11)	6.28 (26)	4.56 (18)	6.28 (36)	4.23 (62)	8.49 (69)	4.53 (63)
72	855	0.560	1-Methylethyl dodecanoate (Isopropyl laurate)	1627	1632	2.21 (15)	2.22 (17)	2.77 (11)	2.08 (31)	3.16 (7)	2.27 (24)	1.60 (2)	-
73	870	0.568	3-Methylbutylpentadecanoate (Isoamyl decanoate)	1651	1655	7.76 (11)	8.14 (13)	20.25 831)	10.59 (20)	4.59 (66)	2.95 (6)	2.00 (9)	3.95 (23)
74	975	0.568	Methylethyl tetradecanoate (Isopropyl myristate)	1824	1834	3.28 (5)	7.10 (29)	4.79 (24)	2.90 (20)	2.40 (23)	4.37 (23)	2.57 (27)	2.70 (17)

75	1035	0.672	Methyl 14-methylpentadecanoate	1887	1919	0.89 (12)	0.63 (18)	0.86 811)	0.65 (16)				
76	1100	0.592	Methylethyl hexadecanoate (Isopropyl palmitate)	1999	2037	3.46 (23	6.33 (31)	6.31 814)	3.75 (4)	5.16 (6)	37.38 (61)	27.67 (27)	21.98 (22)
77			*Aromatics esters*										
	530	1.192	Methyl 2-hydroxibenzoate (Methyl salicylate)	1190	1207	-	1.33 (21)	1.30 816)	1.32 (19)	2.73 (9)	2.60 (36)	-	-
78	720	0.960	2-Phenylethyl butanoate (2-Phenylethyl butyrate)	1442	1439	0.69 (32)	0.74 (29)	0.74 (13)	0.42 (23)	-	-	-	-
79	885	0.936	Hexyl 2-hydroxybenzoate (*n*-Hexyl salicylate)	1682	1678	0.55 (14)	0.58 (27)	0.86 (16)	0.53 (11)	-	-	-	-
			Subtotal (GC Peak Area)			250.61 (7)	218.07 (19)	234.70 (16)	167.82 (7)	220.31 (15)	234.49 (24)	190.34 (28)	221.99 (23)
			Subtotal (Number of Compounds)			20	22	22	22	20	18	17	16
			Subtotal (%)			2.4	1.8	2.9	2.1	2.8	2.4	2.3	2.5
			Alcohols										
			Aliphatics alcohols										
80	80	0.712	Propan-1-ol (Propyl alcohol)	**595**	578	6.92 (22)	8.41 (26)	-	-	28.68 (33)	22.30 (16)	24.52 (6)	17.77 (18)
81	90	0.800	2-Methyl-propan-1-ol (Isobutyl alcohol)	600	602	146.93 (29)	145.09 (33)	163.10 (18)	139.19 (25)	151.29 (4)	134.26 (14)	156.19 (14)	147.66 (9)
82	105	0.816	Butan-1-ol (Butyl alcohol)	653	637	11.15 (18)	11.04 (30)	10.53 (11)	7.76 (6)	19.14 (2)	18.04 (4)	17.60 (2)	17.16 (7)
83	110	0.816	1- Penten-3-ol	656	649	-	-	-	-	1.30[e]	1.28 (4)	1.33 (1)	1.39 (3)
84	120	0.688	Pentan-3-ol (Diethyl carbinol)	710	672	-	-	-	-	0.55 (18)	0.39 (14)	-	-
85	120	0.712	Pentan-2-ol (Methylpropyl carbinol)	706	672	-	-	-	-	0.87 (8)	0.59 (3)	-	-
86	145	0.864	3-Methyl-butan-1-ol (Isoamyl alcohol)	737	731	942.69 (21)	1145.90 (20)	716.02 (5)	697.36 (3)	937.34 (4)	1301.66 (10)	717.12 (17)	725.79 (17)
87	160	0.944	Pentan-1-ol (Amyl alcohol)	768	767	10.62 (26)	11.45 (35)	10.70 (4)	7.87 (15)	5.93 (3)	3.95 (1)	5.06 (27)	4.87 (3)
88	155	4.840	Propane-1,2-diol (Propylene Glycol)	792	764	23.93 (19)	18.95 (8)	21.70 (8)	17.79 (20)	-	-	-	-
89	160	1.200	Pent-2-en-1-ol	769	768	1.80 (34)	1.47 (31)	1.54 (9)	1.67 (12)	-	-	-	-
90	170	3.144	Butane-2,3-diol	806	796	322.84 (18)	560.15 (34)	377.00 (19)	366.79 (2)	341.15 (40)	375.95 (60)	281.59 (9)	353.90 (24)
91	175	3.608	Butane-2,2-diol	806	805	250.97 (14)	242.77 (17)	180.96 (17)	192.07 (14)	155.94 (36)	149.87 (31)	126.28 (7)	169.71 (1)
92	215	0.992	3-Methyl-pentan-1-ol	854	852	70.98 (9)	73.92 (19)	67.96 (5)	55.30 (3)	34.14 (3)	35.25 (4)	36.34 (3)	35.54 (1)
93	215	1.184	3-Ethoxy-propan-1-ol	855	852	8.97 (14)	15.29 (9)	11.53 (15)	5.63 (10)	7.74 (1)	6.94 (23)	8.86 (8)	7.51 (5)
94	220	1.112	Hex-3-en-1-ol isomer	856	858	39.44 (14)	39.52 (19)	38.76 (4)	32.08 (6)	19.26 (4)	19.16 (7)	19.84 (1)	19.66 (3)
95	225	1.168	Hex-3-en-1-ol isomer	864	861	6.86 (20)	8.30 (22)	-	-	5.74 (3)	4.98 (22)	3.98 (21)	2.72 (11)
96	230	1.040	Hexan-1-ol	870	870	189.47 (19)	194.60 (15)	203.55 (16)	165.92 (7)	159.30 (4)	224.09 (4)	167.99 (5)	220.64 (13)
97	260	0.832	Heptan-2-ol	905	906	2.97 (9)	3.35 (24)	2.76 (8)	2.18 (2)	4.29 (2)	3.81 (11)	1.40 (4)	1.22 (15)
98	330	0.928	Heptan-1-ol	969	979	9.02 (16)	11.50 (27)	9.45 (13)	6.90 (5)	12.79 (18)	9.94 (14)	11.03 (6)	12.83 (23)
99	335	0.936	Oct-1-en-3-ol	980	985	3.29 (14)	3.90 (25)	3.09 (15)	2.39 (8)	3.49 (31)	2.97 (12)	2.32 (13)	2.27 (8)
100	365	1.016	3-Ethyl-4-methylpentanol	1020	1018	1.77 (8)	2.23 (26)	1.85 (6)	-	3.27 (10)	2.76 (17)	-	-
101	380	0.88	2-Ethyl-hexan-1-ol	1029	1034	3.97 (12)	5.64 (14)	4.01 (3)	3.55 (16)	12.55 (3)	5.29 (12)	6.48 (2)	5.91 (10)
102	410	1.000	Octan-1-ol	1070	1068	23.28 815)	28.83 (23)	24.07 (5)	18.70 (4)	-	-	-	-

103	445	0.784	Nonan-2-ol	1098	1107	13.40 (12)	16.04 (29)	12.13 (13)	8.75 (5)	22.15 (13)	17.86 (15)	14.27 (32)	9.96 (18)
104	515	0.880	Nonan-1-ol	1171	1189	6.36 (10)	9.32 (27)	7.34 (12)	6.34 (12)	9.30 (16)	4.34 (23)	3.53 (45)	3.81 (32)
105	590	0.664	Dec-2-en-1-ol	1283	1277	2.05 (23)	6.15 (30)	3.53 (18)	4.33 (1)	-	-	-	-
106	595	0.856	Decan-1-ol	1272	1283	9.04 (17)	12.76 (27)	11.72 (7)	7.62 (6)	15.42 (25)	10.86 (21)	8.87 (9)	8.48 (1)
107	615	0.752	Dec-2-en-4-ol (*m/z* = 71,58)	-	1307	0.22 (17)	0.26 (6)	0.26 (16)	0.21 (22)	-	-	-	-
108	620	0.744	Undecan-2-ol	1309	1313	1.89 (17)	2.28 (28)	2.50 (11)	1.58 (1)	4.83 (20)	2.81 (21)	0.97 (14)	1.30 (7)
109	740	0.888	Dodecan-1-ol	1473	1470	8.64 (36)	8.67 (9)	10.69 (24)	11.56 (17)	3.73 (35)	2.65 (5)	-	-
110	770	0.664	Tridec-2-en-1-ol	1585	1511	3.63 (4)	4.10 (22)	4.99 (24)	4.50 (16)	-	-	-	-
			Aromatics										
111	400	2.96	Benzyl alcohol	1043	1059	1.05 (21)	1.49 (14)	-	-	-	-	-	-
112	465	2.296	Phenylethyl alcohol	1116	1132	963.29 (10)	1009.95 (11)	861.99 (4)	1058.63 (13)	413.07 (47)	1059.83 (6)	905.76 (15)	841.07 (22)
			Subtotal (GC Peak Area)			3087.44 (16)	3603.36 (20)	2763.72 (9)	2826.67 (9)	2373.25 (20)	3426.42 (15)	2521.34 (13)	2611.20 (17)
			Subtotal (Number of Compounds)			30	30	27	26	26	26	22	22
			Subtotal (%)			29.3	30.0	33.5	35.7	29.7	34.8	30.2	29.7
			Volatile phenols										
113	430	2.504	2-Methoxy-phenol (Guaiacol)	1090	1092	0.84 (32)	1.54 (5)	-	-	-	-	-	-
114	525	4.040	4-Ethylphenol	1178	1205	-	-	-	-	10.23 (26)	-	-	-
115	600	1.664	4-Ethyl-2-methoxy-phenol (4-Ethylguaiacol)	1288	1290	0.08 (25)	0.10 (17)	-	-	18.05 (21)	-	-	-
116	625	2.560	2-Methoxy-4-vinylphenol (*p*-Vinylguaiacol)	1327	1322	9.01 (24)	16.03 (6)	12.33 (6)	8.03 (7)	-	-	-	-
117	780	0.704	2,6-bis (1,1-dimethylethyl)-4-methyl-phenol	1514	1525	5.51 (5)	5.35 (4)	3.99 (5)	3.84 (9)	1.41 (38)	1.07 (5)	0.87 (11)	0.79 (12)
118	780	1.488	2,4-bis (1,1-dimethylethyl)-phenol	1512	1526	28.86 (24)	40.71 (11)	41.29 (5)	25.58 (4)	27.32 (49)	22.56 (8)	24.97 (7)	25.43 (18)
			Subtotal (GC Peak Area)			44.30 (22)	63.73 (9)	57.62 (6)	37.46 (5)	57.02 (36)	23.65 (8)	25.84 (7)	26.23 (17)
			Subtotal (Number of Compounds)			5	5	3	3	4	2	2	2
			Subtotal (%)			0.4	0.5	0.7	0.5	0.7	0.2	0.3	0.3
			Aldehydes										
			Aliphatics										
119	100	0.472	3-Methylbutanal (Isovaleraldehyde)	652	625	9.44 (19)	7.93 (29)	10.07 (26)	10.09 (16)	3.10 (5)	3.58 (24)	8.07 (21)	8.07 (22)
120	100	0.624	But-2-en-1-al	623	625	-	-	-	-	7.16 (18)	2.66 (43)	8.80 (4)	6.03 (3)
121	175	0.584	Hexanal	780	801	12.49 (9)	-	-	-	2.29 (59)	-	3.04 (11)	2.62 (4)
122	220	0.744	2-Hexenal	855	857	-	-	-	-	-	-	1.63 (4)	1.24 (5)
123	260	0.640	Heptanal	906	906	5.16 (18)	1.63 (4)	4.38 (24)	5.21 (28)	-	-	-	-
124	445	0.632	Nonanal	1106	1106	38.12 (1)	45.09 (23)	37.68 (8)	30.25 (18)	14.64 (6)	9.29 (16)	15.38 (25)	13.72 (11)
125	535	0.632	Decanal	1208	1207	44.51 (15)	55.74 (21)	50.77 (9)	51.04 (22)	26.65 (1)	15.98 (18)	17.71 (33)	25.18 (24)

126	620	0.632	Undecanal	1310	1313	6.85 (16)	7.16 (25)	7.97 (25)	6.19 (32)	1.70 (19)	-	3.97 (27)	2.15 (5)
127	700	0.624	Dodecanal	1409	1415	34.77 (4)	38.17 (25)	33.94 (32)	28.52 816)	4.03 (7)	5.10 (38)	2.20 (5)	2.60 (26)
			Aromatics										
128	310	1.360	Benzaldehyde	962	959	10.23 (5)	8.07 (9)	12.80 (6)	11.47 (4)	5.15 (9)	8.08 (16)	80.57 (5)	127.45 (23)
129	390	1.480	Phenyl acetaldehyde	1049	1046	4.14 (19)	6.45 (3)	4.80 (6)	4.18 (4)	7.60 (24)	5.36 (23)	10.23 (6)	9.16 (14)
			Subtotal (GC Peak Area)			165.71 (9)	170.24 (22)	162.40 (16)	146.94 (18)	72.32 (9)	50.05 (22)	151.59 (12)	198.22 (21)
			Subtotal (Number of Compounds)			9	8	8	8	9	7	10	10
			Subtotal (%)			1.6	1.4	2.0	1.9	0.9	0.5	1.8	2.3
			Ketones										
			Aliphatics										
130	70	0.384	Butan-2-one	600	554	-	8.14 (29)	10.59 (8)	2.32 (36)	-	-	-	-
131	115	0.528	3-Pentanone	650	660	-	-	-	-	-	-	1.78 (5)	2.00 (5)
132	130	1.224	3-Hydroxy-butan-2-one	711	697	4.02 (30)	9.12 (10)	6.17 (14)	5.93[e]	4.51 (7)	11.08 (16)	2.93 (10)	3.31 (14)
133	140	0.712	3-Penten-2-one	729	719	-	-	-	-	-	-	2.16 (3)	1.33 (3)
134	150	0.44	1-(ethenyloxy)-3-methyl-Butane (*m/z* = 114,99,70,55,43)	-	742	-	-	-	-	-	-	11.39 (10)	11.19 (11)
135	170	0.600	3-Ethoxy-butan-2-one (*m/z* = 73,45)	-	790	7.30 (10)	5.94 (22)	6.79 (10)	4.79 (5)	1.87 (13)	2.00 (7)	2.63 (3)	2.54 (4)
136	250	0.632	Heptan-2-one	889	895	14.50 (15)	13.25 822)	11.39 (25)	11.81 (1)	5.54[e]	0.41 (5)	8.00 (18)	8.67 (6)
137	340	0.600	Octan-3-one	985	985	0.68 (16)	1.01 (45)	-	-	-	-	0.44 (1)	0.42 (5)
138	335	0.736	6-Methyl-hept-5-en-2-one (Sulcatone)	986	985	7.63 (17)	10.26 (7)	10.45 (5)	9.50 (8)	5.18 (4)	3.13	-	5.14 (14)
139	430	0.608	Nonan-3-one	1091	1084	1.28 (8)	1.44 (9)	0.97 (15)	0.73 (6)	-	-	0.80 (4)	0.73 (9)
140	435	0.640	Nonan-2-one	1100	1090	43.80 (3)	46.09 (20)	34.89 (17)	25.81 (5)	8.18 (16)	8.28 (9)	24.57 (2)	24.94 (6)
141	455	1.112	Octan-2,5-dione	1102	1119	-	2.68 (7)	2.31 (7)	1.65 (14)	-	-	1.47 (15)	1.45 (9)
142	600	0.648	Undecan-3-one	1283	1289	2.31 (11)	1.54 (33)	1.35 (19)	1.01 815)	-	-	-	-
143	605	0.672	Undecan-2-one	1291	1295	6.61 (13)	7.16 (32)	5.59 (44)	3.63 (7)	1.09 (62)	2.03[e]	0.79 (11)	1.27 (6)
			Aromatics										
144	415	1.304	Acetophenone	1069	1074	2.11 (13)	2.76 (14)	2.43 (7)	1.71 (9)	2.03 (33)	1.48 (12)	1.65 (1)	1.46 (1)
			Subtotal (GC Peak Area)			90.25 (9)	109.37 (19)	93.43 (16)	68.89 (6)	28.39 (12)	28.41 (12)	58.63 (7)	64.45 (8)
			Subtotal (Number of Compounds)			10	12	11	11	7	7	12	13
			Subtotal (%)			0.9	0.9	1.1	0.9	0.4	0.3	0.7	0.7
			Furans										
145	110	0.416	Tetrahydro-furan	623	648	1.79 (20)	12.10 (5)	15.09 (4)	15.45 (8)	26.16 (13)	27.02 (9)	22.07 (11)	17.48 (4)
146	205	1.808	2-Furanylmethanal (Furfural)	830	840	80.33 (6)	63.96 (4)	70.45 (1)	87.86 (3)	20.97 (34)	31.28 (12)	280.13 (1)	166.46 (7)

147	240	3.816	2-Furanylmethanol (Furfuryl alcohol)	866	867	69.93 (21)	103.81 (9)	85.18 (4)	69.35 (4)	44.87 (16)	42.02 (24)	41.90 (25)	29.89 (21)
148	275	1.536	1-(2-furanyl)-ethanone (2-Acetylfuran)	910	917	3.45 (13)	4.13 (1)	3.78 (4)	3.36 (4)	3.51 (29)	2.94 (19)	3.54 (4)	3.33 (21)
149	325	1.504	5-Methylfuran-2-carbaldehyde (5-Methylfurfural)	962	965	3.28 (8)	4.60 (9)	5.33 (13)	5.97 (13)	-	-	4.16 (26)	6.03 (52)
150	325	1.536	Methyl furan-2-carboxylate (Methyl 2-furoate)	983	975	1.78 (7)	1.90 (9)	1.79 (6)	1.72 (4)	1.11 (31)	1.03 (14)	-	-
151	345	0.568	2-Pentyl-furan	992	990	0.76 (9)	0.78 (15)	0.75 (14)	0.60 (19)	0.73 (41)	0.49 (34)	0.41 (50)	0.30 (7)
152	345	1.224	Furfuryl ethanoate (Furfuryl acetate)	998	995	2.20 (1)	2.09 (11)	1.67 (18)	1.26 (2)	-	-	-	-
153	350	1.112	Benzofuran	1006	996	1.42 (24)	1.66 (14)	1.12 (11)	1.08 (17)	1.26 (15)	1.23 (7)	1.22 (8)	1.27 (5)
154	365	1.272	1-(2-Furanyl)-propan-1-one (2-Propionylfuran)	1008	1007	0.93 (13)	1.16 (18)	1.06 (3)	0.87 (9)	0.54 (7)	0.68 (8)	0.85 (11)	0.95 (7)
155	390	1.288	2-Acetyl-5-methylfuran	1039	1046	-	-	-	-	-	-	1.12 (16)	1.18 (7)
156	405	1.224	Ethyl 2-furancarboxylate (Ethyl 2-furoate)	1062	1051	11.36 (12)	13.34 (19)	12.50 (3)	10.00 (2)	14.69 (18)	15.20 (18)	20.72 (6)	18.94 (11)
157	485	0.696	2-*n*-Heptylfuran	1196	1154	-	-	-	-	1.88 (11)	2.11 (8)	1.50 (15)	1.82 (10)
			Subtotal (GC Peak Area)			177.22 (13)	209.52 (8)	198.72 (3)	197.52 (4)	115.72 (20)	123.99 (16)	377.63 (5)	247.64 (10)
			Subtotal (Number of Compounds)			11	11	11	11	10	10	11	11
			Subtotal (%)			1.7	1.7	2.4	2.5	1.4	1.3	4.5	2.8
			Lactones										
158	275	2.336	γ-Butyrolactone	915	918	16.26 (11)	38.50 (19)	30.11 (10)	25.00 (6)	28.79 (2)	27.23 (8)	23.44 (9)	25.23 (10)
159	610	1.144	3-methyl-4-octanolide (Whiskey lactone)	1310	1301	2.37 (12)	4.79 (6)	3.39 (4)	2.32 (9)	1.93 (33)	1.76 (37)	2.11 (11)	1.98 (10)
160	630	1.288	β-Methyl-γ-octalactone	1340	1327	2.52 (30)	5.43 (7)	3.56 (7)	2.70 (6)	1.95 (37)	2.36 (11)	2.07 (11)	1.97 (19)
161	670	1.272	γ-Nonanoic lactone	1360	1377	-	0.53 (20)	-	-	-	-	-	-
			Subtotal (GC Peak Area)			21.15 (14)	49.25 (16)	37.06 (10)	30.01 (6)	32.66 (6)	31.35 (10)	27.62 (9)	29.18 (11)
			Subtotal (Number of Compounds)			3	4	3	3	3	3	3	3
			Subtotal (%)			0.2	0.4	0.4	0.4	0.4	0.3	0.3	0.3
			Acetals										
162	130	0.488	1,1-Diethoxy-ethane	719	695	242.57 (4)	182.53 (20)	204.36 (30)	153.83 (9)	23.07 (18)	105.06 (7)	177.05 (33)	210.75 (12)
163	155	0.512	2,4,5-Trimethyl-1,3-dioxolane	739	754	171.56 (26)	170.68 (11)	274.98 (22)	231.01 (19)	16.40 (32)	140.64 (10)	137.51 (22)	271.64 (10)
164	225	0.440	1,1-Diethoxy-2-methyl-propane (Isobutanal diethyl acetal)	858	863	2.66 (4)	2.20 (21)	1.11 (20)	-	0.57 (14)	0.95 (9)	1.69 (15)	1.82 (12)
165	235	0.456	1-(1-Ethoxyethoxy)-butane	872	876	-	-	-	-	-	-	5.48 (2)	4.36 (9)
166	310	0.464	1,1-Diethoxy-3-methyl-butane	954	953	5.76 (15)	3.76 (20)	2.25 (31)	1.31 (17)	3.30 (26)	2.61 (10)	6.83 (16)	7.23 (15)
167	325	0.480	1-(1-Ethoxyethoxy)-pentane (Acetaldehyde ethyl amyl acetal)	977	974	69.54 (12)	61.58 (21)	39.41 (24)	28.36 (15)	-	15.97 (21)	44.45 (10)	49.17 (7)
168	365	0.696	1,1-Diethoxy-pentane	1016	1017	-	-	-	-	-	-	2.42 (11)	2.11 (9)
			Subtotal (GC Peak Area)			492.08 (13)	420.75 (17)	522.12 (25)	414.51 (15)	43.34 (24)	265.22 (10)	375.43 (25)	547.10 (11)
			Subtotal (Number of Compounds)			5	5	5	4	4	5	7	7

			Subtotal (%)			4.7	3.5	6.3	5.2	0.5	2.7	4.5	6.2
			Thiols and others sulphur compounds										
169	115	0.608	Methyl thiolacetate	701	660	11.11 (9)	10.03 (26)	10.49 (10)	8.94 (12)	3.23 (18)	4.52 (8)	4.70 (8)	4.66 (3)
170	140	0.624	Dimethyl disulfide	722	719	0.78 (4)	1.30 (12)	-	-	-	-	-	-
171	155	0.640	Ethyl ethanethioate (Ethyl thioacetate)	756	754	4.17 (26)	3.80 (21)	3.90 (14)	3.18 (10)	-	1.51 (9)	1.32 (11)	1.57 (5)
172	340	1.216	2-Methyl-tetrahydrotiophen-3-one	994	991	19.06 (9)	20.67 (5)	19.97 (3)	18.44 (5)	13.15 (47)	7.48 (38)	1.16 (9)	1.17 (6)
173	350	2.168	3-(Methylthio)-propan-1-ol (Methionol)	982	1002	9.83 (10)	21.27 (1)	16.83 (16)	10.07 (19)	14.31 (13)	11.17 (5)	13.06 (9)	13.93 (12)
174	445	0.912	Ethyl 3-(methylthio)propionate	1098	1101	-	-	-	-	1.17 (7)	1.31 (12)	1.34 (12)	1.30 (8)
			Subtotal (GC Peak Area)			44.95 (11)	57.07 (8)	51.19 (9)	40.63 (11)	31.86 (27)	25.99 (16)	21.59 (18)	22.63 (20)
			Subtotal (Number of Compounds)			5	5	4	4	4	5	5	5
			Subtotal (%)			0.4	0.5	0.6	0.5	0.4	0.3	0.3	0.3
			Norisoprenoids										
175	405	0.72	3,5,5-Trimethylcyclohex-2-enone (Isophorone)	1118	1062	-	-	-	-	0.30 (39)	-	-	-
176	600	0.600	Vitispirane	1281	1289	3.70 (11)	4.56 (1)	4.30 (24)	2.06 812)	18.31 (11)	22.47 (8)	28.05 (5)	27.43 (2)
177	660	0.744	1,1,6-Trimethyl-1,2-dihydro-naphathalene (TDN)	1354	1363	1.08 (8)	8.80 (31)	0.95 (19)	3.80 (13)	2.93 (20)	3.35 (6)	4.46 (9)	3.98 (9)
178	675	0.848	*β*-Damascenone isomer	1359	1388	12.02 (18)	14.49 (33)	11.69 (22)	7.47 (6)	-	-	-	-
179	685	0.792	*β*-Damascenone isomer	1359	1395	0.62 (21)	0.81 (25)	0.55 (34)	-	7.05 (28)	7.44 (19)	10.23 (7)	8.80 (10)
180	735	0.728	Geranyl acetone	1453	1463	21.03 (34)	28.28 827)	34.39 (15)	18.65 (9)	20.01 (33)	9.04 (20)	8.64 (25)	11.20 (9)
181	880	1.016	Methyl dihydro jasmonate	1650	1671	3.97 833)	5.73 (22)	3.89 (20)	3.45 (33)	1.29 (6)	1.91 (40)	1.05 (34)	1.14 (33)
			Subtotal (GC Peak Area)			42.42 (26)	62.67 (27)	55.77 (18)	35.43 (11)	49.88 (23)	44.21 (14)	52.44 (10)	52.56 (6)
			Subtotal (Number of Compounds)			6	6	6	5	6	5	5	5
			Subtotal (%)			0.4	0.5	0.7	0.4	0.6	0.5	0.6	0.6
			Terpenic compounds										
			Monoterpenic compounds										
182	285	0.448	*α*-Pinene	934	932	0.95 (27)	1.15 (28)	0.76 (7)	0.96 (24)	-	-	-	-
183	340	0.520	*β*-Pinene	980	990	0.52 (12)	0.67 (23)	-	-	-	-	-	-
184	345	0.504	*α*-Terpinene	1018	995	-	-	-	-	1.21 (29)	1.06 (6)	0.87 (11)	0.90 (12)
185	375	0.520	Limonene	1031	1028	42.50 (4)	65.67 (23)	19.78 (2)	35.27 (4)	19.83 (12)	22.55 (2)	15.91 (6)	18.35 (8)
186	410	0.792	Dihydromyrcenol	1072	1068	4.65 (17)	6.48 (24)	5.86 (23)	3.64 (14)	-	-	-	-
187	435	0.896	Linalool	1105	1095	24.42 (13)	27.96 (38)	20.86 (23)	14.07 (3)	28.17 (18)	24.04 (15)	17.21 (32)	21.27 (20)
188	450	0.968	Hotrienol	1110	1112	1.09 (10)	1.21 (29)	1.07 (7)	-	2.23 (54)	1.65 (23)	0.97 (26)	0.99 (26)
189	490	0.656	Nerol oxide	1172	1154	1.50 (11)	1.94 (21)	1.46 (16)	-	2.45 (39)	2.44 (10)	3.43 (8)	2.88 (15)
190	495	1.088	endo-Borneol	1165	1166	0.60 (3)	1.82 (31)	2.02 (19)	1.07 (20)	-	-	-	-

191	520	0.992	*α*-Terpineol	1224	1195	3.66 (32)	7.08 (17)	8.77 (23)	4.10 (8)	5.08 (33)	4.16 (20)	5.16 (10)	5.26 (28)
192	560	0.976	*β*-Citronellol	1234	1242	4.58 (24)	5.43 (14)	5.14 (5)	3.05 (7)	1.71 (11)	2.43 (9)	2.13 (13)	2.22 (7)
193	580	0.552	Myrtenol	1264	1265	2.70 (6)	5.58 (19)	2.58 (22)	1.32 (14)	4.31 (22)	3.09 (11)	-	-
194	580	1.08	Geraniol	1265	1272	2.08 (24)	3.06 (15)	2.29 (4)	1.83 (5)	1.39 (4)	3.72 (28)	-	-
195	605	0.560	Nerol	1245	1295	-	-	-	-	3.34 (4)	3.15 (20)	3.29 (27)	4.43 (32)
196	780	0.944	Limonene dioxide	1294	1304	6.36 (31)	4.79 (28)	4.39 (28)	3.46 (11)	-	4.40 (19)	1.94 (60)	1.85 (35)
			Sesquiterpenic compounds										
197	815	0.784	Nerolidol	1564	1573	2.49 (35)	3.48 (24)	5.72 (5)	4.22 (17)	4.66 (8)	4.92 (14)	2.54 (7)	4.37 (18)
198	995	0.752	Farnesol	1792	1801	1.39 (25)	2.81 (7)	4.37 (2)	2.85 (18)	4.46 (18)	2.40 (18)	3.44 (25)	5.16 (13)
			Subtotal (GC Peak Area)			99.50 (13)	139.15 (22)	85.07 (12)	75.84 (7)	78.84 (18)	80.01 (12)	56.91 (19)	67.67 (17)
			Subtotal (Number of Compounds)			15	15	14	12	12	13	11	11
			Subtotal (%)			0.9	1.2	1.0	1.0	1.0	0.8	0.7	0.8
			Total (GC Peak Area)			10543.20 (13)	12011.51 (16)	8247.38 (15)	7922.09 (12)	7976.33 (16)	9836.17 (16)	8343.84 (16)	8792.76 (17)
			Total (Number of Identified Compounds)			167	172	163	157	148	146	148	148

[a] Retention times for first (^1t_R) and second (^2t_R) dimensions in seconds.
[b] RI, Retention Index reported in the literature for HP-5 GC column or equivalents (Ansorena et al., 2000; Campeol et al., 2003; Cardeal et al., 2008; Engel et al., 2002; Eyres et al., 2005; Fan & Qian, 2006; Högnadóttir & Rouseff, 2003; Jordán et al., 2002; Leffingwell & Alford, 2005; Perestrelo et al., 2011; Petronilho et al., 2011; Pino et al., 2005; Robinson A.L., 2011a; Rocha et al., 2007; Salvador et al., 2013; Silva et al., 2015; Silva et al., 2010, Jalali et al., 2012).
[c] RI: retention index obtained through the modulated chromatogram. [d] Mean of three replicates. [e] The compound was only detected in one replicate. [f] Relative standard deviation, expressed in percentage, in parenthesis.

Table S2. Volatile Compounds Identified by HS-SPME/ GC×GC -ToFMS in the red wine samples (Chapter II.3)

Peak number	^1t_R [a] (s)	^2t_R [a] (s)	Compound	$RI_{lit.}$ [b]	RI_{cal} [c]	After 2 months of storage				After 9months of storage			
						Untreated	SO_2	425 MPa	500 MPa	Untreated	SO_2	425 MPa	500 MPa
						Peak Area[d] ($x10^5$) and RSD [f] (%)							
			Carboxylic Acids										
1	95	3.176	Acetic acid	619	600	729.59 (15)	681.43 (12)	269.35 (12)	201.66 (9)	297.91 (19)	255.73 (13)	134.46 (20)	243.18 (17)
2	155	3.856	2-Methylpropanoic acid (Isobutyric acid)	762	767	21.38 (3)	19.58 (16)	21.88 (17)	29.50 (3)	-	-	-	-
3	175	4.632	Butanoic acid (Butyric acid)	808	806	9.04 (22)	6.71 (23)	9.24 (28)	16.55 (13)	-	-	-	-
4	225	3.896	2-Methyl butanoic acid	867	878	23.20 (18)	22.50 (30)	35.65 (11)	31.10 (6)	-	-	-	-
5	235	3.680	3-Methylbutanoic acid (Isovaleric acid)	880	876	30.86 (13)	24.79 (41)	33.21 (1)	27.17 (30)	30.05 (2)	26.36 (23)	27.77 (6)	23.58 (35)
6	360	3.864	Hexanoic acid (Caproic acid)	1015	1017	89.02 (10)	109.65 (16)	100.30 (22)	143.71 (11)	75.70 (29)	75.35 (10)	79.96 (10)	71.68 (21)
7	525	2.704	Octanoic acid (Caprylic acid)	1203	1179	108.03 (27)	131.33 (26	96.42 (18)	141.13 (11)	85.56 (16)	62.11 (5)	49.66 (12)	51.63 (27)
8	690	2.056	*n*-Decanoic acid (*n*-Caprinic acid)	1380	1372	17.59 (19)	15.67 (3)	-	-				
9	950	1.624	Tetradecanoic acid	1779	1768	-	-	-	-	10.62 (43)	5.76 (18)	-	7.31 (26)
10	1070	1.488	*n*-Hexadecanoic acid (Palmitic acid)	1985	1984	-	11.98 (21)	-	-	20.03 (24)	24.58 (33)	-	18.66 (37)
			Subtotal (GC Peak Area)			1028.69 (16)	1023.64 (16)	566.05 (15)	591.81 (11)	519.88 (20)	449.89 (13)	291.85 (15)	416.03 (21)
			Subtotal (Number of Compounds)			8	9	7	7	6	6	4	6
			Subtotal (%)			10.6	10.3	7.2	7.0	6.9	7.9	5.3	7.3
			Esters										
			Aliphatics ethyl esters										
11	95	0.456	Ethyl ethanoate (Ethyl acetate)	613	613	577.06 (26)	126.67 (1)	330.65 (10)	3030.71 (7)	292.36 (22)	288.11 (13)	208.89 (6)	234.88 (8)
12	125	0.496	Ethyl propanoate (Ethyl proprionate)	714	684	75.20 (11)	86.36 (12)	61.17 (22)	68.20 (4)	64.33 (9)	51.56 (8)	40.41 (5)	44.56 (15)
13	150	0.488	Ethyl 2-methylpropanoate (Ethyl isobutyrate)	762	742	31.47 (14)	33.44 (11)	28.14 (31)	32.57 (2)	53.80 (12)	42.99[e]	43.74 (2)	42.84[e]
14	165	0.632	Diethyl carbonate	785	778	1.58 (7)	1.41 (13)	1.22 (18)	1.39 85)	1.36 (4)	0.74 (56)	1.41 (15)	1.21 (6)
15	180	0.536	Ethyl butanoate (Ethyl butyrate)	800	807	186.27 (9)	199.73 (11)	175.33 (20)	219.00 (3)	175.32 (28)	142.67 (9)	116.62 (21)	144.12 (3)

16	190	1.184	Ethyl 2-hydroxypropanoate (Ethyl lactate)	815	820	33.87 (6)	27.34 (5)	32.15 811)	33.74 (5)	255.38 (10)	-	45.11 (3)	50.34 (4)
17	210	0.656	Ethyl but-2-enoate	844	845	13.35 (5)	13.95 (3)	11.75 (17)	13.89 (1)	9.60 (5)	7.64 (3)	8.09 (4)	7.88[e]
18	215	0.512	Ethyl 2-methylbutanoate (Ethyl 2-methylbutyrate)	849	851	13.53 (14)	19.08 (14)	18.92 (6)	21.34 (9)	21.65 (4)	17.85 (6)	18.40 (17)	16.68 (2)
19	220	0.528	Ethyl 3-methylbutanoate (Ethyl isovalerate)	856	857	34.65 813)	41.56 (28)	37.46 (35)	32.52 (3)	40.69 (6)	34.04 (4)	32.27 (15)	35.05 (1)
20	260	0.552	Ethyl pentanoate (Ethyl valerate)	898	906	11.64 (24)	11.73 (14)	10.88 (22)	12.72 (8)	6.89 (17)	4.76 (27)	4.87 (7)	4.91 (15)
21	300	1.352	Ethyl 3-hydroxy-butanoate	949	949	10.56 (6)	9.07 (14)	10.68 (20)	11.98 (10)	5.82 (7)	4.88 (15)	5.63 (12)	5.54 (7)
22	315	0.568	Ethyl 4-methyl-pentanoate	964	968	2.21 (6)	2.03 (4)	2.92 (29)	2.90 (20)	-	-	-	-
23	320	0.936	Ethyl 2-hydroxyisovalerate	968	969	-	-	-	-	6.65 (4)	4.15 (11)	-	-
24	340	0.632	Ethyl hex-5-enoate	975	990	20.02 (17)	23.02 (10)	19.01 (32)	24.27 (3)	13.10 (18)	12.39 (2)	12.56 (10)	9.79 (3)
25	350	0.576	Ethyl hexanoate	1001	1001	417.89 (35)	518.75 (22)	279.76 (18)	359.73 (26)	257.93 (5)	301.05 (12)	370.05 (17)	389.38 (9)
26	360	0.632	Ethyl hex-3-enote	1006	1012	17.06 (40)	18.59 (25)	16.96 (6)	16.84 (20)	6.01[e]	6.19 (16)	10.99 (9)	10.79 (8)
27	390	0.64	Ethyl hex-2-enoate	1040	1045	56.39 (12)	57.12 (7)	51.70 (34)	66.49 (4)	32.00 (10)	32.05 (2)	39.36 (28)	32.50 (1)
28	405	0.952	Ethyl 2-hydroxy-4-methyl-pentanoate	1060	1062	11.40 (8)	10.64 (10)	13.12 (20)	14.30 (3)	29.21 (12)	29.40 (11)	13.14 (16)	14.24 (11)
29	420	1	Diethyl malonate	1069	1079	-	-	-	-	1.30 (58)	0.65 (4)	0.62 (31)	0.57 (8)
30	420	2.040	Ethyl 4-hydroxybutanoate	1039	1080	-	-	-	-	61.59 (10)	38.88 (9)	28.29 (7)	33.99 (25)
31	435	0.616	Ethyl hept-4-enoate	1090	1095	-	-	-	-	4.45 (32)	3.85 (3)	3.66 (10)	2.78 (6)
32	440	0.658	Ethyl heptanoate	1104	1100	47.40 (22)	42.47 (10)	39.77 (17)	42.17 (12)	26.88 (8)	22.72 (6)	20.18 (9)	20.44 (6)
33	450	1.048	Ethyl methyl succinate	1120	1113	-	-	-	.	5.91 (6)	4.24 (6)	1.58 (18)	1.30 (7)
34	515	0.936	Diethyl butanedioate	1182	1189	78.03 (17)	62.09 (9)	63.87 (35)	76.31 (5)	153.81 (1)	170.88 (15)	143.98 (2)	161.06 (24)
35	520	0.632	Ethyl 7-octenoate	1186	1195	-	-	-	-	10.61 (18)	9.18 (5)	9.10 (15)	7.77 (28)
36	530	0.576	Ethyl octanoate	1199	1207	500.57 (36)	557.02 (2)	280.71(1)	236.28 (13)	287.78 (31)	275.19 (17)	208.40 (14)	230.55 (39)
37	570	0.64	Ethyl oct-2-enoate	1246	1254	-	-	-	-	0.80 (20)	0.74 (15)	-	-
38	595	0.880	Ethyl-*n*-propyl butanedioate (*m/z* = 129,101,73,43)	-	1283	-	-	-	-	2.93 (21)	-	-	-
39	610	0.576	Ethyl nonanoate	1294	1301	34.04 (33)	35.53 (5)	38.18 (16)	37.96 (11)	7.94 (30)	12.08 (13)	3.77 (40)	2.25 (17)
40	640	0.816	Ethyl butyl butanedioate (*m/z* = 147,129,101,56)	-	1339	-	-	-	-	9.83 (10)	8.27 (4)	1.96 (19)	1.43 (15)
41	675	0.672	Ethyl dec-9-enoate	1382	1389	18.00 (29)	8.95 (13)	24.92 (8)	17.77 (37)	-	-	-	-
42	690	0.584	Ethyl decanoate (Ethyl caprinate)	1394	1401	305.35 (25)	346.68 (11)	186.32 (6)	189.93 (35)	120.76 (7)	102.17 (9)	80.92 (13)	87.85 (7)
43	715	0.816	Ethyl 3-methylbutyl succinate	1429	1435	3.74 (21)	2.87 (5)	2.71 (34)	3.89 (5)	65.52 (12)	63.22 (7)	14.83 (23)	12.41 (15)
44	835	0.592	Ethyl dodecanoate (Ethyl laurate)	1593	1601	30.46 (15)	20.31 (11)	17.74 (26)	20.99 (11)	18.42 (47)	6.09 (28)	3.95 (15)	3.46 (14)
45	965	0.600	Ethyl tetradecanoate (Ethyl myristate)	1793	1801	1.95 (27)	1.81 (30)	3.56 (11)	2.07 (7)	0.95 (9)	1.79 (25)	1.01 (13)	0.89 (21)
46	1085	0.616	Ethyl hexadecanoate (ethyl palmitate)	1994	2010	5.31 (27)	5.68 (25)	3.45 (20)	5.09 (26)	4.03 (18)	4.11 (10)	4.44 (19)	3.00 (27)
			Aromatics ethyl esters										

47	510	0.96	Ethyl benzoate	1179	1184	3.78 (8)	3.72 (8)	3.65 (33)	4.49 (3)	5.59 (17)	2.86 (10)	2.56 (9)	2.71 (7)
48	570	1.016	Ethyl 2-phenylethanoate	1251	1254	4.84 (17)	4.83 (6)	5.00 (28)	5.75 (5)	3.46 (33)	3.01 (7)	3.65 (28)	3.31 (13)
49	655	0.968	Ethyl dihydrocinnamate	1347	1357	0.97 (12)	0.89 (3)	1.17 (15)	1.04 (3)	1.09 (10)	0.75 (10)	-	-
			Subtotal (GC Peak Area)			2548.58 (26)	2293.34 (11)	1771.87 (14)	1879.33 (14)	2065.75 (15)	1711.16 (12)	1504.41 (12)	1620.47 (14)
			Subtotal (Number of Compounds)			30	30	30	30	37	35	33	33
			Subtotal (%)			26.3	23.0	22.1	22.1	27.9	30.1	27.3	28.5
			Aliphatic acetate esters										
50	75	0.456	Methyl ethanoate	559	566	10.35 (18)	11.40 (12)	12.11 (31)	9.43 (10)	12.12 (50)	5.55 (53)	9.19 (26)	11.16 (7)
51	125	0.52	*n*-Propyl ethanoate	712	684	18.49 (5)	20.24 (12)	16.03 (12)	19.13 (8)	10.49 (24)	10.74 (15)	6.10 (5)	6.44 (6)
52	145	1.328	Methyl 2-hydroxypropanoate	748	732	-	-	-	-	1.15 (17)	1.49 (2)	-	-
53	160	0.52	2-Methylpropyl ethanoate	767	766	109.77 (9)	112.75 (6)	92. 25 (15)	101.47 (7)	53.17 (6)	44.26 (2)	80.69 (4)	74.64 (1)
54	190	0.56	Butyl ethanoate	812	819	22.26 (2)	23.82 (1)	21.60 (16)	22.11 (18)	6.49 (15)	5.80 (15)	5.35 (16)	4.74 (13)
55	235	0.704	1-Methoxy-2-propyl acetate	870	876	-	-	-	-	3.21 (4)	3.17 (23)	2.77[e]	3.15 (1)
56	235	3.208	2-Methylbutyl ethanoate	885	879	-	-	-	-	8.21 (11)	6.55 (5)	2.90 (50)	3.54 (13)
57	240	0.544	3-Methylbutyl ethanoate	876	882	489.58 (4)	362.34 (23)	339.37 (10)	536.13 (14)	243.43 (7)	165.74 (21)	204.71 (35)	155.55 (2)
58	360	0.64	Hexyl ethanoate	1014	1006	97.87 (7)	92.36 (10)	83.30 (23)	93.37 (1)	46.70 (14)	35.33 (6)	36.89 (3)	39.48 (7)
59	455	0.592	Heptyl ethanoate	1118	1118	6.48 (27)	5.24 (10)	5.00 (30)	6.17 (17)	-	2.21 (40)	1.19 (9)	1.70 (20)
60	535	0.624	Octyl ethanoate	1213	1211	3.89 (23)	-	-	-	-	-	-	-
			Acetates aromatics esters										
61	580	1.064	2-Phenylethyl ethanoate	1256	1260	73.93 (18)	66.43 (6)	63.81 (28)	82.47 (5)	33.43 (10)	27.41 (5)	24.79 (15)	25.91 (9)
			Subtotal (GC Peak Area)			832.61 (7)	694.57 (16)	633.48 (15)	870.28 (11)	418.42 (10)	308.24 (15)	374.56 (23)	326.31 (4)
			Subtotal (Number of Compounds)			9	8	8	8	10	11	10	10
			Subtotal (%)			8.6	7.0	7.9	10.2	5.6	5.4	6.8	5.7
			Aliphatic esters										
62	170	0.568	3-Methylbutyl methanoate	792	780	6.03 (6)	5.34 (10)	5.83 (32)	5.58 (1)	0.99 (77)	0.71 (30)	1.25 (4)	1.44 (3)
63	280	0.600	Methyl hexanoate (Methyl caproate)	934	927	17.17 (20)	16.66 (11)	14.38 (27)	10.40 (28)	7.76 (8)	5.94 (11)	6.51 (6)	4.75 (26)
64	300	1.352	2-Methylpropyl butanoate (Isobutyl butyrate)	961	949	-	1.54 (14)	1.13 (29)	1.70 (8)	1.17 (13)	0.85 (7)	0.55 (26)	0.52 (1)
65	320	0.560	Pentyl propanoate (Amyl propionate)	969	972	19.42 (20)	25.46 (37)	31.60 (15)	39.66 (35)	-	-	-	-
66	365	0.520	3-Methylpropyl butanoate (Isoamyl butyrate)	1013	1017	11.07 (18)	11.18 (3)	7.35 (22)	9.13 (16)	14.81 (25)	14.83 (20)	16.11 (16)	18.98[e]
67	405	0.544	Butyl 3-methylbutanoate (*n*-Butyl isovalerate)	1048	1062	-	-	-	-	11.02 (7)	9.00 (1)	7.43 (11)	7.33 (4)
68	410	1.080	3-Methylbutyl 2-hydroxypropanoate (Isoamyl lactate)	1047	1068	8.32 (8)	7.90 (3)	7.90 (12)	9.40 (13)	78.38 (18)	65.90 (8)	5.80 (3)	8.33 (25)
69	420	0.936	Octy methanoate (Octyl formate)	1104	1079	-	-	-	-	69.60 (11)	41.87 (1)	36.53 (3)	37.11 (9)

70	440	0.560	Propyl hexanoate (Propyl caproate)	1093	1101	2.41 (19)	2.27 (4)	2.39 (33)	2.60 (12)	2.82 (14)	2.16 (10)	1.67 (12)	1.61 (12)
71	445	0.528	2-Methylbutyl 2-methylbutanoate	1103	1107	-	-	-	-	1.98 (11)	1.71 (15)	1.07 (6)	1.12[e]
72	465	0.600	Methyl octanoate (Methyl caprylate)	1126	1130	29.65 (16)	33.78 (9)	34.24 (28)	36.76 (6)	18.26 (10)	17.60 (6)	15.47 (9)	16.01 (7)
73	490	0.536	2-Methylpropyl hexanoate (Isobutyl caproate)	1148	1160	7.75 (21)	8.97 (21)	6.52 (28)	8.76 (5)	8.31 (23)	6.28 (3)	7.07 (10)	6.79 (8)
74	515	0.592	Butyl hexanoate (Butyl caproate)	1189	1188	2.15 (36)	-	0.74 (13)	0.85 (5)	-	-	-	-
75	560	0.544	Hexyl 2-methyl-butanoate (Hexyl 2-methyl-butyrate)	1234	1242	8.68 (21)	-	6.31 (19)	7.93 (8)	6.05 (29)	3.00 (7)	3.74 (30)	6.47 (11)
76	575	0.552	3-Methylbutyl hexanoate (Isoamyl caproate)	1250	1259	54.02 (22)	81.10 (17)	69.31 (11)	76.75 (9)	28.33 (12)	25.65 (10)	24.09 (11)	24.36 (6)
77	600	0.600	Propyl octanoate (Propyl caprylate)	1289	1296	2.51 (27)	2.99 (8)	2.56 (25)	3.97 (20)	-	-	-	-
78	635	0.600	Methyl decanoate (Methyl caprinate)	1326	1332	1.58 (29)	2.03 (8)	2.05 (30)	2.37 (3)	0.50 (26)	0.61 (12)	0.40 (7)	0.37 (11)
79	650	0.552	2-Methylpropyl octanoate (Isobutyl caprylate)	1348	1351	-	3.87 (9)	4.04 (29)	4.35 811)	2.63 (3)	4.86 (3)	2.66 (30)	3.00 (31)
80	730	0.552	3-Methylbutyl octanoate (Isoamyl caprylate)	1444	1456	24.10 (23)	25.76 (7)	25.19 (16)	25.85 (2)	16.87 (14)	14.20 (14)	10.91 (14)	10.44 (9)
81	840	0.608	1-[2-(Isobutyryloxy)-1-methylethyl]-2,2-dimethylpropyl 2-methylpropanoate (*m/z* = 243,159,111,83,71,43)	-	1607	-	-	-	-	2.04 (38)	4.57 (55)	2.60 (22)	2.42 (34)
82	850	0.600	1-Methylethyl dodecanoate (Isopropyl laurate)	1624	1627	-	1.51 (20)	2.99 (23)	1.96 818)	-	-	-	-
83	870	0.568	3-Methylbutylpentadecanoate (Isoamyl decanoate)	1651	1655	2.17 (36)	3.43 (23)	2.32 (43)	1.34 (9)	0.81 (29)	0.91 (13)	-	-
84	975	0.568	Methylethyl tetradecanoate (Isopropyl myristate)	1824	1834	-	-	2.41 (15)	2.94 (4)	1.95 (27)	2.42 (12)	2.13 (7)	2.51 (24)
85	1100	0.592	Methylethyl hexadecanoate (Isopropyl palmitate)	1999	2037	-	-	2.35 (3)	3.24 (51)	17.89 (29)	14.80 (30)	7.25 (9)	3.47 (3)
			Aromatics esters										
86	505	1.328	2-Phenylethyl methanoate (2-Phenylethyl formate)	1178	1178	0.90 (18)	0.75 (3)	1.10 (22)	0.91 (6)	-	-	-	-
87	530	1.192	Methyl 2-hydroxibenzoate (Methyl salicylate)	1190	1207	1.68 (30)	1.24 (11)	1.75 (8)	1.39 (6)	12.07 (12)	3.08 (9)	-	-
88	720	0.960	2-Phenylethyl butanoate (2-Phenylethyl butyrate)	1442	1439	-	-	0.81 (6)	0.69 (1)	-	-	-	-
89	885	0.936	Hexyl 2-hydroxybenzoate (*n*-Hexyl salicylate)	1682	1678	-	-	0.68 (26)	0.68 (17)	-	-	-	-
			Subtotal (GC Peak Area)			199.63 (20)	235.78 (15)	235.94 (19)	259.21 (13	304.23 (15)	240.92 (10)	282.48 816)	303.48 (10)
			Subtotal (Number of Compounds)			17	18	24	24	22	22	20	20
			Subtotal (%)			2.1	2.4	2.9	3.0	4.1	4.2	5.1	5.3
			Alcohols										
			Aliphatics alcohols										
90	80	0.712	Propan-1-ol (Propyl alcohol)	595	578	18.06 (26)	10.10 (37)	15.47 (15)	15.39 (10)	50.99 (48)	19.75 (56)	21.91 (6)	22.21 (15)
91	90	0.800	2-Methyl-propan-1-ol	600	602	405.98 (27)	367.88 (8)	306.10 (15)	307.66 (4)	282.60 (9)	188.87 (19)	229.63 (37)	282.24 (7)

92	105	0.816	Butan-1-ol (Butyl alcohol)	653	637	40.38 (28)	45.97 (16)	48.98 (17)	55.78 (1)	29.76 (25)	21.39 (22)	23.26 (3)	23.37 (1)
93	110	0.816	1- Penten-3-ol	656	649	10.07 (1)	10.15 (13)	9.85 (26)	11.39 (8)	5.35 (23)	4.04 (21)	4.76 (3)	4.74 (3)
94	120	0.688	Pentan-3-ol (Diethyl carbinol)	710	672	-	-	1.45 (5)	1.36 (4)	2.65 (4)	1.14 (32)	-	-
95	120	0.712	Pentan-2-ol (Methylpropyl carbinol)	706	672	-	-	-	-	4.78 (14)	3.34 (16)	-	-
96	145	0.864	3-Methyl-butan-1-ol (Isoamyl alcohol)	737	731	891.56 (9)	1333.04 (21)	612.80 (39)	776.30 (23)	1041.42 (8)	801.47 (9)	432.38 (15)	407.61 (22)
97	160	0.944	Pentan-1-ol (Amyl alcohol)	768	767	25.17 810)	22.89 (6)	23.52 (16)	26.86 (4)	15.18 (29)	13.39 (7)	-	-
98	160	1.200	Pent-2-en-1-ol	769	768	5.79 (18)	5.50 (10)	5.72 (15)	6.51 (4)	-	-	-	-
99	170	3.144	Butane-2,3-diol	806	796	476.57 (11)	583.96 (25)	487.99 (30)	553.80 (14)	-	-	-	-
100	175	3.608	Butane-2,2-diol	806	805	311.74 (28)	275.07 (20)	302.10 (25)	277.47 (27)	-	-	-	-
101	215	0.992	3-Methyl-pentan-1-ol	854	852	82.61 (6)	79.91 (9)	85.52 (17)	94.90 (4)	34.29 (25)	28.32 (27)	25.05 (3)	28.21 (6)
102	215	1.184	3-Ethoxy-propan-1-ol	855	852	5.09 (13)	4.57 (18)	4.19 (27)	5.10 (14)	8.43 (29)	3.16 (13)	-	2.43 (9)
103	220	1.112	Hex-3-en-1-ol isomer	856	858	14.40 (11)	11.75 (27)	1.78 (3)	2.03 (8)	8.01 (33)	5.39 (25)	6.56 (14)	5.72 (12)
104	225	1.168	Hex-3-en-1-ol isomer	864	861	-	-	-	-	2.62 (16)	3.46 (3)	3.71 (7)	4.40 (7)
105	230	1.040	Hexan-1-ol	870	870	408.68 (3)	285.46 (25)	383.73 (23)	407.20 (14)	483.24 (35)	359.44 (15)	537.14 (11)	465.83 (11)
106	230	1.296	Hex-2-en-1-ol	862	870	4.24 (38)	3.11 (10)	3.61 (32)	4.66 (10)	-	-	-	-
107	260	0.832	Heptan-2-ol	905	906	9.74 (9)	9.56 (10)	10.14 (22)	12.07 (7)	14.73 (9)	6.74 (7)	4.12 (7)	4.30 (4)
108	315	1.200	Hept-4-en-1-ol	-	964	8.63 (6)	7.92 (4)	8.99 (17)	10.42 (6)	-	-	-	-
109	330	0.928	Heptan-1-ol	969	979	88.73 (3)	94.03 (11)	147.17 (25)	125.70 (16)	68.14 (21)	76.56 (20)	68.55 (20)	90.17 (8)
110	335	0.936	Oct-1-en-3-ol	980	985	11.33 (10)	11.71 (16)	13.65 (23)	16.08 (12)	11.04 (15)	8.26 (12)	5.61 (47)	4.19 (16)
111	340	0.960	6-Methyl-hept-5-en-2-ol (Sulcatol)	992	990	1.59 (26)	3.47 (17)	1.77 (35)	1.52 (20)	-	-	-	-
112	350	0.832	Octan-2-ol	997	1001	2.30 (7)	2.77 (13)	2.31 (8)	3.72 (24)	-	-	-	-
113	355	0.752	Octan-3-ol	994	1006	3.65 (27)	3.85 (35)	2.99 (32)	3.42 (8)	4.91 (7)	2.86 (5)	1.64 (7)	1.67 (3)
114	365	1.016	3-Ethyl-4-methylpentan-1-ol	1020	1018	3.57 (8)	3.40 (9)	3.74 (26)	3.85 (18)	-	-	-	-
115	380	0.88	2-Ethyl-hexan-1-ol	1029	1034	5.25 (15)	4.45 (5)	5.89 (15)	7.04 (33)	5.74 (8)	-	5.55 (8)	5.49 (5)
116	410	1.000	Octan-1-ol	1070	1068	65.01 (12)	62.82 (6)	76. 34 (21)	75.94 (1)	-	-	-	-
117	420	1.104	2-Octen-1-ol	1069	1079	3.09 (10)	2.83 (7)	3.06 (28)	3.43 (11)	5.92 (20)	3.05 (5)	-	-
118	445	0.784	Nonan-2-ol	1098	1107	5.40 (15)	5.68 (24)	5.66 (22)	6.96 (4)	10.74 (15)	8.36 (15)	3.02 (21)	2.56 (29)
119	515	0.880	Nonan-1-ol	1171	1189	42.11 (13)	39.74 (10)	44.18 (28)	48.55 (3)	26.71 (36)	14.67 (18)	35.60 (21)	25.40 (19)
120	575	1.016	Dec-4-en-1-ol	1257	1260	3.51 (32)	3.23 (9)	3.52 (31)	4.45 (9)	-	-	-	-
121	590	0.664	Dec-2-en-1-ol	1283	1277	-	3.88 (1)	3.83 (28)	5.42 (32)	-	-	-	-
122	595	0.856	Decan-1-ol	1272	1283	16.33 (18)	14.59 (6)	16.64 (31)	19.33 (5)	13.89 (2)	8.72 (25)	10.01 (1)	11.00 (1)
123	740	0.880	Dodecan-1-ol	1473	1470	6.56 (32)	6.69 (9)	7.42 (22)	7.43 (11)	-	-	-	-
124	770	0.664	Tridec-2-en-1-ol	1585	1511	3.32 (20)	2.64 (11)	4.18 (8)	3.35 (9)	-	-	-	-

			Aromatics										
125	400	2.96	Benzyl alcohol	1043	1059	35.33 (12)	30.35 (9)	30.22 (24)	37.95 (3)	134.38 (18)	58.35 (4)	-	-
126	465	2.296	Phenylethyl alcohol	1116	1132	1112.69 (10)	1177.32 (13)	1039.46 (5)	832.36 (2)	1042.86 (10)	607.99 (19)	554.59 (12)	522.77 (14)
			Subtotal (GC Peak Area)			4128.49 (12)	4530.26 (18)	3723.93 (21)	3775.43 (12)	3308.37 (15)	2248.73 (15)	1973.10 (16)	1914.32 (14)
			Subtotal (Number of Compounds)			33	34	35	35	24	23	18	19
			Subtotal (%)			42.6	45.5	46.4	44.4	44.7	39.5	35.9	33.7
			Volatile phenols										
127	780	0.704	2,6-bis(1,1-dimethylethyl)-4-methyl-phenol	1514	1525	4.51 (11)	4.88 (7)	4.90 (34)	5.37 (9)	1.06 (18)	1.65 (14)	1.10 (7)	1.43 (18)
128	780	1.488	2,4-bis(1,1-dimethylethyl)-phenol	1512	1526	6.57 (31)	5.25 (8)	5.52 (12)	5.83 (9)	4.01 (34)	4.42 (21)	5.66 (15)	4.04 (20)
			Subtotal (GC Peak Area)			11.09	10.13	10.42	11.20	5.07 (30)	6.07 (19)	6.76 (14)	5.47 (19)
			Subtotal (Number of Compounds)			2	2	2	2	2	2	2	2
			Subtotal (%)			0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
			Aldehydes										
			Aliphatics										
129	100	0.472	3-Methylbutanal (Isovaleraldehyde)	652	625	14.62 (9)	7.55 (19)	11.65 (14)	8.15 (11)	1.86 (13)	5.57 (3)	3.64 (7)	4.90 (29)
130	175	0.584	Hexanal	780	801	-	-	-	-	1.85 (11)	2.54 (15)	9.01 (2)	9.73 (7)
131	445	0.632	Nonanal	1106	1106	22.75 (24)	26.33 (12)	28.83 (24)	29.61 (9)	11.13 (19)	10.57 (25)	9.96 (10)	8.10 (10)
132	535	0.632	Decanal	1208	1207	30.47 (21)	33.66 (16)	39.31 (10)	47.15 (20)	26.81 (23)	34.38 (16)	31.19 (10)	15.25 (5)
133	620	0.632	Undecanal	1310	1313	4.94 (37)	5.37 (23)	4.95 (31)	5.27 (4)	1.76 (17)	2.90 (10)	1.98 (7)	-
134	700	0.624	Dodecanal	1409	1415	19.00 (50)	16.14 (7)	11.29 (13)	6.12 (23)	2.97 (30)	2.45 (11)	3.35 (11)	2.47 (7)
			Aromatics										
135	310	1.360	Benzaldehyde	962	959	147.71 (4)	321.62 (8)	366.01 (2)	350.26 (2)	197.75 (8)	214.41 (3)	444.94 (10)	473.16 (10)
136	390	1.480	Phenyl acetaldehyde	1049	1046	10.01 (11)	9.09 (10)	10.98 (13)	12.19 (4)	6.66 (10)	6.50 (7)	12.55 (4)	15.51 (11)
			Subtotal (GC Peak Area)			249.48 (14)	419.77 (9)	473.03 (5)	458.74 (5)	250.79 (10)	279.31 (5)	516.62 (9)	529.13 (10)
			Subtotal (Number of Compounds)			7	7	7	7	8	8	8	7
			Subtotal (%)			2.6	4.2	5.9	5.4	3.4	4.9	9.4	9.3
			Ketones										
			Aliphatics										
137	110	0.512	3-Methyl-butan-2-one	600	554	5.53 (29)	4.90 (9)	3.63 (11)	3.28 (5)	-	-	-	-
138	115	0.528	3-Pentanone	650	660	6.52 (22)	6.54 (15)	6.10 (34)	8.17 (12)	-	-	5.28(2)	5.27 (2)
139	115	0.632	2,3-Pentanedione	658	660	19.16 (2)	23.59 (10)	13.47 (5)	14.51 (13)	-	-	26.98 (9)	27.78 (6)
140	130	1.224	3-Hydroxy-butan-2-one	711	697	10.36 (6)	5.56 (9)	5.94 (6)	6.15 (10)	-	1.27 (11)	4.50 (38)	5.81 (2)

141	140	0.712	3-Penten-2-one	729	719	-	0.59 (36)	0.36 (21)	0.63 (4)	9.46[e]	8.40 (17)	5.12 (3)	4.39 (3)
142	165	0.664	Hexane-2,3-dione	786	778	0.90 (4)	3,66 (5)	3.77 (8)	4.04[e]	-	-	2.75 (5)	2.05 (2)
143	175	0.664	Hexane-3,4-dione	793	801	-	0.80 (15)	1.20 (9)	1.11 (13)	-	-	-	-
144	250	0.632	Heptan-2-one	889	895	5.50 (3)	5.23 (6)	5.30 (25)	5.16 (14)	-	0.36 (15)	1.93 (19)	2.23 (7)
145	260	0.688	4-Ethoxy-2-pentanone	900	906	-	-	-	-	1.47 (14)	1.12 (5)	0.74 (5)	0.86 (19)
146	335	0.600	Octan-3-one	985	985	5.42 (13)	5.66 (8)	4.99 (30)	6.30 (3)	-	-	2.46 (5)	2.32 (4)
147	335	0.736	6-Methyl-Hept-5-en-2-one (Sulcatone)	985	988	13.16 (6)	14.67 (13)	15.07 (7)	15.70 (3)	-	-	-	-
148	430	0.608	Nonan-3-one	1091	1084	1.00 (8)	1.10 (2)	0.68 (1)	1.34 (12)	-	-	2.46 (5)	2.32 (4)
149	435	0.640	Nonan-2-one	1100	1090	11.16 (14)	8.26 (7)	10.04 (23)	10.98 (8)	0.64 (15)	1.15 (6)	4.50 (16)	4.77 (5)
150	600	0.648	Undecan-3-one	1283	1289	1.50 (19)	1.32 (36)	1.53 (11)	1.70 (23)	-	-	-	-
151	605	0.672	Undecan-2-one	1291	1295	0.47 (23)	0.66 (15)	0.90 (1)	0.86 (2)	-	-	-	-
			Aromatics										
152	415	1.304	Acetophenone	1069	1074	2.84 (19)	2.52 (6)	2.64 (15)	3.10 (6)	0.56 (11)	0.85 (19)	1.59 (13)	1.34 (9)
			Subtotal (GC Peak Area)			83.52 (10)	85.03 (10)	75.63 (14)	83.02 (8)	12.13 (4)	13.14 (15)	58.32 (11)	59.15 (5)
			Subtotal (Number of Compounds)			13	15	15	15	4	6	11	11
			Subtotal (%)			0.9	0.9	1.0	1.0	0.2	0.2	1.1	1.0
			Furans										
153	110	0.416	Tetrahydro-furan	623	648	-	-	-	-	11.48 (10)	11.55 (6)	10.28 (30)	10.54 (27)
154	205	1.808	2-Furanylmethanal (Furfural)	830	840	-	3.04 (3)	3.68 (9)	3.47 (7)	2.65 (11)	5.46 (4)	28.32 (5)	28.17 (2)
155	325	0.488	2,6,6-trimethoxy-2-vinyltetrahydropyran	971	974	-	-	-	-	12.86 (11)	4.36 (12)	5.03 (3)	4.78 (2)
156	345	0.568	2-Pentyl-furan	992	990	0.93 (35)	1.27 (24)	0.97 (15)	0.80 (13)	1.90 (39)	0.85 (22)	0.28 (37)	0.65 (28)
157	350	1.112	Benzofuran	1006	996	-	-	-	-	1.08 (9)	0.62 (5)	-	-
158	405	1.224	Ethyl 2-furancarboxylate (Ethyl 2-furoate)	1062	1051	1.20 (10)	1.03 (3)	1.16 (26)	1.32 (1)	1.37 (5)	1.11 (7)	1.38 (12)	1.43 (12)
			Subtotal (GC Peak Area)			2.12 (21)	5.33 (8)	5.81 (14)	5.59 (7)	31.34 (12)	23.95 (7)	45.30 (11)	45.58 (8)
			Subtotal (Number of Compounds)			2	3	3	3	6	6	5	5
			Subtotal (%)			0.1	0.1	0.1	0.1	0.4	0.4	0.8	0.8
			Lactones										
159	275	2.336	γ-Butyrolactone	915	918	53.59 (10)	53.56 (14)	45.81 (18)	56.22 (14)	47.02 (16)	48.16 (6)	26.72 (6)	32.01 (4)
160	670	1.272	γ-Nonanoic lactone	1360	1377	1.45 (30)	1.13 (17)	1.44 (21)	1.58 (13)	1.08 (22)	0.76 (13)	0.87 (26)	0.80 (7)
			Subtotal (GC Peak Area)			55.04 (11)	54.69 (14)	47.25 (18)	57.80 (14)	48.10 (16)	48.92 (7)	27.59 (7)	32.80 (4)
			Subtotal (Number of Compounds)			2	2	2	2	2	2	2	2
			Subtotal (%)			0.6	0.5	0.6	0.7	0.6	0.9	0.5	0.6

			Acetals										
161	130	0.488	1,1-Diethoxy-ethane	719	695	206.64 (23)	212.12 (35)	248.89 (23)	269.12 (55.9)	30.10 (15)	50.13 (18)	97.57 (3)	101.77 (2)
162	155	0.512	2,4,5-Trimethyl-1,3-dioxolane	739	754	9.75 (17)	154.81 (27)	18.99 (23)	24.45 (2)	21.92 (6)	44.34 (28)	107.74 (3)	83.11 (10)
163	225	0.440	1,1-Diethoxy-2-methyl-propane (Isobutanal diethyl acetal)	858	863	2.24 (24)	1.11 (25)	-	-	0.58 (16)	0.93 (12)	1.80 (14)	1.89 (1)
164	235	0.456	1-(1-ethoxyethoxy)-Butane	872	876	10.45 (7)	-	-	-	-	-	2.09 (20)	2.77 (22)
165	310	0.464	1,1-Diethoxy-3-methyl-butane (Isovaleraldehyde diethyl acetal)	954	953	8.43 (17)	3.19 (14)	3.04 (26)	3.20 (8)	1.97 (22)	1.97 (19)	5.69 (18)	6.14 (5)
166	325	0.480	1-(1-Ethoxyethoxy)-pentane (Acetaldehyde ethyl amyl acetal)	977	974	88.27 (11)	39.86 (12)	36.74 (30)	49.95 (9)	-	10.82 (18)	22.68 (8)	24.15 (7)
167	365	0.696	1,1-Diethoxy-pentane (Varealdehyde diethyl acetal)	1016	1017	0.34 (17)	0.48 (19)	0.46 (23)	0.51 (21)	-	-	-	-
			Subtotal (GC Peak Area)			326.12 (19)	411.57 (30)	59.22 (27)	78.12 (7)	54.57 (12)	108.19 (22)	237.57 (4)	219.83 (6)
			Subtotal (Number of Compounds)			7	6	5	5	4	5	6	6
			Subtotal (%)			3.4	4.1	3.1	3.1	0.7	1.9	4.3	3.9
			Thiols and others sulphur compounds										
168	140	0.624	Dimethyl disulfide	722	719	-	-	-	-	3.83 (30)	1.47 (24)	1.27 (1)	0.98 (16)
169	340	1.216	2-Methyl-tetrahydrotiophen-3-one (Dihydro-2-methyl-3(2H)-thiophenone)	994	991	4.60 (4)	3.47 (4)	3.25 (8)	3.55 (5)	-	-	-	-
170	350	2.168	3-(Methylthio)-propan-1-ol (Methionol)	982	1002	33.42 (4)	27.13 (28)	30.34 (28)	40.02 (10)	25.83 (26)	23.22 (13)	12.62 (9)	14.65 (10)
171	445	0.912	Ethyl 3-(methylthio)propionate	1098	1101	-	1.95 (14)	1.95 (21)	2.22 (5)	2.49 (27)	2.34 (6)	1.51 (13)	1.28 (5)
			Subtotal (GC Peak Area)			38.02 (4)	32.55 (25)	35.54 (26)	45.79 (9)	32.15 (27)	27.03 (13)	15.41 (9)	16.91 (10)
			Subtotal (Number of Compounds)			2	3	3	3	3	3	3	3
			Subtotal (%)			0.4	0.3	0.4	0.5	0.4	0.5	0.3	0.3
			Norisoprenoids										
172	380	0.64	2,6,6-Trimethylcyclohexanone	1035	1034	2.28 (10)	2.36 (4)	2.36 (23)	2.64 (7)	0.85 (21)	0.89 (12)	1.45 (4)	1.98 (12)
173	405	0.72	3,5,5-Trimethylcyclohex-2-enone (Isophorone)	1118	1062	-	.	0.61 (19)	0.74 (2)	0.41 (15)	0.34 (9)	0.36 (6)	0.36 (6)
174	600	0.600	Vitispirane	1281	1289	-	-	-	-	16.65 (11)	13.13 (7)	6.77 (5)	8.05 (3)
175	660	0.744	1,1,6-Trimethyl-1,2-dihydro-naphathalene (TDN)	1354	1363	-	-	-	-	0.82 (22)	0.78 (19)	0.43 (4)	0.43 (6)
176	685	0.792	*β*-Damascenone	1359	1395	4.95 (20)	4.81 (4)	4.10 (28)	5.94 (2)	8.54 (13)	7.19 (7)	7.96 (12)	8.06 (10)
177	735	0.728	Geranyl acetone	1453	1463	-	-	37.22 (22)	16.92 (28)	7.16 (31)	3.72 (29)	4.02 (14)	6.02 (13)
178	880	1.016	Methyl dihydro jasmonate	1650	1671	5.06 (32)	6.29 (23)	5.40 (12)	7.16 (26)	-	-	-	-
			Subtotal (GC Peak Area)			12.29 (23)	13.46 (13)	49.69 (21)	33.40 (16)	34.42 (16)	26.05 (11)	20.99 (9)	24.90 (9)
			Subtotal (Number of Compounds)			3	3	5	5	6	6	6	6
			Subtotal (%)			0.1	0.1	0.6	0.4	0.5	0.5	0.4	0.4

			Terpenic compounds										
			Monoterpenic compounds										
179	285	0.448	*α*-Pinene	934	932	0.77 (17)	0.97 (22)	0.80 (21)	0.79 (20)	-	-	-	-
180	340	0.520	*β*-Pinene	980	990	1.02 (24)	1.16 (21)	0.92 (20)	1.27 (36)	-	-	-	-
181	345	0.504	*α*-Terpinene	1018	995	-	-	-	-	2.83 (6)	1.56 (3)	1.16 (9)	1.13 (7)
182	375	0.520	Limonene	1031	1028	50.16 (19)	30.75 (12)	33.12 (28)	31.25 (24)	27.93 (6)	26.46 (12)	22.26 (15)	35.60 (18)
183	395	0.536	*β*-Ocimene	1050	1051	-	-	-	-	1.22 (5)	0.66[e]	-	-
184	400	0.632	m-Cymene	1033	1056	0.77 (24)	0.96 (5)	0.85 (29)	1.13 (5)	-	-	-	-
185	410	0.792	Dihydromyrcenol	1072	1068	4.02 (22)	4.02 (24)	5.15 (3)	4.96 (22)	-	-	-	-
186	425	0.560	*α*-Terpinolene	1096	1084	-	1.10 (11)	-	1.20 (9)	-	-	-	-
187	435	0.896	Linalool	1105	1095	36.35 (17)	34.43 (8)	31.91 (23)	42.82 (2)	115.80 (13)	72.54 (7)	54.16 (6)	58.11 (8)
188	445	0.608	Rose oxide	1115	1107	2.24 (30)	3.31 (23)	2.03 (20)	3.61 (7)	-	-	-	-
189	450	0.968	Hotrienol	1110	1112	2.32 (12)	2.27 (15)	1.56 (8)	3.33 (6)	2.30 (29)	1.15 (9)	1.33 (18)	1.22 (11)
190	490	0.656	Nerol oxide	1172	1154	1.42 (22)	1.36 (11)	-	-	5.37 (9)	3.24 (4)	3.47 (17)	3.47 (11)
191	495	1.088	endo-Borneol	1165	1166	0.63 (29)	0.53 (4)	0.64 (4)	0.46 (17)	-	-	-	-
192	515	0.808	Terpinen-4-ol	1177	1189	5.97 (13)	5.81 (8)	5.58 (13)	7.29 (3)	10.35 (12)	4.63 (10)	3.71 (15)	3.60 (12)
193	520	0.992	*α*-Terpineol	1224	1195	5.10 (19)	6.21 (11)	6.13 (2)	6.78 (8)	71.02 (5)	29.56 (9)	10.98 (12)	10.99 (9)
194	525	0.944	Pinocarveol	1195	1201	1.80 (9)	0.84 (6)	-	-	-	-	-	-
195	535	0.856	1-*p*-Menthen-9-al	1217	1213	1.11 (1)	0.89 (10)	1.28 (7)	1.03 (16)	-	-	-	-
196	545	0.816	Piperitol	1220	1225	2.06 (12)	1.83 (9)	2.24 814)	2.25 (1)	-	-	-	-
197	560	0.976	*β*-Citronellol	1234	1242	31.77 (21)	28.60 (15)	32.00 (18)	37.58 (5)	12.12 (26)	11.59 (13)	12.03 (10)	15.50 (20)
198	580	0.552	Myrtenol	1264	1265	2.12 (17)	2.27 (16)	1.93 (1)	2.20 (2)	13.16 (10)	9.86 (10)	9.00 (9)	9.43 (10)
199	580	1.08	Geraniol	1265	1272	6.15 (21)	5.76 (3)	4.61 (16)	1.51 (12)	16.33 (1)	6.87 (3)	-	-
200	585	0.552	Geranyl vinyl ether	1275	1271	-	-	-	-	3.49 (7)	3.26 (4)	3.18 (6)	2.99 (5)
201	585	0.600	Mentha-1,8-dien-7-ol	1330	1271	-	-	-	-	18.76 (19)	11.18 (35)	15.23 (16)	18.45 (8)
202	605	0.560	Nerol	1245	1295	5.00 (17)	4.00 (8)	4.40 (4)	5.71 (9)	12.53 (15)	9.63 (9)	7.73 (15)	7.64 (14)
203	780	0.944	Limonene dioxide	1294	1304	6.08 (25)	7.00 (21)	2.50 (6)	2.86 (3)	-	-	-	-
			Sesquiterpenic compounds										
204	695	0.584	Longifolene	1402	1408	4.22 (16)	4.52 (30)	6.86 (28)	3.79 (12)	-	-	-	-
205	815	0.784	Nerolidol	1564	1573	-	-	-	-	3.62 (39)	2.79 (26)	-	-
206	995	0.752	Farnesol	1792	1801	3.05 (2)	5.62 (19)	3.12 (17)	3.74 (14)	2.60 (30)	3.35 (92)	1.02 (16)	2.54 (21)
			Subtotal (GC Peak Area)			*174.15 (19)*	154.20 (13)	147.64 (19)	165.58 (9)	319.42 (11)	198.34(12)	145.27 (11)	170.68 (12)
			Subtotal (Number of Compounds)			22	23	20	21	16	16	13	13

Subtotal (%)	1.8	1.5	1.8	1.9	4.3	3.5	2.6	3.0
Total (GC Peak Area)	9689.84 (16)	9964.32 (16)	8025.17 (17)	8505.29 (12)	7404.65 (15)	5689.96 (13)	5500.24 (14)	5685.05 (12)
Total (Number of Identified Compounds)	157	163	166	167	150	151	141	142

[a] Retention times for first (^1t_R) and second (^2t_R) dimensions in seconds.

[b] RI, Retention Index reported in the literature for HP-5 GC column or equivalents (Ansorena et al., 2000; Campeol et al., 2003; Cardeal et al., 2008; Engel et al., 2002; Eyres et al., 2005; Fan & Qian, 2006; Högnadóttir & Rouseff, 2003; Jordán et al., 2002; Leffingwell & Alford, 2005; Perestrelo et al., 2011; Petronilho et al., 2011; Pino et al., 2005; Robinson A.L., 2011a; Rocha et al., 2007; Salvador et al., 2013; Silva et al., 2015; Silva et al., 2010, Jalali et al., 2012).

[c] RI: retention index obtained through the modulated chromatogram.[d] Mean of three replicates. [e] The compound was only detected in one replicate. [f] Relative standard deviation, expressed in percentage, in parenthesis.