Universidade de Aveiro Departamento de Química
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Composição volátil da casta *Vitis vinifera* L. Fernão-Pires da Região Demarcada da Bairrada: 1. Comparação com as outras principais castas brancas; 2. Desenvolvimento de metodologias de análise

Volatile composition of *Vitis vinifera* L. Fernão-Pires variety from Bairrada Appellation: 1. Comparison with other major white grape varieties; 2. Development of methodologies of analysis

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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 da Doutora Sílvia Maria Simões da Rocha Carriço, Professora Auxiliar do Departamento de Química e do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro

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palavras-chave

Fernão-Pires, castas brancas *Vitis vinifera* L., compostos voláteis, libertação de aroma por tratamento enzimatico, capacidade de retenção de compostos voláteis, assinatura volatil

resumo

A Bairrada é uma das regiões vitivinícolas mais antigas de Portugal, apesar de a Região Demarcada da Bairrada só ter sido oficialmente criada em 1979. A casta Fernão-Pires (FP) *Vitis vinifera* L. é a principal casta branca cultivada nesta região, onde é conhecida pelo nome de Maria-Gomes. As castas Bical (Bic), Arinto (Ari) e Cerceal (Cer), são outras castas brancas relevantes igualmente cultivadas na Região Demarcada da Bairrada. Estas quatro castas representam, respectivamente, 70%, 10%, 10% e 5% do total do encepamento de castas brancas nesta região.

O conhecimento da composição volátil destas quatro variedades pode oferecer um meio de avaliar o seu potencial de aroma e melhorar a qualidade do aroma dos seus vinhos. No entanto, a composição volátil destas variedades ainda não se encontra caracterizada.

Neste trabalho, o estudo foi centrado na casta FP, devido à sua importância no contexto da Região Demarcada da Bairrada, mostrando que esta casta apresenta um perfil significativamente diferente das outras castas brancas mais representativas (Bic, Ari e Cer), contendo um maior número de compostos voláteis e em maior concentração. As potencialidades de aroma da casta FP foram avaliadas pela análise da composição volátil das uvas, mostos e vinho. Nas uvas, os compostos voláteis varietais encontram-se principalmente associados às películas (69,3%) e parte líquida da polpa (25,3%), e encontram-se distribuídos pelas formas livre (66,7%) e glicosidicamente ligada (33,3%). Esta variedade apresenta terpenóides, álcoois aromáticos, norisoprenóides em C_{13} e álcoois em C_6. Ela é caracterizada principalmente pela presença de monoterpenóides, um deles presente numa concentração acima do seu limite de percepção sensorial (geraniol), e pela presença de um terpendiol odorante na forma glicosidicamente ligada (*Z*-2,6-dimetil-2,7-octadieno-1,6-diol). De forma semelhante, os mostos são caracterizados pela presença de monoterpenóides, alguns deles em concentrações acima do seu limite de percepção sensorial (hotrienol e linalol), e pela presença de terpendióis, odorantes ou não, na forma de potencial (*Z*-2,6-dimetil-2,7-octadieno-1,6-diol e 3,7-dimetil-1,5-octadieno-3,7-diol), que podem representar uma importante fonte de monoterpenóides. O potencial em terpenóides das uvas, que se encontra principalmente associado às formas glicosidicamente ligadas das películas, assim como a presença de álcoois em C_6 nas películas na forma livre, sugerem que as alterações/melhoramentos tecnológicos, relacionados principalmente com as preparações enzimáticas usadas durante a vinificação para libertar os compostos ligados, e o tempo de contacto com as películas devem ser tidos em conta como estratégias para aumentar a qualidade do aroma da casta FP.

De facto, o efeito da utilização de uma enzima libertadora de aroma no vinho Fernão-Pires foi testado neste trabalho e confirmou a melhoria do seu aroma, ao contrário do que se verificou com a Bic. Essa melhoria traduziu-se pelo aumento de 9% no total dos compostos voláteis desta casta devido principalmente ao aumento do geraniol (67%), dos terpendiois (96%), fenóis e álcoois aromáticos (26%) e ésteres (32%). Alguns deles foram identificados em concentrações acima do seu limite de percepção sensorial, podendo contribuir com notas florais e frutadas.

O facto das quatro castas estudadas exibirem composições voláteis diferentes permite dizer que, para a melhoria da qualidade do seu vinho, devem ser desenvolvidas tecnologias de vinificação especificamente para cada uma. Uma vez que estas castas crescem todas na mesma região (Bairrada), o conhecimento da sua composição volátil pemitirá aos vinicultores planear o seu uso em vinhos monovarietais ou em misturas, juntando o potencial em ácidos orgânicos voláteis das castas Ari e Cer (não mostrado neste estudo) às características terpénicas da casta FP, e em alcoois aromáticos da casta Bic.

A variabilidade da composição volátil dos mostos da casta FP ao longo dos anos, foi também avaliada pela análise dos mostos de 4 colheitas. Com base nos dados obtidos, usando cromatografia de gás-espectrometria de massa seguida da análise de componentes principais (GC-MS-PCA), foram estabelecidas relações entre a composição varietal volátil dos mostos e a classificação da qualidade do aroma dos vinhos brancos das referidas colheitas, fornecidas pela câmara de prova da Comissão Vitivinícola da Região da Bairrada (CVRB). Os resultados das análises mostraram-se consistentes com a classificação da qualidade dos vinhos dada pela CVRB, indicando que a qualidade do aroma do vinho está claramente ligada com a composição varietal volátil na forma livre. Por outro lado, a fracção potencialmente volátil permitiu distinguir os mostos de acordo com a sua composição em precursores de aroma na forma potencial. Este estudo fornece informação aos vinicultores sobre as metodologias de vinificação que podem ser implementadas para a melhoria da qualidade do aroma dos vinhos.

O crescente aparecimento de técnicas para a rápida caracterização de produtos alimentares, nomeadamente com recurso à espectrometria de massa, levou ao desenvolvimento de uma metodologia para a distinção rápida de vinhos através da análise da sua fracção volátil. Esta técnica baseada na microextracção em fase sólida, em modo de espaço de cabeça acoplada à cromatografia de gás- espectrometria de massa seguida da análise de componentes principais (HS-SPME-GC-MS-PCA), permitiu avaliar a assinatura global volátil do espaço de cabeça do vinho (perfil cromatográfico e padrões de fragmentação *m/z* para cada varrimento) sem uma completa separação cromatográfica dos seus componentes. Aos dados resultantes da fracção volátil não resolvida foi aplicada uma PCA para poder extrair dos dados a maior informação química possível e para extrair os fragmentos *m/z* (marcas) para a caracterização e distinção das variedades de vinhos. Esta metolodologia foi testada em dois vinhos monovarietais diferentes (FP e Ari *Vitis vinifera* L. var). Associada à rapidez desta metodologia pelo tempo de extracção e à robustez, é ainda importante realçar a elevada sensibilidade e o baixo efeito da humidade das amostras na resposta do detector MS, quando comparado com os “narizes-electrónicos” convencionais.

As interacções que os compostos voláteis podem estabelecer com as macromoléculas do vinho e a importância deste fenómeno para a qualidade sensorial dos vinhos levou ao devenvolvimento de uma metodologia de microextracção em fase sólida em modo espaço de cabeça, acoplada à cromatografia de gás (HS-SPME-GC) para o estudo, em vinhos modelo, das interacções de 3 ésteres (hexanoato, octanoato e decanoato de etilo) e diferentes quantidades de fracção polimérica extraída do vinho FP (1.0 g/L, 10.0 g/L e 30 g/L). Esta técnica permite calcular o índice de retenção (RI) para cada composto, que é definido como sendo a capacidade de retenção da fracção polimérica de cada vinho em relação aos três ésteres. A maior capacidade de retenção foi observada para o composto mais hidrofóbico, o decanoato de etilo, e para a concentração mais elevada de fracção polimérica. No entanto, o decanoato de etilo é também retido para a concentração de fracção polimérica do vinho de 1.0 g/L. Este estudo sugere que os compostos retidos podem ser doseados para o espaço de cabeça promovendo a percepção do seu aroma por um longo período de tempo.

keywords

Fernão-Pires, white *Vitis vinifera* L. varieties, volatile compounds, aroma release enzymatic treatment, volatile retention capacity, volatile signature

summary

Bairrada is one of the ancient winemaking regions in Portugal, although Bairrada Appellation was only officially created in 1979. *Vitis vinifera* L. Fernão-Pires (FP) is the main white grape variety in Bairrada, where it is known as Maria-Gomes. Other varieties, such as Bical (Bic), Arinto (Ari) and Cerceal (Cer), are also relevant white varieties cultivated in Bairrada Appellation. They represent 70%, 10%, 10% and 5% of the white vineyard, respectively.

The knowledge of the volatile composition of these four varieties may offer a means of evaluating their potential aroma and to improve the quality of wine aroma. Nevertheless, the volatile composition of these varieties is not yet characterized.

In this work, particular attention is devoted to Fernão-Pires variety due to its importance in the Bairrada Appellation context, showing that this variety exhibits a volatile profile significantly different from those of the other most representative white varieties (Bic, Ari and Cer), with a higher number and concentration of volatile compounds.

The aroma potentialities of *Vitis vinifera L.* FP were estimated by the analysis of the volatile composition of its grapes, musts and wine. In grapes, the varietal volatile content is mainly associated to the skin (69.3%) and liquid pulp (25.3%) and is distributed by free (66.7%) and glycosidically-linked (33.3%) forms. This grapes variety exhibited terpenoids, aromatic alcohols, C_{13} norisoprenoids and C_6 alcohols. It is mainly characterized by the presence of monoterpenoids, one of them over their sensory perception limit (geraniol), as well as the presence of an odour terpendiol in the glycosidically-linked form (*Z*-2,6-dimethylocta-2,7-dien-1,6-diol). Similarly, the musts are characterized by the presence of monoterpenoids, some of them over their sensorial perception limits (hotrienol and linalool), as well as the presence of odour/odourless terpendiols in the potential form (*Z*-2,6-dimethylocta-2,7-dien-1,6-diol and 3,7-dimethylocta-1,5-dien-3,7-diol), which may also represent an important source of monoterpenoids. The terpenoid potential of grapes, which is mainly associated to the glycosidically-linked form of skin, as well as the presence of C_6 alcohols in the free form of skin, suggests that both technological changes/improvements, related mainly to enzymatic preparations used during winemaking to release the linked compounds and skin contact time must be taken into consideration as strategies to increase the aroma quality of wine from FP variety.

In fact, the effect of an aroma release enzyme in FP wine was tested in this work, demonstrating the real improvement of its aroma, contrarily to Bic. There was an increase of 9% in the total amount of volatile compounds, due mainly to the increase of geraniol (67%), terpendiols (96%), phenols and aromatic alcohols (26%) and esters (32%). Some of them were within their sensory perception limits and may have a contribution to the floral and fruity notes.

As a consequence of the fact that the four varieties under study exhibit different volatile composition patterns, winemaking technologies should be specifically developed for each variety for the improvement of wine aroma quality. As these varieties are all grown in the same region (Bairrada Appellation), knowledge of their varietal composition will allow winemakers to plan their use in monovarietal wines or in blends, providing the potential of the high volatile organic acid composition of Ari and Cer (data not shown) together with the terpenic characteristics of FP and the aromatic alcohols of Bic.

The harvest variability of the varietal composition of FP grape variety was also evaluated from the musts across four harvests. Based on the data obtained, using gas chromatography- mass spectrometry (GC-MS) tandem with principal component analysis (PCA), relationships were established between the varietal volatile composition of the musts and the white wine aroma quality classification conferred by the wine taster chamber of Comissão Vitivinícola da Região da Bairrada (CVRB). The results of the volatile analysis were consistent with the wine quality classification given by CVRB, indicating that the wine aroma quality is clearly linked with the musts free varietal volatile composition. On the other hand, the PVC fraction allowed the distinction of the musts according to their potential aroma precursor's composition. The proposed approach provides information to winemakers concerning the winemaking methodologies that can be implemented to improve the wine aroma quality.

The emergency of techniques for the rapid characterization of food products, with the use of mass spectrometry, leaded us to the development of a methodology for the rapid distinction of wines by volatile fraction analysis. This technique based on headspace solid-phase microextraction- gas chromatography- mass spectrometry- principal component analysis (HS-SPME-GC-MS-PCA), allows one to evaluate the global volatile signature of the wine headspace (chromatographic profile and *m/z* pattern of fragmentation in each scan) without complete chromatographic separation of its components. In order to retrieve from the data as much chemical information as possible and to extract *m/z* fragments (markers) for the characterization and distinction of the wines varieties, a PCA was applied to the data resultant from the unresolved volatile fraction. Two different monovarietal white wines (*Vitis vinifera* L. var FP and Ari) were tested. Associated to the fast character of the proposed methodology and robustness taking into account the extraction time, it is also important to highlight the higher sensibility and lower effect of the sample moisture of the MS sensor response when compared to the conventional *e*-noses.

The interactions that volatile compounds can establish with the wine macromolecules and the importance of this ocurrence for the sensory quality of wines leaded us to the development of a methodology (HS-SPME-GC) for the study in model wines of the interactions between three ethyl esteres (ethyl hexanoate, ethyl octanoate and ethyl decanoate) and diferent amounts of polymeric fraction extracted from the FP wine (1.0 g/L, 10.0 g/L and 30 g/L). This methodology allowed to calculate the retention index (RI) for each compound, which is the retention capacity of each wine polymeric fraction towards the three esters established. The higher retention indexes were observed for ethyl decanoate, the more hydrophobic compound, and for the wine polymeric material with higher concentration. Ethyl decanoate was found to be retained even for the wine polymeric fraction concentration of 1.0 g/L. Furthermore, this study also suggested that the retained compounds are dosed to the headspace, which may promote the perception of their aroma for a longer period of time.

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LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers:

Sílvia M. Rocha, **Paula Coutinho**, António Barros, Manuel A. Coimbra, Ivonne Delgadillo, A. Dias Cardoso. 2000. Aroma potential of two Bairrada white grape varieties: Maria Gomes and Bical. *J. Agric. Food Chem.* **48**:4802–4807.

Sílvia M. Rocha, **Paula Coutinho**, Ivonne Delgadillo, A. Dias Cardoso, Manuel A. Coimbra. 2005. Effect of enzymatic aroma release on the volatile compounds of white wines presenting different aroma potentials. *J. Sci. Food Agric.* **85**:199-205.

Sílvia M. Rocha, **Paula Coutinho**, António Barros, Ivonne Delgadillo, Manuel A. Coimbra. 2006. Rapid tool for distinction of wines based on the global volatile signature. *J. Chromatogr. A* **1114**:188–197.

Sílvia M. Rocha, **Paula Coutinho**, Ivonne Delgadillo, Manuel A. Coimbra. 2007. Headspace-solid phase microextraction-gas chromatography as a tool to define an index that establishes the retention capacity of the wine polymeric fraction towards ethyl esters. *J. Chromatogr. A* **1150**:155-161.

Sílvia M. Rocha, **Paula Coutinho**, António Barros, Ivonne Delgadillo, Manuel A. Coimbra. 2007. Establishment of the varietal volatile profile of musts from white *Vitis vinifera* L. varieties. *J. Sci. Food Agric.* (*in press).*

I

Introduction

Summary

The Portuguese Bairrada Appellation is succinctly described, identifying some of its particularities (geography, soil, climate and white grape varieties). *Vitis vinifera L.* Fernão-Pires is the most cultivated white variety in this region, representing 70% of the total white Bairrada vineyard. This section also cover the state of art on the volatile composition of grapes and wines, *i.e.* the main chemical groups of volatile compounds, modifications that can happen during winemaking, aroma descriptors, sensory perception limits and their importance to the wine aroma quality. The volatile compounds can interact with the wine polymeric fraction, which affects its wine aroma. Thus, the interactions, as well as the retention phenomena between volatiles and wine macromolecules are also addressed. Other aspects, such as the main methodologies concerning extraction, characterization and data treatment of volatile compounds are reviewed. Particular attention is devoted to the methodologies/techniques used in this work. At last, the main goals of this work are presented.

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I.1. The Bairrada Appellation

Bairrada is one of the Portuguese Appellations and was legally established in 1979 (Portaria nº. 709-A/79, from December 28). The name "Bairrada" has its origins in the nature of the soils of this region, located between the Vouga and Mondego rivers, characterized by strong argillaceous (from the Portuguese word "barro") and calcareous soils, very different from the sandy soils in the coast.

I.1.1. Geographical area of Bairrada Appellation

The Bairrada Appellation area includes the councils of Anadia, Mealhada, Oliveira do Bairro, Águeda (Aguada de Baixo, Aguada de Cima, Águeda, Barrô, Belazaima do Chão, Borralha, Espinhel, Fermentelos, Óis da Ribeira, Recardães and Valongo do Vouga), Aveiro (Nariz), Cantanhede (Ançã, Bolho, Cadima, Camarneira, Cordinhã, Corticeiro de Cima, Covões, Febres, Murtede, Ourentã, Outil, Pocariça, Portunhos, Sanguinheira, São Caetano, Sepins and Vilamar), Coimbra (Botão, Souselas, Torre de Vilela, Trouxemil and Vil de Matos) and Vagos (Covão do Lobo, Ouca, Santa Catarina and Sosa) (Figure I.1.1).

This region has an estimated total vineyard area of 12,000 ha (Menezes de Almeida, 2003). The red and white vineyard corresponds to 70% and 30% of the total, respectively. The Bairrada Appellation produces annually 9 millions litres of white wine.

I.1.2. Natural factors

I.1.2.1. Climate

Bairrada Appellation is near the Atlantic Ocean, so the climate is essentially Atlantic. However, it may present some Mediterranean characteristics during Summer (July-August), which is very dry. The occurrence of frost in the Spring is common until the middle of April or, rarely, until the first days of May, conditioning the regional white varieties that are very temporary. In some years, the abundance of rain in the second half of September may cause rotten in some regional varieties

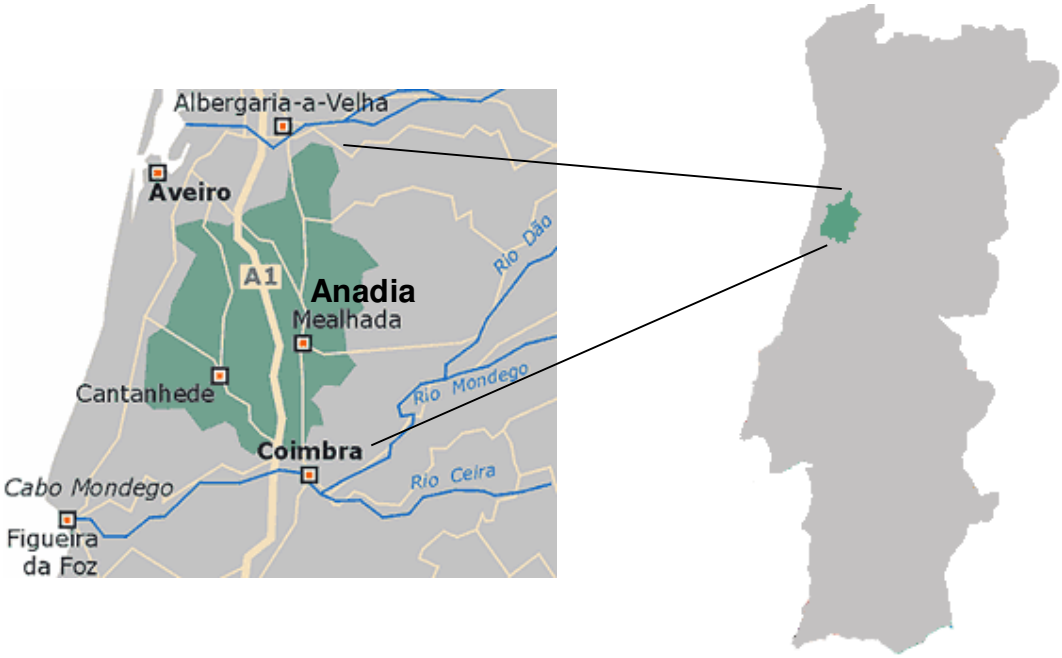


Figure I.1.1. Bairrada Appellation

I.1.2.2. Soils

The Bairrada Appellation presents a soft wave relief, with a 50 to 150 m quota. The vineyards were planted mostly in soils from inferior and medium Jurassic, which are argil-calcareous soils. According to Dec.-Lei nº 72/98 from March 26, the vineyards for the Bairrada wines production must be planted in red calcareous soils, humid or not humid litholic soils and low consolidated sand material. Because the water amount during Summer is scarce, these soils are very dry, which favours a good maturation of grapes, especially for Baga red variety.

I.1.3. White varieties

For each Portuguese Appellation there are specific recommended and authorized varieties. The *Vitis vinifera* L. white varieties recommended for the Bairrada Appellation are: Fernão-Pires (Maria-Gomes), Bical, Arinto, Cerceal, Chardonnay, Pinot-Blanc, Rabo-de-Ovelha, Sauvignon, Sercialinho and Verdelho (Dec.-Lei nº. 301/2003, from December 4).

Vitis vinifera L. Fernão-Pires is a commonly found variety in Portuguese Appellations and it is the most cultivated white variety in Portugal, as well as in Bairrada. In Bairrada

Appellation it is known as Maria-Gomes, name also legally recognized (Dec.-Lei nº. 301/2003, from December 4). In other Portuguese regions it is also named as "Gaeiro". This white grape variety is recommended for VQPRD of Bairrada ("vinho de qualidade produzido em região denominada"- quality wine produced in a denominated region) (Dec.-Lei nº72/98 from March 26 and Dec.-Lei 301/2003 from December 4). This variety represents about 70% of the total white Bairrada vineyard. Bical, Arinto and Cerceal, some of the other varieties, represent 10%, 10% and 5% of the white vineyard, respectively.

The main characteristics of *Vitis vinifera* L. Fernão-Pires variety are described as following:

Phenological dates (indicative dates):

Growth of thorns– second week of March,

Flower– first to second week of June,

Paint (*véraison*)– fourth week of July,

Maturation – first week of September.

Morphologic aspects:

The leaves have a medium size, green colour and have a characteristic wavy shape.

Their bunch has a medium size and is low compacted (Figure I.1.2). The berry is of medium size, with a round shape and a green-yellow colour. The port is semi-erect and has a strong vigour.

Vineyard particularities:

It has a medium/high production and a precocious maturation. Because it is an extemporaneous variety, it reaches a high alcoholic degree and shows a poor acid character.

Aroma characteristics:

The Fernão-Pires wine has been described by the Interprofessional Association of Bairrada Appellation (Comissão Vitivinícola da Região da Bairrada) as exhibiting mature

citrus and floral notes, such as orange, orange-tree and mimosa. Other white variety, such as Bical, has been described as presenting buttery and fruity notes, mainly melon, and is also rich in alcohol. Wines prepared from these varieties can be consumed immediately or can be kept for some years without losing their balance and aroma (Menezes de Almeida, 2003).

Figure I.1.2. *Vitis vinifera* L. Fernão-Pires variety

I.2. Volatile compounds of grape, must and wine

The aroma is one of the most important factors to determine the wine character and quality and, hence, consumer acceptance. Several studies recognized a relationship between the wine character and the grape and musts volatile composition (Cordonnier and Bayonove, 1974; Wilson *et al.*, 1986). Studies focused on the determination of the varietal character reported that it might be determined, in red grapes, by aromatic alcohols and norisoprenoids (López-Tamames *et al.*, 1997; Rosillo *et al.*, 1999; López *et al.*, 2004, Salinas *et al.*, 2004) and, in white grapes, by monoterpenoids (Cordonnier and Bayonove, 1974; Gunata *et al.*, 1985ab; Wilson *et al.*, 1986; Strauss *et al.*, 1986; Scheider *et al.*, 2001; Vázquez *et al.*, 2002; Diéguez *et al.*, 2003; Fernández-González and Di Stefano, 2004). Additionally, the wine aroma is composed by several other compounds from several chemical groups (alcohols, esters, acids, aldehydes, lactones, etc), many of them produced during fermentation.

The compounds that can potentially contribute to the wine aroma are volatile and/or semi-volatile compounds. They are usually small molecules, with small molecular weight and certain hydrophobicity. Aroma substances can then be defined as volatile compounds which are perceived by the odour receptor sites of the nose, *i.e.* the olfactory tissues of the nasal cavity. They reach the receptors when drawn in through the nose (nasal detection) and via the throat after being released by chewing (retronasal detection) (Belitz *et al.*, 2004). Thus, the intensity of an olfactive sensation depends not only on the concentration of these substances in the liquid phase but also on its volatility (vapour pressure) and sensory perception limit (section I.2.3). Furthermore, there is not only one compound responsible, by itself, for the organoleptic character of a wine.

The diversity of the white wines is due not only to soil, sun exposure, winemaking technology etc, but mostly to the different volatile compounds present in the different varieties used in wine production. In particular, the *Muscat* variety has been object of several studies due to its existence all over the world and its significant aroma composition (Williams *et al.*, 1980; Wilson *et al.*, 1984, 1986; Gunata *et al.*, 1985ab, 1986; Park *et al.*, 1991; Belancic *et al.*, 1997; Cordonnier *et al.*, 1998). For instance, its character is known to be due to the presence of monoterpenoids, such as linalool, geraniol, nerol, α-terpineol and hotrienol.

The aroma is the product of several biochemical and technological modifications that originally arises from grapes. Thus, the knowledge of the volatile composition of grapes and/or musts and its localization offers a means of evaluating the potential aroma of a variety, allowing the improvement of the quality of wine aroma.

I.2.1. Classification of wine aromas

The classification of the wine aroma was earlier proposed (Cordonnier and Bayonove, 1978) and can be described according to the stage where it is formed: *varietal*, *pre-fermentative*, *fermentative* and *post-fermentative* (Figure I.2.1).

The *varietal* aromas are directly associated to the grape variety, biosynthesized during the grape development. They depend on other factors such as type of soil, climate, healthy state and degree of grape maturity, but are independent of the winemaking techniques. The terpenic compounds, C_{13} norisoprenoids, thiols and methoxypyrazines are some groups of compounds associated to the varietal aromas of wines. The majority of grape varieties have low amounts of free volatile compounds responsible for the aroma. However, they have precursors in their composition in the form of glycosides, fatty acids, carotenoids and phenolic compounds that can originate aroma compounds. According to Cordonnier and Bayonove (1978), they can yet be divided in three categories: fruity, floral and herbaceous aromas.

The *pre-fermentative* aromas result from several mechanic or technological operations (transport, crushing, maceration and clarification) effectuated before the beginning of the fermentation process. This aroma results mainly from C_6 alcohols and aldehydes.

The *fermentative* aromas result from yeasts during alcoholic fermentation. They depend on the temperature and yeast species used. In this stage, the majority of the volatile compounds responsible for the wine aroma are formed. The esters are the main chemical group resulting from fermentation, but higher alcohols, ketones, aldehydes and fatty acids are also formed.

Lastly, the *post-fermentative* aromas or *bouquet* are developed during conservation and evolution of wines as a result of physicochemical, oxidation and reduction reactions of the wine compounds. They depend on the bottle maturation conditions. The phenols and C_{13} norisoprenoids are some groups of compounds formed during the wine evolution. The white wines are normally consumed in the first years after their production, thus the post-fermentative aromas result essentially from the early stages of the processes of

conservation and storage. During that time, the groups of volatile compounds that may suffer modifications are terpenoids, C_{13} norisoprenoids, esters, volatile phenols and sulfur compounds.

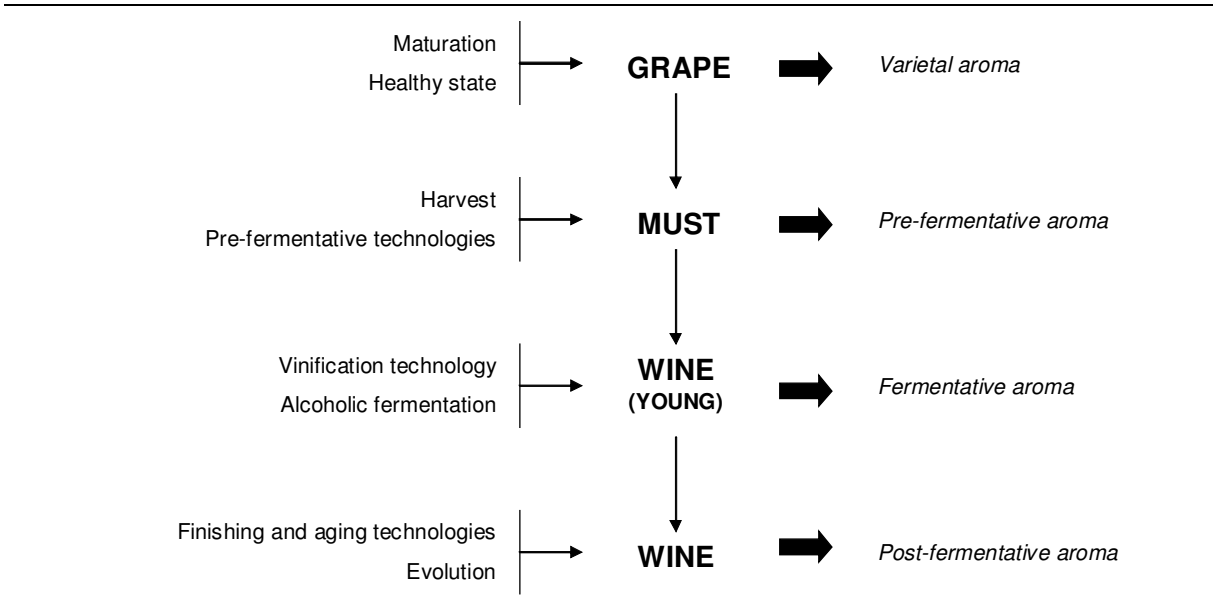


Figure I.2.1. Classification of aroma according to the stage where they are formed

The formation of volatile compounds responsible for the aroma occurs in most fruits during maturation and over maturation basically through catabolic reactions. The aroma of wine was found to be more complex than the aroma of grape and must. This is due to the transformations that occur during the yeast metabolism, which are responsible for the majority of the volatile compounds present in the fermented drinks. It is known that they result from fatty acids, amino acids, isoprene and cinnamic acids metabolisms (Ribéreau-Gayon *et al.*, 2000). During the evolution of white wines some modifications on aroma composition can happen due to the reactions between different wine constituents.

I.2.2. Aroma precursors

The maturity state of the grape determines largely the wine quality, in particular the aroma. Therefore, it is useful for the winemakers to know what occurs during grape ripeness. Other important aspects to be aware of are the volatile chemical composition of the grape (free and/or glycosidically-linked forms) and its localization and distribution

through the grape. This will allow to understand not only their characteristics but also the possible transformations that occur during winemaking.

The volatile compounds of grapes, musts and wines may arise from: *(i)* Free and volatile odourous substances; *(ii)* Non-volatile and non-odourous precursors (glycosides, cysteine derivatives, fatty acids, organic acids, polyphenols, etc.); *(iii)* Odourous or non-odourous volatile compounds which, due to their instability, can be transformed into other odour compounds (terpenoids, C_{13} norisoprenoids, etc) (Ribéreau-Gayon *et al.*, 1998). All these items represent the aroma potential of a wine.

I.2.2.1. The grape and its aroma potential localization

The grape is classed in a group of several fleshy fruits and belongs to the *Vitis* genus; its inflorescences organized into clusters are a bunch of berries. Each berry is attached to the stale, a small pedicel containing the vessels, which supply the berry with water and nutritive substances (Riberau-Gayon *et al.*, 1998). Cluster structure depends on the length of the pedicels: if they are long and thin, the grapes are spread out; if they are short, bunches are compact and the grapes are packed together. The varieties used for winemaking often belong to the latter category. Cluster compactness is one of the factors affecting rot sensitivity. The bunch is composed by two different parts: the lignified part (stalk) and the grape berries. Genetic factors and environmental conditions, that characterize berry formation, greatly influence its development and its composition at maturity.

In general, the evolution of grapes is divided in three phases (Ribéreau-Gayon *et al.*, 2000). The first is characterized by an intense metabolic activity, with an elevated respiratory intensity and a rapid accumulation of acids. In this first period the chlorophyll is the predominant pigment. The second is characterized by a slowed growth phase during which *véraison* occurs. *Véraison* is characterized by the appearance of colour in coloured varieties and a translucent skin in white varieties. The third is characterized by a second growth phase corresponding to maturation. The respiratory intensity decreases, whereas certain enzymatic activities sharply increase. The grape accumulates free sugars, potassium, amino acids and phenolic compounds. When the grape achieved its complete development, it has a more or less round shape, with a variable colour and consistence, depending on the grape variety.

Each grape berry is formed by skin, pulp and seeds (Figure I.2.2) (Kanellis *et al.*, 1993). The pulp is a fragile material of big swollen cells which rupture produces the juice or must. The material that results from the grape crushing represents the solid part of grapes, skin and seeds and the husks (when the pulling grape from stalks is not made). The must, which is obtained mechanically by crushing or pressing grapes, becomes turbid due to several suspension materials.

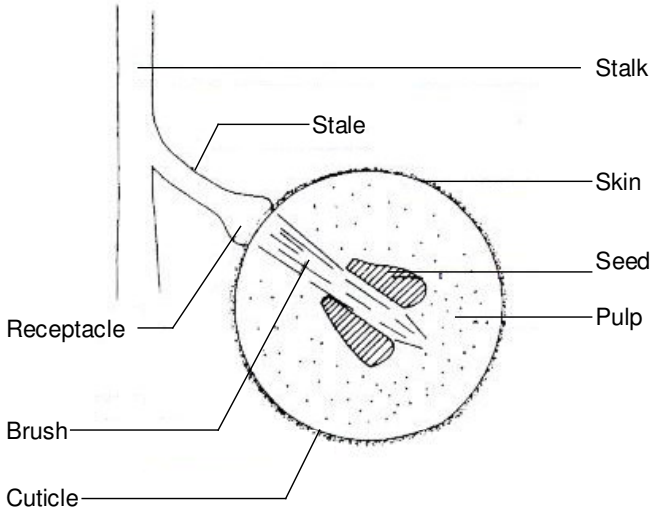


Figure I.2.2. Grape berry structure (based on Kanellis *et al.*, 1993)

The skin is formed by epidermis, one or two cell layers above which is the cereous substance, a fine powder cover (the cuticle), and by hypoderm with 5 to 10 polyhedral cells containing colour and flavour material, responsible respectively for colour and odour/taste of grape (Kanellis *et al.*, 1993). The skin represents about 5-12% of the total berry weight. The pulp is formed by 25 to 30 layers of cells containing the cell sap, the main constituent of the juice. Inside the pulp, it is possible to observe a more voluminous intermediate zone and an internal zone witch contains the seeds. This fraction accounts for 64-90% of the berry weight. The seeds can vary from 4 to 11, but some varieties are seedless. It is in the seeds that tannins, responsible for the astringency, and oils, that damage the wine quality, can be found. Thus, the crushing of the seeds during the mechanical treatments of the grapes must be avoided. This part constitute up to 10% of weight of the fruit.

Most studies concerning the localization and distribution of the free and glycosidically-linked volatile compounds have been carried out in varieties considered highly odourants, such as the *Muscat* variety. The less odourant varieties, or neutral varieties, have been less studied even though they are economically more important – about 90% of the vineyard production is made with less odourant varieties. Furthermore, the study of the aroma potential of neutral varieties can give important information about the technology that can be used in order to improve the wine aroma quality.

During grape ripening, the glycosidically-linked fraction is accumulated in the fruit and usually can be found in higher amounts than the free forms. All this aroma potential was found to be distributed through the different parts of the grape but their distribution is not the same for all varieties (Gómez *et al.*, 1994). Several studies show that the volatile compounds are mainly located in the solid parts of the grape (internal cells of skins and in the pulp solid parts inside vacuoles) where they are synthesized and/or stored (Cordonnier and Bayonove, 1978; Gunata *et al.*, 1985ab; Wilson *et al.*, 1986; Bayonove *et al.*, 1998a; Gómez *et al.*, 1994). However, the distribution of the compounds between the liquid and solid parts can be different. The main localization of these compounds in the skin suggests that it would be possible to increase the wine aroma fraction if a step that promotes their transference to juice is applied (Cabaroglu and Canbas, 2002; Selli *et al.*, 2006). However, an exhaustive time of contact can have an unpleasant effect due to the extraction of undesirable compounds, like the C_6 alcohols, responsible for herbaceous aromas (Baumes *et al.* 1986, Cordonnier, 1989). Thus, it seems necessary to attempt to optimal conditions.

The higher amount of free geraniol, observed on skins of three different varieties studied by Gómez *et al.* (1994), suggests that the hypodermic cells of the fruit have specific locations where the biosynthesis occurs and/or this compound is stored. It also seems likely that the geraniol has a fundamental role in the monoterpenols metabolism. On the other hand, the casual distribution of linalool through grapes of two Muscat varieties indicates an independent function for this compound in other sites that not exclusively the exocarp.

In other studies concerning the development of monoterpenols in Muscat grapes, the linalool has been proposed also as the substrate for the conversion of compounds with higher oxidation state, like 3,7-dimethylocta-1,5-dien-3,7-diol, known as terpendiol I (Wilson *et al.*, 1984). The fact that linalool is located apart from the places where there is a higher concentration of other monoterpenols, in the same oxidation state, and the co-

existence in similar levels with terpendiol I seems to corroborate that. However, this work did not indicate if the oxidation occurred exclusively in the solid part of the pulp or if it also occurred in the skin. The relatively low levels of terpendiol I found in the skin indicate that this compound is stored in the vacuolated cells of the solid pulp instead of being produced in other place (Wilson *et al.*, 1986).

Besides monoterpenoids, also C_{13} norisoprenoids are important for wine varietal aroma. Usually, these compounds are not present in grapes in the free form but as precursors, appearing later by their hydrolysis (Wintherhalter *et al.*, 1990; Skouroumounis *et al.*, 1994). Strauss *et al.* (1987) shows that the precursors of these compounds are mainly located in the juice of grapes. Different types of precursors were observed and apparently are developed in fruit by sugar accumulation.

I.2.2.2. Glycosydically-linked form

As mentioned before, the chemical compounds responsible for the grape and wine aromas are found in the free form, volatile and odourant, and in the glycosylated form, non-volatile and odourless, as aglycones of β-D-glucopyranose (β-D-Glc*p*). When the volatile compounds are linked to sugars they are known as aroma precursors (Figure I.2.3). The aglycones can be terpenoids, aliphatic or aromatic alcohols and C_{13} norisoprenoids. The study of the volatile components originated from the non-volatile precursors has been object of several studies (Cordonnier and Bayonove, 1974; Williams *et al.*, 1982ab; Gunata *et al.*, 1985ab; Voirin *et al.*, 1992ab; Cabrita *et al.*, 2006). Bound aroma, potentially developed during winemaking, is unknown for the majority of grape cultivar appellations. French varieties such as Chardonnay (Sefton *et al.*, 1993; Arrhenius *et al.*, 1996), Cabernet Sauvignon (Gómez *et al.*, 1995), and Muscat (Carro-Mariño *et al.*, 1995), German varieties such as Riesling (Skouroumounis and Winterhalter, 1994; Guth, 1997a) and several Spanish varieties (Versini *et al.*, 1995; López-Tamames *et al.*, 1997; Rosillo *et al.*, 1999; Falqué *et al.*, 2002) have been studied in order to establish databases of flavour compounds.

The glycosydically-linked forms are abundant in grapes. Four types of glycosides were identified: three diglycosides (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides or rutinoside, 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides or arabinosylglucoside, 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside or apioglucoside) and one monoglucoside (β-D-glucopyranose). The most abundant glycosides are arabinosylglucoside (32 to 58%) and

apioglucoside (28 to 46%), followed by rutinoside (6 to 13%). Monoglucosides represent only 4 to 9% of the total glycosidic forms (Bayonove *et al.*, 1992). However, it is known that even though they are likely to be present, they don't appear in all varieties and their proportion differs greatly depending on the variety. This aroma potential is naturally revealed during fruit maturation by endogenous enzymes identified as *β*-glucosidases (Gunata *et al.*, 1990ab, Gunata *et al.*, 1998). However, due to the unfavourable pH of musts and wines (2.8-3.5); and high variability within harvests, these enzymes show low activities and cannot liberate the whole aroma potential. Also, the yeasts and *Botrytis cinerea* fungus have β-glucosidase activities but, in a similar way to natural glucosidases of grapes, have very low activities in the conditions found in musts and wines (Delcroix *et al.*, 1994).

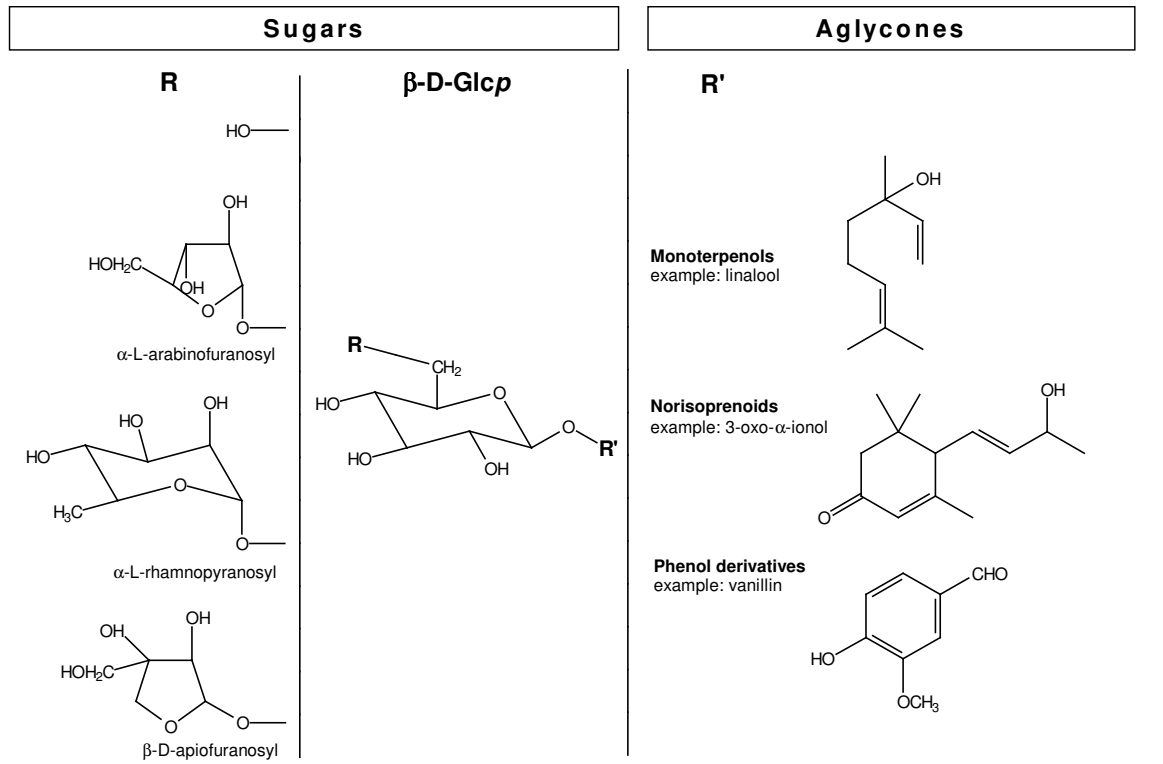


Figure I.2.3. Mono- and disaccharides identified as flavour precursors in grapes and wines (based on Park and Noble, 1993 and D'Incecco *et al.*, 2004)

The hydrolysis of the glycosidically-linked compounds may be promoted by acids or enzyme addition (Williams *et al.*, 1982c; Darriet *et al.*, 1988; Schwab *et al.*, 1988; Gunata *et al.*, 1990a; Shoseyov *et al.*, 1990; Di Stefano *et al.*, 1991; Razungles *et al.*, 1993;

Gueguen *et al.*, 1996; Villettaz *et al.*, 1996; Schneider *et al.*, 2001, Cabaroglu *et al.*, 2003). The acidic hydrolysis reveals polyols and terpenoids but it has some disadvantages like promoting terpenoid rearrangement and benefiting the hydrolysis of certain compounds present in small amounts. The enzymatic hydrolysis is the most used method because it doesn't promote significant alterations in the natural composition of grapes, mainly in monoterpenes (Aryan *et al.*, 1987; Gunata *et al.*, 1990a).

The release of the volatile compounds by enzymatic activity occurs in two sequential steps (Gunata *et al.*, 1990b) (Figure I.2.4). Firstly, α-L-arabinofuranosidase (EC 3.2.1.55), α-L-rhamnopyranosidase (EC 3.2.1.40) or β-D-apiofuranosidase act cutting the (1→6) glycosidic linkage. Secondly, the β-D-glucopyranosidase (also referred as β-D-glucosidase) (EC 3.2.1.21), acts cutting the glucose-aglycone link, releasing the aglycone (Ribéreau-Gayon *et al.*, 2000). The enzymatic hydrolysis of aglycones depends on the variety of the grape, wine pH, glycosides nature, aglycones structure and type of enzyme used.

Several hydrolytic experiments have been performed with exogenous *β*-glucosidases (Dubourdieu *et al.*, 1988; Shoseyov *et al.*, 1988; Cordonnier *et al.*, 1989; Cordonnier, 1989; Gunata *et al.*, 1990ab Vasserot *et al.*, 1993; Gueguen *et al.*, 1996). The use of these *β*-glucosidases allowed the release of volatile compounds, producing more fruity and floral wines, with more intense and complex aroma (Cordonnier *et al.*, 1989; Cordonnier, 1989; Bayonove *et al.*, 1992; Gueguen *et al.,* 1996; Cabaroglu *et al.*, 2003). These winemaking treatments were applied especially to aroma-rich varieties to increase their aroma, but this strategy is also particularly valuable for neutral varieties and/or varieties with poor floral and fruity aroma (Vázquez *et al.*, 2002). However, they should be used on the basis of knowledge of the activity of the commercial enzymes, as well as knowledge of the aroma potential of the grape varieties. *β*-Glucosidase activity was reported to have different specificities for different aglycones (Gunata *et al.*, 1990a; Gunata *et al.*, 1990b; Cordonnier, 1989). Cordonnier *et al.* (1989) have studied the activity of 34 commercial enzyme preparations in the Muscat de Frontignant variety, and have shown that the amount of volatile compounds released varies with the enzymatic preparation used. Also, it has been reported that the action of aroma release enzymes in wines varies according to the wine's sugar content (Gunata *et al.*, 1990a).

The C_{13} norisoprenoids of wines may arise from grape non-volatile precursors, (carotenoids and glycosides) (Skouroumounis *et al.*, 1994). Some C_{13} norisoprenoids, as vomofoliol, 3-oxo-α-ionol and 3-hydroxydamascone were identified in glycosidically-linked

form (Winterhalter *et al.*, 1990; Razungles *et al.*, 1993). The glycosides of C_{13} norisoprenoids are all monoglucosides that are not susceptible of being hydrolysed by grapes and yeast glucosidases, but may be revealed by exogenous fungal glucosidases. However, the volatile compounds thus revealed are not highly odouriferous.

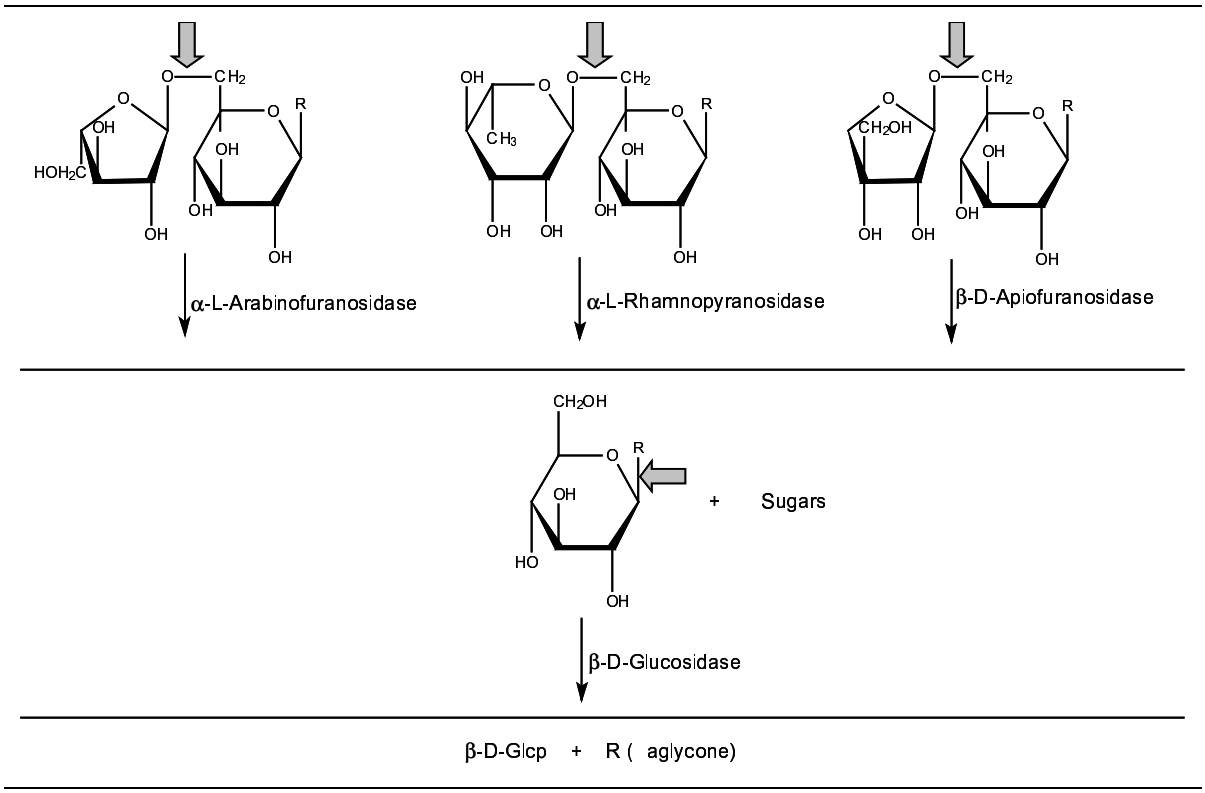


Figure I.2.4. Scheme of the hydrolysis of terpene glycosides; R- aglycone (based on Gunata *et al.*, 1990b); the arrows point out the glycosidic linkage where the enzymes act

I.2.3. Sensory properties

The effort developed in the last years, mainly through techniques like gas chromatography linked to olfactometry (GC-O), allowed relating many wine volatile compounds with their aroma descriptors (term used to described an aroma). This study has been made trying to relate the exhaled aroma of wines with the one perceived by the olfactory memory. Noble *et al.* (1987) proposed a wine aroma wheel that comprises the aromas exhibited by the wines (Figure I.2.5). This wheel is made of three tiers: it has very general terms located in the centre (*e.g.* fruity or chemical), going to the most specific terms in the outer tier (*e.g.* grapefruit or strawberries).

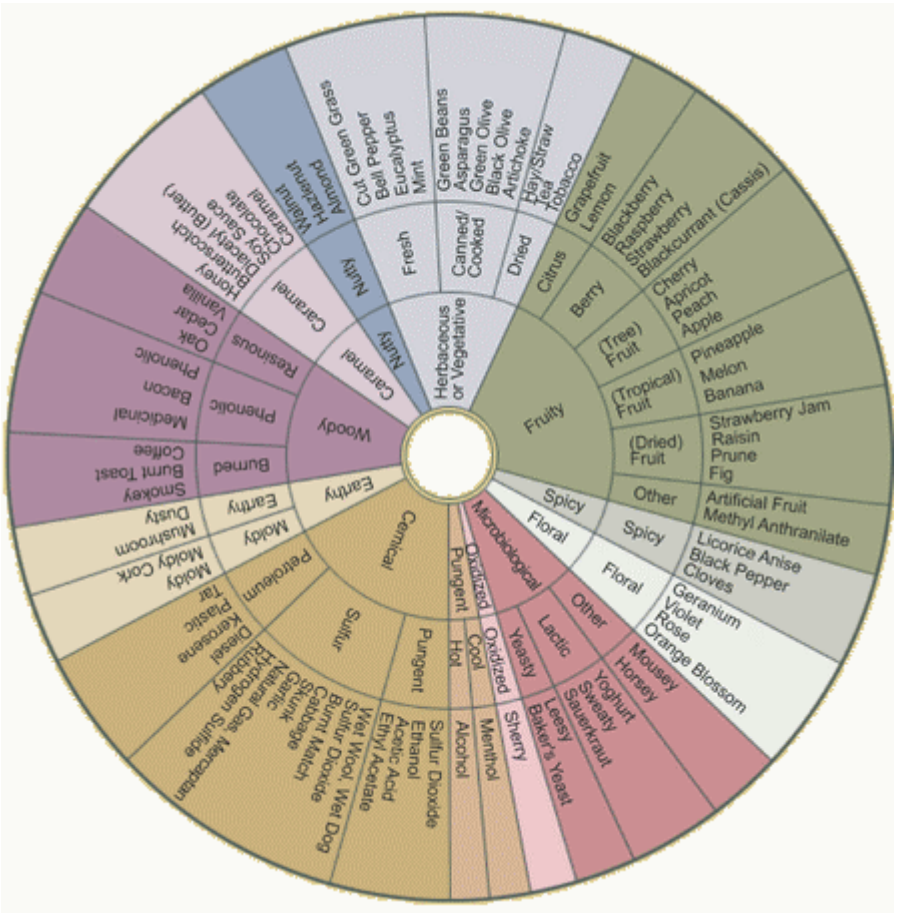


Figure I.2.5. Wine aroma wheel (based on Noble *et al.,* 1987)

It is important to know not only the aroma descriptor of each compound but it is also necessary to take into account the sensory perception limit (SPL) concept. If a certain volatile compound with a certain aroma descriptor was identified in a wine, it doesn't mean that it will contribute individually for the wine aroma. So, a synergistic effect between these two concepts, aroma and SPL (also known as odour threshold), may be considered. Hence, a compound contributes to the aroma if its concentration in wine is above its SPL. The SPL of a compound can be defined as the lowest concentration of a compound that is just enough for the recognition of its odour by, at least, 50% of the elements of a tasting panel (Boidron *et al.*, 1988). The threshold values of aroma compounds are dependent on their vapour pressure, which is affected by both temperature and medium (Belitz *et al.*, 2004); the same compound can have a different SPL in water, in ethanolic solution and in wine and it depends also on the presence of others compounds. The presence of certain compounds can, in some cases, make others less perceptible. The frequent discrepancies concerning the threshold values in the literature are basically due to such differences.

The individual contribution of a volatile compound to the aroma of a wine can be evaluated by the aroma index (*I*) that is the relation between the concentration of a compound in wine and its SPL (Belitz *et al.*, 2004), according to the following formula:

$$I = C_x / SPL_x$$

where: C_x is the concentration of a compound (x) in wine, and SPL_x is the sensory perception limit of a compound (x) in wine.

According to the aroma index value obtained for a compound in a matrix, it is possible to distinguish between active aroma compounds (compounds that contribute individually to the wine aroma) and inactive aroma compounds (compounds that do not contribute individually to the wine aroma). If $I>1$, the aroma compounds are known as active.

I.2.4. Main chemical groups

Several studies have identified a considerable number (600-800) of volatile and semi-volatile compounds in grapes and wines, representing several chemical groups, namely, terpenoids, aliphatic and aromatic alcohols, esters, aldehydes, ketones, acids and phenols (Slingsby *et al.*, 1980; McLellan and Race, 1993; Park and Noble, 1993; Sefton *et al.*, 1993; Zhou *et al.*, 1996, Shreier, 1997; Rapp, 1998; Kotseridis and Baumes, 2000). Generally, each chemical group is responsible for conferring characteristic aromas. In wines, the total concentration of volatile and semi-volatile compounds can achieve 0.8-1.3 g/L, which represents 1% of ethanol concentration.

I.2.4.1. Terpenoids

About 70 terpenic compounds have been identified: forty in grapes and thirty in wines (Ribéreau-Gayon *et al.*, 2000). Most of them have important contributions to the aroma of grapes and wines.

The 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 sesqui-terpenoids (C_{15}). The monoterpenoids, built with two isoprenic base units, can occur in both free and glycosidically-linked forms, as previously discussed (section I.2.2) and can be odourants. They occur in the form of hydrocarbons (limonene, myrcene, etc), aldehydes (linalal, neral, geranial, etc), alcohols (linalool, geraniol etc), acids (geranic acid)

and esters (linalyl acetate). From these, the most odourant monoterpene compounds are the alcohols, in particular linalool, α-terpineol, nerol, geraniol, citronellol and hotrienol, which may have a positive contribution to the wine aroma, mostly floral (Figure I.2.6). The aldehydes are more odouriferous but also more aggressive than the respective alcohols. The monoterpene alcohols can also be grouped in the monohydroxylated forms of the monoterpenes (monoterpenols) and polyhydroxylated forms of the monoterpenes (monoterpendiols and monoterpentriols) (Table I.2.1). The sesquiterpenoids (C_{15}), built with three isoprenic base units, such as farnesol and nerolidol, are other odourant molecules that have also been described in grapes and wines (Figure I.2.7) (Salinas *et al.*, 2004; Rocha *et al.*, 2006; Coelho *et al.*, 2006).

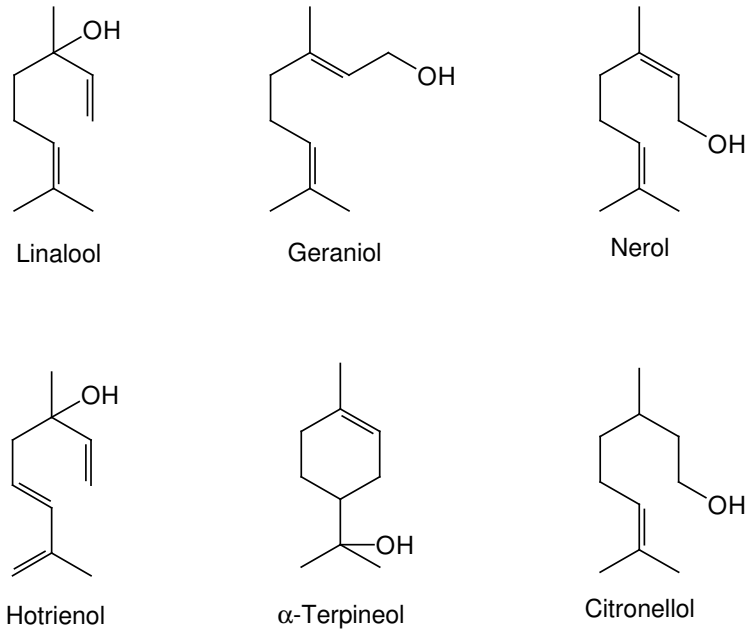
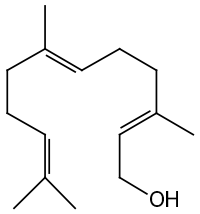


Figure I.2.6. Main monoterpenols identified in grapes and wines

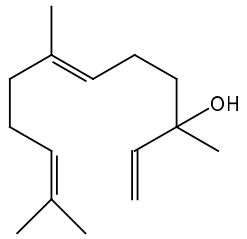
Monoterpenoids are the main group of compounds responsible for the varietal aroma of some wines, as they are associated with several varieties, such as *Muscat*, *Riesling*, etc. Gunata *et al.* (1985b) reported that, for *Muscat* variety, monoterpenoids represent about 40 to 50% of the quantified volatile compounds.

Table I.2.1. Chemical names of some monoterpene alcohols (Williams *et al.*, 1981)

Common name	Chemical name
Monoterpenols	
Linalool	3,7-dimethylocta-1,6-dien-3-ol
α-Terpineol	α,α,4-trimethyl-3-cyclohexene-1-methanol
Nerol	(*Z*)-3,7-dimethylocta-2,6-dien-1-ol
Geraniol	(*E*)- 3,7-dimethylocta-2,6-dien-1-ol
Hotrienol	3,7-dimethylocta-1,5,7-trien-3-ol
Monoterpendiols	
Terpendiol I	3,7-dimethylocta-1,5-dien-3,7-diol
Terpendiol II	3,7-dimethylocta-1,7-dien-3,6-diol
Endiol	3,7-dimethylocta-1-en-3,7-diol
Monoterpentriol	
Entriol	3,7-dimethyloct-1-en-3,6,7-triol



Farnesol



Nerolidol

Figure I.2.7. Sesquiterpenoids identified in grapes and wines

The terpenoid compounds present in wines arises mainly from the natural plant biosynthesis or from the microbial activity production (Ribéreau-Gayon *et al.*, 1998), related with sugars, fatty acids and amino acids metabolisms (Belitz *et al.*, 2004). They can also suffer modifications at the wine pH and later during the evolution of wine.

Modifications during winemaking

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. Besides linalool, geraniol and nerol, several 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 1.2.8 shows the linalool oxygenated products) (Luan *et al.*, 2004).

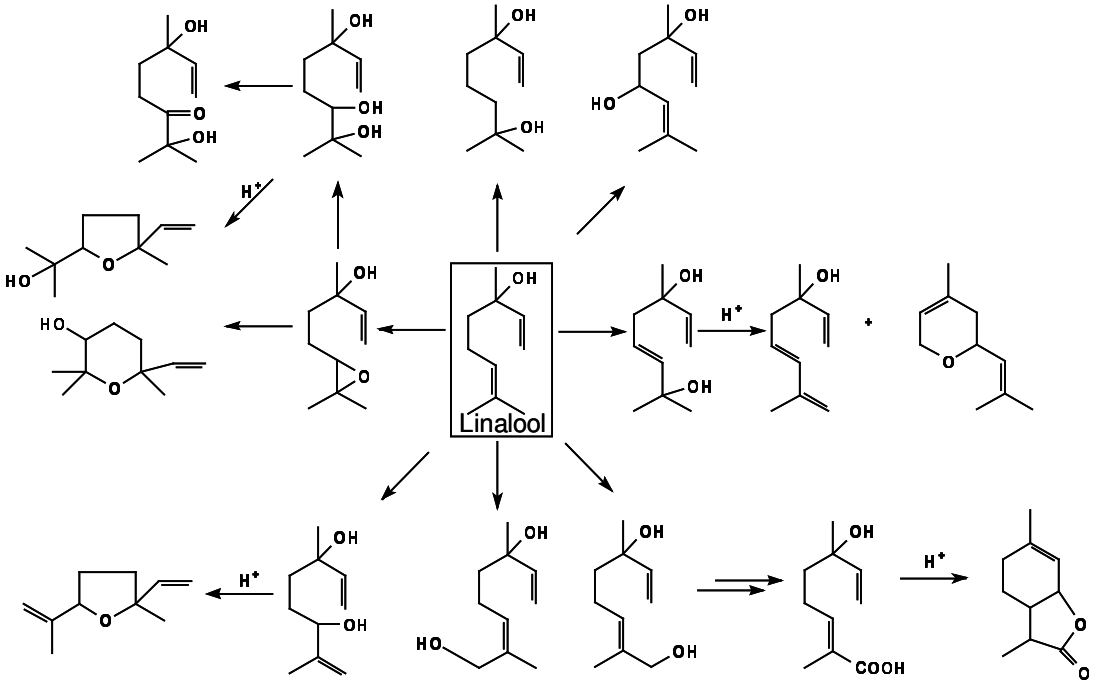


Figure I.2.8. Oxygenation reaction products of linalool in musts (Luan *et al.*, 2004)

Citronellol may be produced during fermentation from geraniol and nerol (Dugelay *et al.*, 1992). The non-odourant monoterpenic polyols (diols and triols) may originate other odourant monoterpenoids by dehydration reactions at the acidic pH of wine. For example, in acid medium, the 3,7-dimethylocta-1,5-dien-3,7-diol may produce hotrienol (Figure I.2.9).

During the white wine conservation and aging, modifications in the terpenoid fraction may occur. Linalool, for example, in an aqueous acid medium, can originate hydroxylinalool through hydration in the C7 position, α-terpineol by cyclization and geraniol and nerol by isomerization (Figure I.2.10) (Rapp, 1990; Bayonove *et al.*, 1998a).

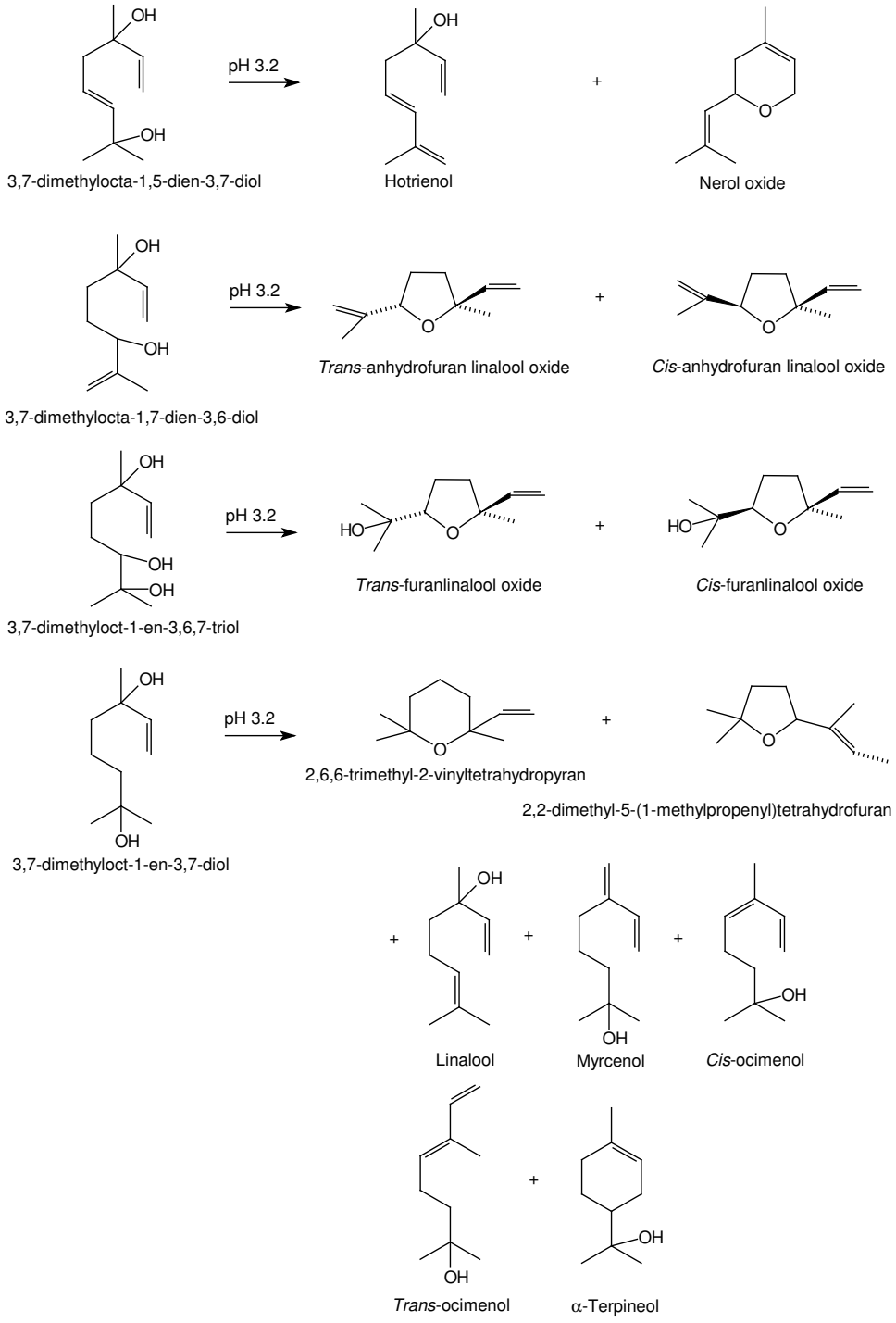


Figure I.2.9. Acid reactions of four terpenic polyols (Williams *et al.*, 1980)

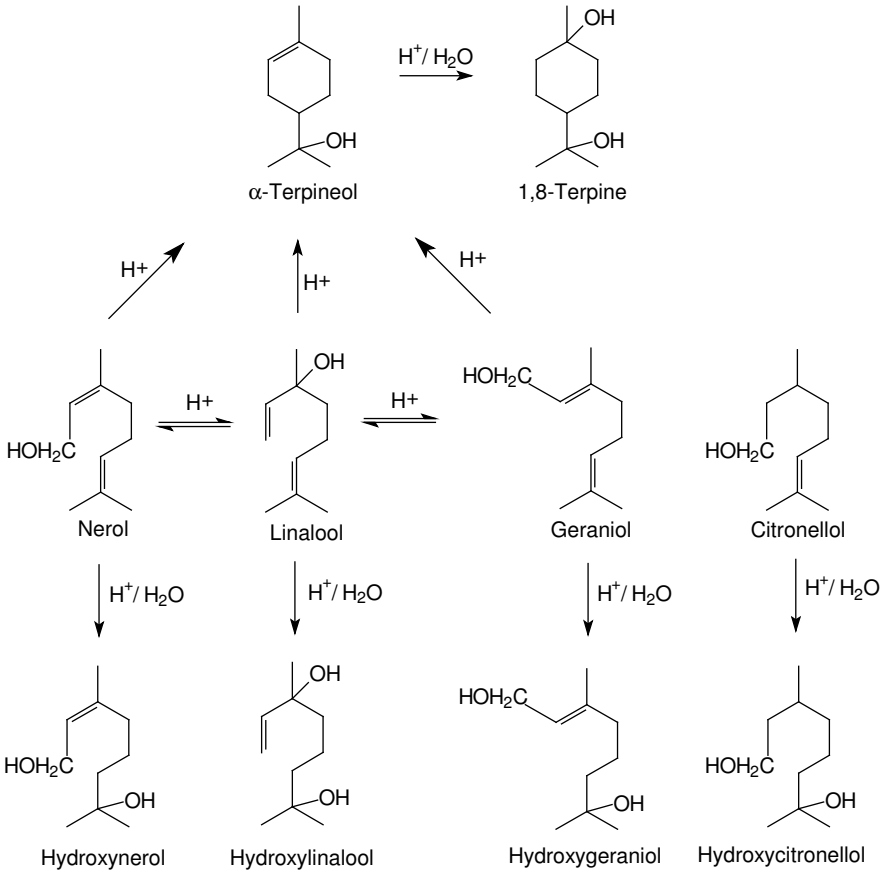


Figure I.2.10. Possible reactions of monoterpenols in acid medium during conservation and evolution of white wine (Rapp, 1990)

The sesquiterpenoids may arise directly from grape and/or have their origin due to rearrangement processes of some sesquiterpenoids during winemaking process and/or wine evolution. Few studies report the occurrence of sesquiterpenoids as components of grapes (Salinas *et al.*, 2004; Coelho *et al.*, 2006). These compounds have been related with medicinal plants with different health applications, mainly anti-inflamatory, antibacterial and antitumor activity (Rocha *et al.*, 2006). In particular, farnesol and nerolidol have been described to disrupt the normal barrier function of the bacterial cell membrane, allowing the permeation into the cell of exogenous solutes such as antibiotics, and exhibit antioxidant and insecticide activities. These compounds have a varietal origin but can also be metabolism products from yeasts originated during the alcoholic fermentation of the musts.

Sensory properties

As earlier mentioned, the terpenoids were found to be mainly associated to floral and fruity aromas (Table I.2.2). The sensory perception limits values are low, between 0.010 to 0.500 mg/L. The most odourants seem to be citronellol, linalool and nerol oxide. Sesquiterpenoids may contribute to woody, spicy, sweety, floral, clove, oily, musty and fresh like odours.

Table I.2.2. Sensory properties of monoterpenoids

Terpenic compounds	Aroma descriptor	SPL [a] (mg/L)
Linalool pyranic oxide	---	3-5 [c]
Linalool	Citrus-like, sweet, flowery (coriander, lavender, rose)	0.10 [b]
Nerol	Rose	0.4-0.5 [b]
α-terpineol	Flowery (lilac /lily), sweet	0.4-0.5 [b]
Hotrienol	Flowery (linden), sweet	0.11 [b]
Geraniol	Rose	0.13 [c]
Citronellol	Rose, lemon	0.018 [b]
Nerol oxide	Camphor	0.10 [c]
Limonene	Lemon, orange, sweet	---

[a]- SPL- sensory perception limits in wines; [b]- Ribéreau-Gayon *et al.*, 1998; [c]- Marais, 1983.

I.2.4.2. C_{13} norisoprenoids

The carotenoids, belonging to the terpenic family, are composed of 40 carbon atoms (tetraterpenes). Their oxidative degradation produces compounds with 9, 10, 11 or 13 carbon atoms (Figure I.2.11). In particular, the 13 carbon atoms derivatives are named C_{13} norisoprenoids. In grapes, either the carotenoids synthesis and/or their degradation, conducting to C_{13} norisoprenoids appearance, are promoted by sun exposure (Razungles and Bayonove, 1996).

This group of compounds may be divided into two main forms: the megastigmane and the non-megastigmane, both containing a large number of volatile compounds (Figure I.2.12) (Ribéreau-Gayon *et al.*, 2000). The first is characterized by a cyclohexadiene or a cyclohexene substituted on carbons 1, 5 and 6. The carbon 6 is attached to an unsaturated aliphatic chain with four carbon atoms. In general, it is possible to find C_{13}

norisoprenoids with an oxygenated skeleton on carbon 7 (damascone series) or on carbon 9 (ionone series). The non-megastigmane forms are considered all the other C_{13} norisoprenoids derivatives, including some odouriferous compounds. C_{13} norisoprenoids can be present in grapes and because of that they may contribute to the varietal aroma of wines.

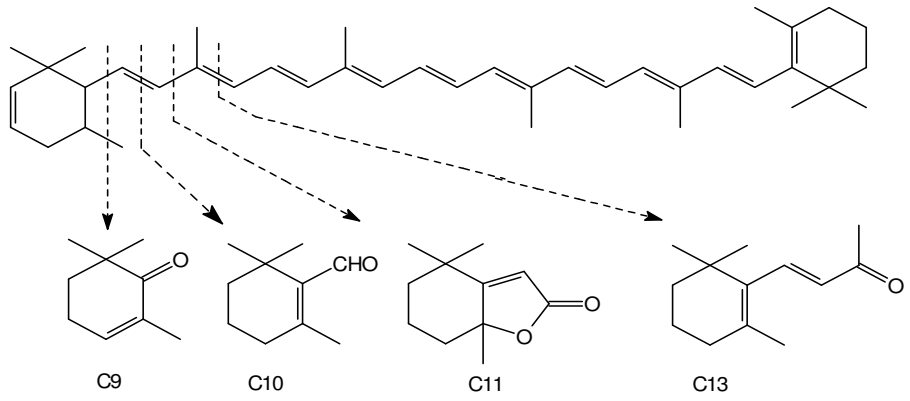


Figure I.2.11. Breakdown of carotenoids leading to the formation of C_9, C_{10}, C_{11} and C_{13} norisoprenoids

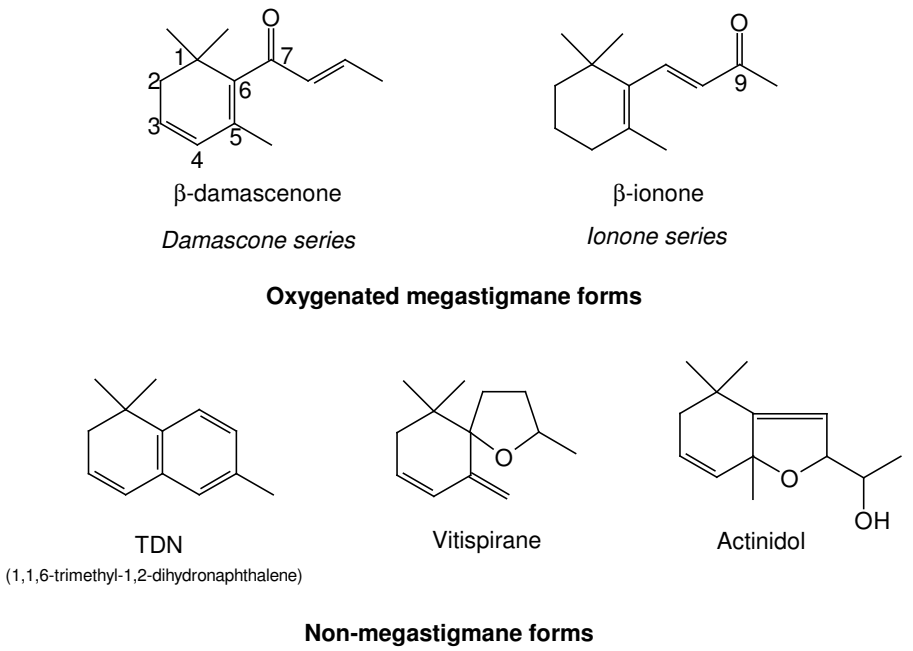


Figure I.2.12. Main families of C_{13} norisoprenoid derivatives in grapes (Ribéreau-Gayon *et al.*, 2000)

Modifications during winemaking

In grapes, the presence of C_{13} norisoprenoids in the free form is very small. However, their precursors are abundant, mainly the glycosidically-linked forms. The chemical or enzymatic hydrolysis of glucosides, that may occur during winemaking, lead to the formation of C_{13} norisoprenoids (Williams *et al.*, 1982b; Simpson and Miller, 1983; Strauss *et al.*, 1987; Skouroumounis *et al.*, 1994; Baumes *et al.*, 2002). In acid medium, chemical modifications can occur in the polyoxygenated C_{13} norisoprenoids (less odourant), conducing, for example, to β-damascenone (Figure I.2.13) (Winterhalter *et al.*, 1990). This compound can also be formed by acid-catalyzed conversion of polyols (diols or allene triols) derived from the enzymatic transformation of the carotenoid neoxanthin (Chevance *et al.*, 2002). The polyol precursors of β-damascenone often accumulate as glycosides in the skin, to be released later under acidic conditions.

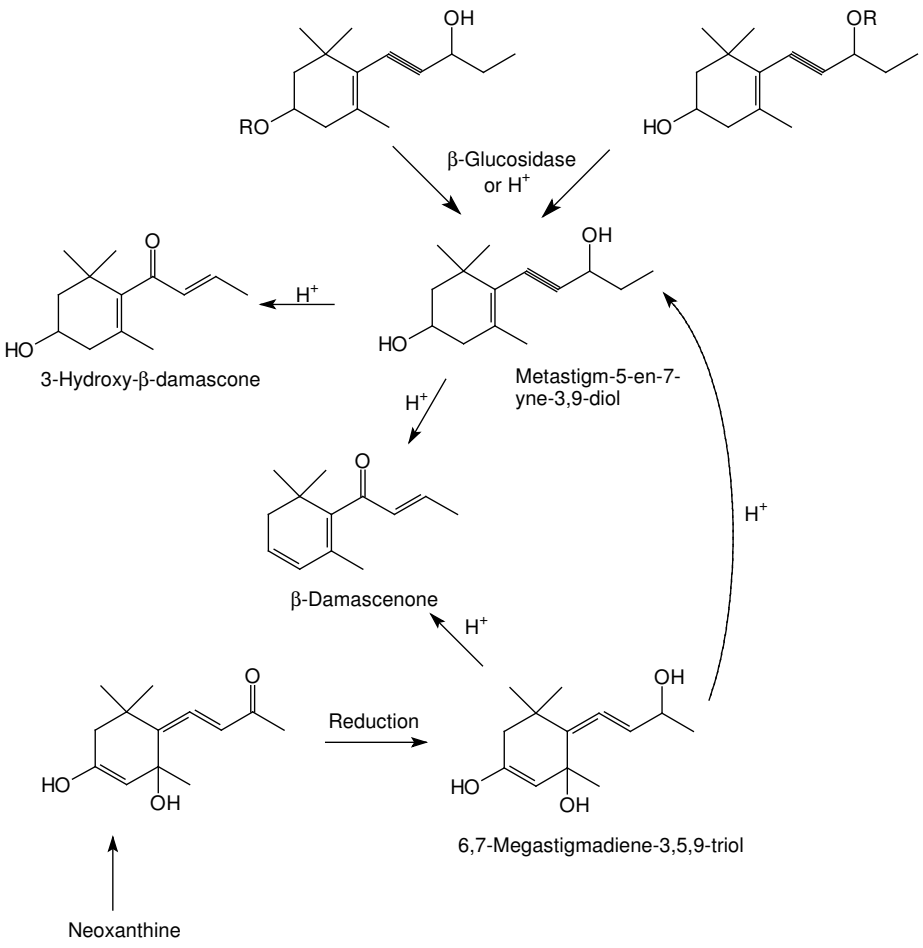


Figure I.2.13. β-damascenone formation in grapes and wines (R=β-D-Glc)

The TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) seems to be derived from the megastigmane forms by chemical modifications in acid medium. The 3,6-dihydroxy-7,8-dihydro-α-ionol (megastigm-4-ene-3,6,9-triol) has been described as vitispirane precursor (Winterhalter, 1992). The same product can lead to the formation of actinediols, when not reduced in lateral chain (Strauss *et al*., 1996). TDN, vitispirane and β-damascenone may also be formed during the evolution of wines by hydrolysis of precursors accumulated during physiologic maturation of the berries, with the sugar accumulation (Strauss *et al*., 1987). More recently, the identification of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene has been reported as a potent odourant in pH 3.2 hydrolysates of crude glycosidic extracts of grapes and identified in several white wines (Janusz *et al*., 2003). Apparently, this compound seems to belong to the C_{13} norisoprenoids chemical group.

Sensory properties

Table I.2.3 shows the main C_{13} norisoprenoids identified in wines, their aroma descriptors and respective sensory perception limits.

β-Damascenone has very low SPL, and can contribute individually to the wine aroma due to the reached concentrations of 89-1505 ng/L in white wines (Ribéreau-Gayon *et al*., 2000). β-Ionone has low SPL but reach concentrations of 0-59 ng/L in white wines. Thus, its contribution to the aroma of white wines is many times negligible. On the other hand, others have higher SPL's, such as 3-oxo-α-ionol, 3-hydroxy-β-damascone and damascene, and their olfactory impact in wines is many times negligible in spite of higher concentrations in some cases. TDN can reach concentrations of 200 μg/L in white wines and, considering its SPL, can have an individual contribution to the wine aroma.

Table I.2.3. Sensory properties of C_{13} norisoprenoids

C_{13} norisoprenoids	Aroma descriptors	SPL [a]
β-damascenone	Flowery, exotic fruit (cassis), baked apple jam	2 ng/L in water [b, c] 45 ng/L hydro alcoholic solution[e]
TDN	Kerosene/petrol	20 μg/L [d]
Vitispirane	Camphor/eucalyptus	800 μg/L in water[b, d, f]
β-ionone	Violet	120 ng/L in water; 800 ng/L hydro alcoholic solution; 1.5 μg/L in wine [e]
3-oxo-α-ionol	Tobacco	---
3-hydroxy-β-damascone	Tea, tobacco	---
Damascene	Tobacco, fruity	---
(*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene	floral/ geranium/ tobacco	40 ng/L in wine [g]

[a]- SPL- sensory perception limits; [b]- Belitz *et al.*, 2004; [c]- Winterhalter *el al.*, 1990; [d]- Strauss *el al.*, 1987; [e]- Ribéreau-Gayon *et al.*, 1998; [f]- Rapp and Pretorius 1990; [g]- Janusz *et al.*, 2003 (aroma descriptor at low concentrations, below 270 ng/L). β-damascenone is also known as 8*E*-megastigma-3,5,8-trien-7-one.

I.2.4.3. Esters

Esters are abundant in wines as a result of the fermentation process and are considered the main chemical group of the volatile fraction of wine. By definition, an ester results from the reaction between an alcohol and an acid with the elimination of a water molecule (Figure I.2.14). This reaction is slow and reversible in aqueous medium.

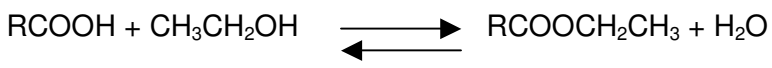


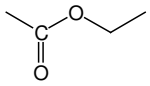
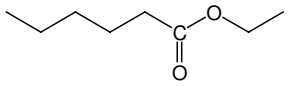
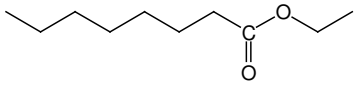
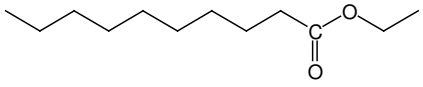
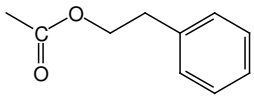
Figure I.2.14- Esterification reaction between ethanol and a carboxylic acid

A considerable number of different alcohols and acids are present in wine, so the number of esters that can be formed is very large. Since the primary alcohols are the most reactive and due to the presence of ethanol, the ethyl esters are the most abundant ones.

The esters play an important role on the aroma composition of wine because usually the larger the amount of esters present the more accentuated the aroma will be (Dubois,

1994a; Ribéreau-Gayon *et al.*, 2000). Table I.2.4 represents some of the esters identified in wines and ranges of their concentrations in white wines.

Table I.2.4. Some esters identified in wines

Structure formula	Ester name	Concentration in white wines (mg/L) [a]
	Ethyl acetate	0.15-150
	Ethyl hexanoate	0.03-1.3
	Ethyl octanoate	0.05-2.3
	Ethyl decanoate	0-2.1
	2-Phenylethyl acetate	0-18.5

[a]- Etiévant, 1991 and Belitz *at al.*, 2004

Formation and modifications during winemaking

Three different origins are known for esters. They can be originated from grapes (in a very small extension); from the yeast metabolic activity, during the fermentative process by enzymatic esterification between free alcohols and the active form of carboxylic acids, the acyl-S-CoA (Dubois, 1994a); and from chemical esterification between ethanol and a considerable number of different acids in wine, during wine long time storage (Soles *et al.*, 1982). The same esters can be synthesized by both ways, enzymatic and chemical esterification.

The ethyl acetate has been recognized as the most important and more abundant ester in wine. Its origin seems to be related to yeast metabolic activity during fermentation,

where it is formed. As other esters, the ethyl acetate exists in an equilibrium balance of ethanol and acetic acid, in aqueous medium.

The ethyl esters of fatty acids, mainly ethyl caproate (ethyl hexanoate) and ethyl caprylate (ethyl octanoate), are produced by yeasts during the alcoholic fermentation (Ribéreau-Gayon *et al*., 1998). Acetic esters from higher alcohols (3-methyl-1-butyl acetate, also known as isoamyl acetate, 2-phenylethyl acetate, etc) are equally produced during the fermentation process.

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 leads to a decreased/difficulty in the esters formation (Killian and Ough, 1979).

Sensory properties

The esters are usually associated to the fermentative aroma of wines, conferring floral and fruity aromas. In this group, the esters of aliphatic monocarboxylic acids exhibit high volatility and therefore may have a great contribution to the wine aroma (Table I.2.5). On the other hand, the esters of hydroxyl and oxo acids present lower volatility. The esters of polycarboxylic acids present lower volatility and may not have any contribution to wine aroma.

The aroma conferred by esters is related with the length of the carbon chain. Therefore, the esters with a short chain have generally pleasant and fruity aromas. With the increase of the molecular weight the fruity characteristics are lost and a soap and rancid odour arises. The ethyl esters of fatty acids, present in a total concentration of a few mg/L, are responsible for very pleasant odours and participate on the aroma character of white wines.

Ribéreau-Gayon *et al*. (1998) report that the SPL of ethyl acetate in wine is 160 mg/L. Below this value it may have a contribution to the global wine aroma, by masking the bouquet of wine, conferring a spicy unpleasant note. Even in small doses (50 to 80 mg/L), ethyl acetate contributes to the global wine aroma.

Acetic esters of higher alcohols (3-methyl-1-butyl acetate, also known as isoamyl acetate, 2-phenylethyl acetate, etc) contribute with intense odours to the global wine aroma but may mask some varietal aromas of certain varieties.

Table I.2.5. Sensory properties of esters

Esters	Aroma descriptor	SPL [a] (mg/L)
3-Methyl-1-butyl acetate (Isoamyl acetate)	Banana	0.23 in hydro alcoholic solution [b]
Ethyl hexanoate	Fruit, apple, banana, brandy	0.014 in hydro alcoholic solution [c]
		0.23 in beer [b]
		0.08 in wine [e]
Ethyl octanoate	Ripe fruits, pear, sweet, banana, pineapple	0.005 in hydro alcoholic solution [c]
		0.9 in beer [b]
		0.58 in wine [e]
Ethyl decanoate	Sweet, fruit, dry fruit, grape, brandy	0.2 in hydro alcoholic solution [c]
		1.5 in beer [b]
		0.51 in wine [e]
Hexyl acetate	Apple, pear, cherry, floral	0.67-2.4 in wine [e]
		3.5 in beer [b]
2-Phenylethyl acetate	Honey, rose	0.70 in hydro alcoholic solution; 3.8 in beer [b]
		1.8 in wine [e]
Ethyl acetate	Fruity, pineapple, spicy note	40 in hydro alcoholic solution [d]
		12.3 in wine [e]
Ethyl lactate	Butter, sour milk	150 in wine [e]

[a]- SPL- sensory perception limits; [b]- Soles *et al.*, 1982; [c]- Ferreira *et al.*, 2000; [d]- Ribéreau-Gayon *et al.*, 1998; [e]-Dubois, 1994a and Belitz *et al.*, 2004

I.2.4.4. Alcohols

Some alcohols can appear in grapes, where they can be biosynthetized (C_6 alcohols, aromatic alcohols), others are formed during pre-fermentation operations (C_6 alcohols) and others are formed during fermentation (ethanol, 2,3-butanediol, 2-phenylethanol, etc).

Formation and modifications during winemaking

The C_6 alcohols (and/or C_6 aldehydes) are mainly formed during pre-fermentative operations (crushing, pressing and skin contact). The mechanical operations promote the enzymes contact with linoleic and linolenic acids, *i.e.* C_6 alcohols (1-hexanol, *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol) formation is due to the oxidation of linolenic and linoleic acids, by the action of two enzymes, alcohol dehydrogenase (ADH) and lipooxygenase, and by air contact with the musts (Joslin and Ough, 1978, Cayrel *et al.*, 1983, Cordonnier, 1989)

(Figure I.2.15). Recently, Oliveira *et al.* (2006) have shown the importance of these compounds as varietal markers for assessment of wine origin. From C_6 alcohols, hexanol is the most abundant. During fermentation, changes in the C_6 alcohols fraction can also occur (Herraiz *et al.*, 1990). The presence of 1-hexanol in wines arises from the 1-hexanol present in must as well as from reduction of hexanal, *trans*-2-hexenal, *cis*-2-hexen-1-ol and *cis*-2-hexen-1-ol. The *cis*-3-hexen-1-ol and *trans*-3-hexen-1-ol seem to be not metabolized by *S. cerevisiae*.

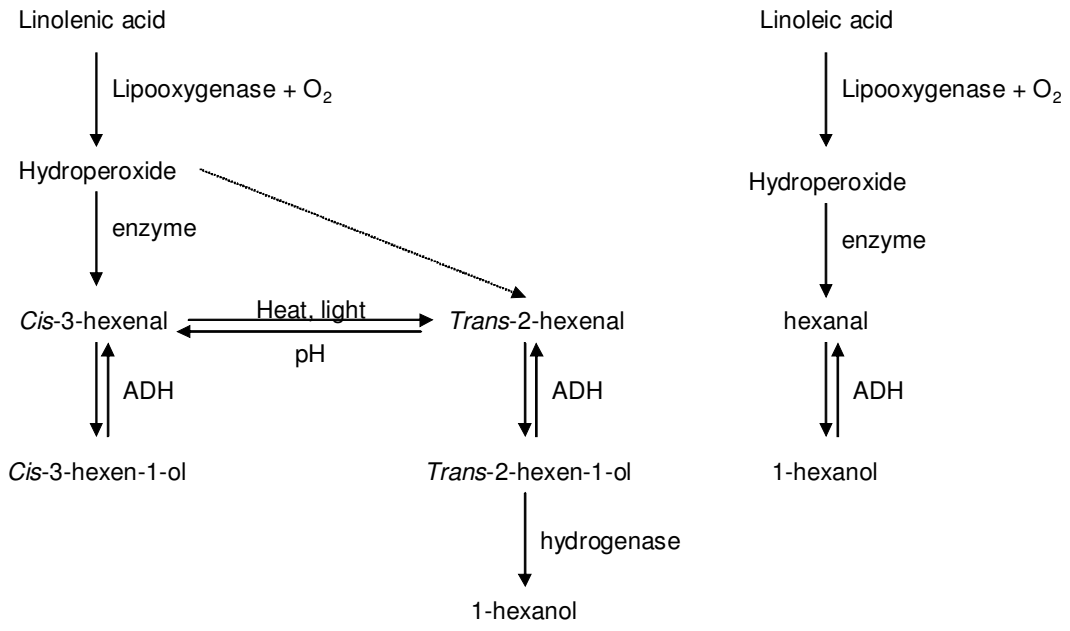


Figure I.2.15. Formation of C_6 alcohols, as proposed by Joslin and Ough (1978)

During fermentation, ethanol and glycerol are the main alcohols formed. Other alcohols, such as 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol are formed in small amounts.

- Ethanol

Besides water, ethanol is the most abundant component of wine. It is produced during the alcoholic fermentation from the sugars present in the must (Ribéreau-Gayon *et al.*, 2000). This compound is responsible by the alcoholic degree (alcohol volume in wine, expressed in percentage) and affects the organoleptic characteristics of the wine even though it is not considered an aroma compound (Dubois, 1994a). However, it is sensory

perceived due to their presence over the SPL. Ethanol plays also an important role on solubilization of compounds, not only for phenolic compounds after winemaking, but also for some aroma compounds, contributing to the expression of global aroma in wine. Studies demonstrate the decrease on the amount of many important volatile compounds when a dealcoholization of wine was made (Gómez-Plaza *et al.*, 1999).

The ethanol, due to its alcohol function, can participate in some reactions, such as esterification reactions with tartaric acid. It can equally react with aldehydes, especially ethanal, producing the diethoxyethane.

- Higher alcohols

The higher alcohols are known as having more than two carbon atoms with just one alcohol function. These alcohols have been found in wines in concentrations between 150 to 550 mg/L. In spite of being present in lower amounts then ethanol, the higher alcohols can play an important role in wine aroma.

The higher alcohols present in wines are formed during fermentation, resulting mainly from the metabolic activity of yeasts. The main higher alcohols from fermentative origin are 2-methyl-1-propanol (isobutyl alcohol), 2-methyl-1-butanol (amyl alcohol) and 3-methyl-1-butanol (isoamyl alcohol). These alcohols have been found in wines in concentrations higher to 50 mg/L (Ribéreau-Gayon *et al.*, 2000). Other compounds, such as 1-propanol, 1-butanol, 1-hexanol, 3-methyl-2-butanol, 2-decanol and 2-butanol have been found in concentrations between 1 and 50 mg/L (Mauricio *et al.*, 1997).

Higher alcohols, during fermentation, are formed by two pathways:

- From some amino acids by Erhlich reaction (catabolic pathway). In this process, the alcohols are formed by amino acids degradation following mechanisms like deamination, decarboxylation and reduction (Figure I.2.16). By this pathway, leucine leads to the formation of 3-methyl-1-butanol and isoleucine to 2-methyl-1-butanol, for example.

- From sugars metabolism by piruvate pathway, having the ketonic acids as intermediates (anabolic pathway).

According to Bayonove *et al.* (1998b), each one of these two pathways represents 25 and 75%, respectively. But, it seems that for alcohols with more carbon atoms, the Erhlich

reaction is preferential. These values vary, from must to must, with the amounts of nitrogen and sugars. In both pathways oxoacids (α-oxobutyric acid, α-oxo-β-methylvaleric, α-oxoisovaleric and α-oxoisocaproic) can be found as precursors of 1-propanol, 2-methyl-1-butanol, 2-methyl-1-propanol, and 3-methyl-1-butanol.

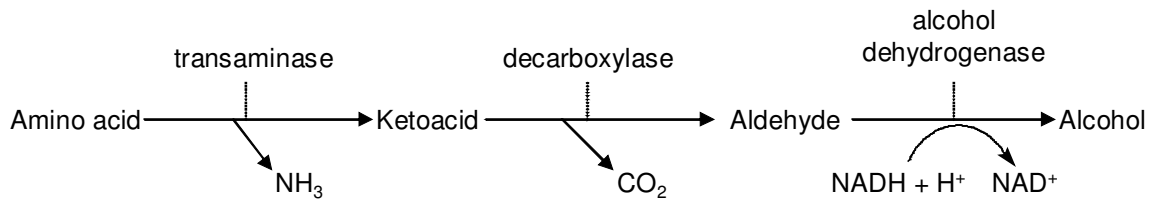
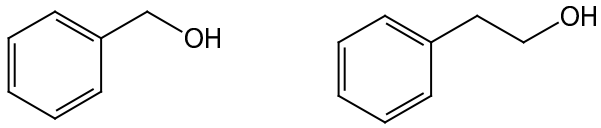


Figure I.2.16. Simplified scheme of Ehrlich reaction (Ribéreau-Gayon *et al.*, 1998)

The content of higher alcohols formed during fermentation varies according to the fermentation conditions, especially the type of yeast. In general, yeast biomass, oxygenation, high temperature and presence of suspension material that promotes fermentation increase the formation of higher alcohols (Ribéreau-Gayon *et al.*, 2000). The higher alcohol content in wine may increase also due to the microbial spoilage involving yeast or bacteria.

- Aromatic alcohols

The 2-phenylethanol and benzyl alcohol are the aromatic alcohols found in wines (Figure I.2.16). These compounds are responsible for important sensory marks. In grapes, they can appear in small amounts. They can be present in the glycosidically-linked form, being released during winemaking (section I.2.2.2). During fermentation, the 2-phenylethanol is formed in considerable amounts, being one of the main flavour compounds involved in the wine aroma. It can be formed from phenylalanine via Ehrlich pathway (Fabre *et al.*, 1998; Hernández-Orte *et al.*, 2002; Etschmann *et al.*, 2002) (Figure I.2.17). Its production depends on the healthy state of grapes and on the type of yeasts used. So, the amounts produced of this alcohol are higher when the grapes are infected.



Benzyl alcohol 2-Phenylethanol

Figure I.2.17. Aromatic alcohols identified in wines

- *Polyalcohols*

Polyalcohols are characterized by the presence of two or more hydroxyl groups in the same molecule. In general, the increase of the -OH groups in the molecule leads to a considerable increase of the boiling point and viscosity due to the increasing number of hydrogen bonds that can be formed. Similar increases are observed in solubility and sweetness. Glycerol and 2,3-butanediol are considered the polyalcohols more abundant in wines. Both, 1,2-propanediol and 2,3-pentanediols, are also abundant and are formed by yeast intervention (Bayonove *et al.*, 1998b).

The 2,3-butanediol can appear in three forms; D-(-)-2,3-butanediol or (*2R, 3R*)-butanediol, *meso*-2,3-butanediol or (*R,S*)-2,3- butanediol and L-(+)-2,3-butanediol or (*2S,3S*)-butanediol (Herold *et al.*, 1995). The D and L forms are optically active and the *meso* is optically inactive. The D-(-)-2,3-butanediol and *meso*-butanediol are secondary products from yeast metabolism during alcoholic fermentation.

The 2,3-butanediol has little odour and its flavour is slightly sweet and bitter at the same time, but does not have much organoleptic impact in wine (Webb *et al.*, 1967; Radler and Zorg, 1986; Ribéreau-Gayon *et al.*, 2000). Its importance comes from the oxidation-reduction equilibrium that is established with acetoin and diacelyl (Figure I.2.18). It is produced by the reduction of acetoin, which is obtained by the condensation of two ethanal molecules (diacetyl).

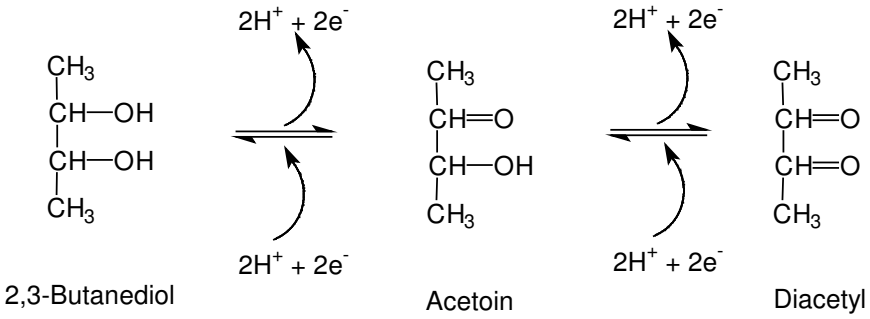


Figure I.2.18. Oxidation-reduction balance of 2,3-butanediol (Ribéreau-Gayon *et al.*, 2000)

Sensory properties

The sensory properties of alcohols are related with the chemical structure of compounds and can be separated in two groups, the C_6 aliphatic alcohols and the aromatic alcohols (Table I.2.6). The first are responsible for herbaceous odours and the second are responsible for pleasant and floral aromas. Although the C_6 alcohols are related with the negative aspects of wine aromas, certain herbaceous notes are appreciated by some consumers (Dubois, 1994b).

At concentration less than 300 mg/L, the higher alcohols can participate in the global wine aroma (I<1) or can contribute individually to the wine aroma (*I*>1). However, at higher concentrations, they can mask other aromas responsible for the sensory properties of the wine.

Table I.2.6. Sensory properties of alcohols

Alcohols	Aroma descriptor	SPL (mg/L) [a]
3-Methyl-1-butanol (isoamyl alcohol)	Alcohol, banana, sweet	70 in beer; 40-60 in wine [b, d]
1-Hexanol	Herbaceous, greasy	4 in beer; 4.8 in water [b], 8.0 in wine [d]
trans-2-Hexen-1-ol	Herbaceous, greasy	15 in beer; 6.7 in water [b]
cis-3-Hexen-1-ol	Herbaceous, greasy	13 in beer; 0.07 in water [b], 0.4 in wine [d]
Benzyl alcohol	Sweet, flower	200 in wine [d]
2-Phenylethanol	Flower, rose, honey	10 in ethanolic solution [c], 14 in wine [d]

[a]- SPL- sensory perception limits; [b]- Marais, 1983; Dubois, 1994b; [c]- Guth, 1997a; [d]-Aznar *et al.*, 2003

I.2.4.5. Aliphatic acids

The acids identified in wines can appear in grapes and can also be formed by metabolic activity of yeasts, during fermentation (Dubois, 1994a).

Formation during winemaking

The acetic acid is the major acid in wines and it is usually present in concentrations rounding 250 mg/L, superior to its SPL (Ribéreau-Gayon *et al.*, 2000). It is the essential constituent of the volatile acidity and its amount (limited by legislation – 200-700 mg/L) indicates the intervention or not of bacterial activity and the consequent spoilage of the wine.

The acids formation is closely related with the higher alcohols biosynthesis (section I.2.4.4). Propanoic, 2-methyl-1-propanoic (isobutyric), 2-methyl-1-butanoic, 3-methyl-1-butanoic (isovaleric) and 2-phenylacetic acids are formed by α-ketoacids (deamination of amino acids followed by the decarboxylation of the homolog oxoacids). Isobutyric and isopentenoic acids come from valine and leucine degradation, respectively. Other compounds, such as butanoic (butyric), hexanoic (caproic), octanoic (caprilic) and decanoic (capric) acids are formed by oxidation of fatty acids. Table I.2.7 indicates the most common acids identified in wines.

Table I.2.7. Most common volatile acids identified in wines

Formula	Acid	Concentration in white wines (mg/L)[a]
CH_3-COOH	Acetic	4.7-400
CH_3-CH_2-COOH	Propanoic	0.1-1.7
CH_3-CH_2-CH_2-COOH	Butanoic	0.04-1.1
CH_3 | CH_3-CH-COOH	2-Methyl-1-propanoic	0.01-2.1
CH_3 | CH_3-CH-CH_2-COOH	3-Methyl-1-butanoic	0.1-1.6
$CH_3CH_2CH_2CH_2CH_2COOH$	Hexanoic	0.02-5.7
$CH_3CH_2CH_2CH_2CH_2CH_2CH_2COOH$	Octanoic	0.07-20.9
$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$	Decanoic	0-4.5

[a] Etiévant, 1991

The C_3 acids (propionic) and C_4 acids (butyric) are also associated with the bacterial spoilage. The C_6, C_8 and C_{10} are formed by yeast and are considered inhibitors of the fermentation at concentration of only a few mg/L.

Sensory properties

In wines, some acids have pleasant aromas and others have unpleasant ones (Table I.2.8). These differences are due to the length of the carbonated chain. Thus, the fatty acids of short chains (C_2 to C_5) seem to be related with unpleasant notes, such as acetic acid (vinegar), propanoic acid (goat) and butanoic acid (rancid butter). The fatty acids with more than 5 carbon atoms are related with pleasant aromas, such as hexanoic acid (sweet aroma), octanoic acid (sweet fruit aroma) and decanoic acid (sweet fruit aroma, vanillin). Acetic acid is usually present over its SPL (Dubois, 1994a).

Table I.2.8. Sensory properties of acids

Acid	Aroma descriptors	SPL [a] (mg/L)
Acetic	Vinegar	200 in hydro alcoholic solution [b]
Propanoic	Goat, rancid	8.1 hydro alcoholic solution [b]
Butanoic	Cheese, rancid, sweet	2.2 in water [c];
		10 in hydro alcoholic solution [b]
2-methyl-1-propanoic (isobutyric)	Rancid butter	200 in hydro alcoholic solution [b]
Hexanoic (caproic)	Cheese, earthy, sweet, rancid	8 in water [c]
		3 in hydro alcoholic solution [b]
		8 in wine [d]
Octanoic (caprylic)	Sweet fruit, rancid	13 in water [c]
		10 in wine [d]
Decanoic (capric)	Sweet, sickening, vanillin, rancid, citric	10 in water [c]
		15 in hydro alcoholic solution [b]
		6 in wine [d]

[a]- SPL- sensory perception limit; [b]- Guth, 1997a; [c]- Dubois, 1994a; [d]- Etiévant, 1991

I.2.4.6. Lactones

The lactones are present in several foods as γ- and δ-lactones. The γ- and δ-lactones result from the cyclization of the hydroxy acids in the 4 or 5 position, respectively (Figure I.2.19). In wines, the γ-lactones are more frequent and can be grouped in: γ-butyrolactones (substituted or not in the 4 position) with alkoxy groups, acetyl and 1-hydroxyethyl groups, and ethoxycarbonyl group; and in 4-acyl-γ-lactones (from γ-C_5 to C_{10} and C_{12}), 3-hydroxy-4,4-dimethyl-2(3H)-furanone (pantolactone) and 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon). However, it is possible to found some δ-lactones in wines (δ-octalactone, δ-nonalactone, δ-decalactone) (Dubois, 1994b).

γ-lactone δ-lactone

Figure I.2.19. General structure of γ- and δ-lactones (R-lateral chain)

As other chemical groups, lactones can appear in grapes or may be formed during winemaking or during the evolution and wine aging.

In grapes, lactones are less representative but can be identified in some cases. For example, some lactones were found in *Riesling* variety where they have a decisive contribute to the varietal aroma.

Formation during winemaking

Figure I.2.20 shows some lactones that have been identified in wines. Inside this group of compounds, several are volatile compounds formed during the fermentative process and may contribute to the wine aroma (Ribéreau-Gayon *et al.*, 2000).

The most well known lactone is the γ-butyrolactone, which results from the lactonization of the γ-hydroxybutyric acid, an unstable molecule produced by deamination and decarboxylation of glutamic acid, according to the Ehrlich reaction (Figure I.2.21). The γ-butyrolactone, 4-ethoxycarbonyl-γ-butyrolactone and 4-ethoxy-γ-butyrolactone are the

most abundant lactones in wines. The first is found in concentrations around 1 mg/L and the others from a few to some tens of μg/L (Bayonove *et al.*, 1998b).

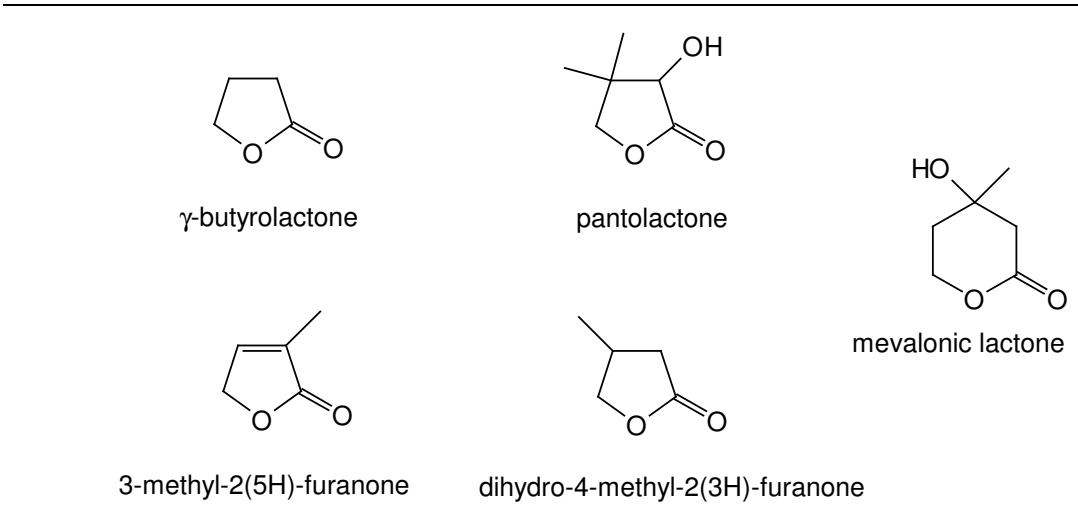


Figure I.2.20. Lactones identified in wines

Lactones are usually produced by enzymatic activity however it is possible their chemical appearance due to the wine and must pH (3.5). The acid medium will give preferentially γ-lactones (Muller *et al.*, 1973). The γ-lactones are formed from 4-oxoacids that come from the carbonated chain along of the fatty acids synthesis or from the deamination of amino acids during the fermentation process.

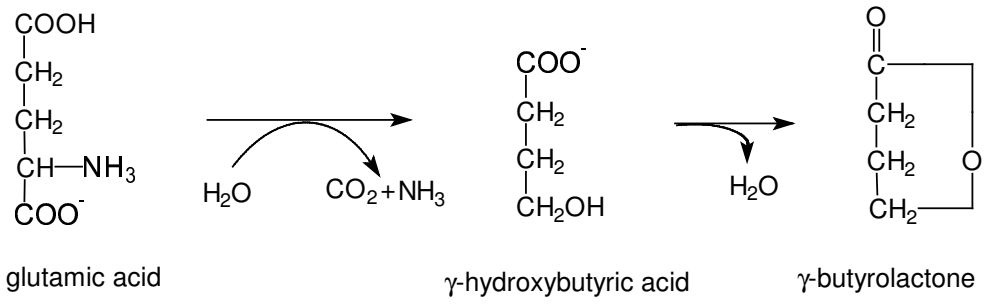


Figure I.2.21. Formation of γ-butyrolactone (Ribéreau-Gayon *et al.*, 2000)

Other compounds, such as solerone (5-oxo-4-hexanolide) (Häring *et al.*, 1997), 3a,4,5,7a-tetrahydro-3,6-dimethylbenzofuran-2(3H)-one (known as wine lactone) an acid-catalyzed cyclization of a linalool derivative (Guth, 1997b; Bonnlander *et al.*, 1998), and

γ-octalactone, γ-undecalactone and γ-dodecalactone (Ferreira *et al.*, 2004) has been also identified in wines.

Several mechanisms were proposed for the formation of some volatile lactones, such as sotolon (Cutzach *et al.*, 1998, Cutzach *et al.*, 2000, Pham *et al.,* 1995, Câmara *et al.*, 2004), ethoxybutirolactone and pantolactone (Cutzach *et al.*, 1999). Figure I.2.22 shows the scheme for the possible formation of pantolactone. These lactones can also be produced during storage and aging of wine.

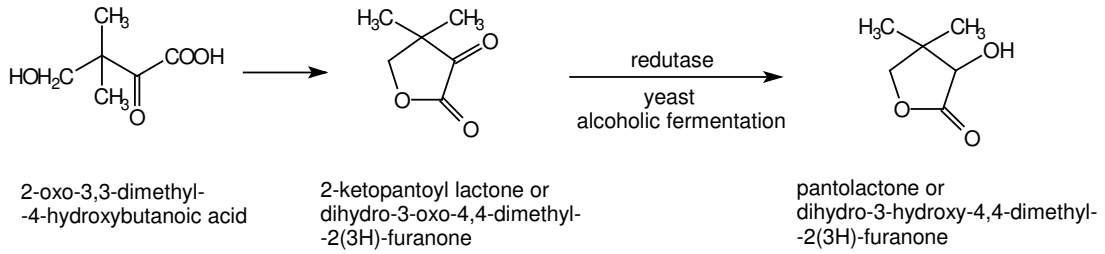


Figure I.2.22. Possible formation pathway of pantolactone proposed by Cutzach *et al.* (1999)

Sensory properties

Lactones are usually responsible for pleasant and fruity aromas. Despite their low SPL, these values vary with the ring type (γ- or δ-), length of lateral chain, the presence of double bounds and presence of ring or chain side groups. The γ-lactones have generally an SPL lower than δ-lactones and the SPL values tend to be lower with the increase of the length of the chain (Dufossé *et al.*, 1994) (Table I.2.9).

The γ-butyrolactone is usually present in wine in a concentration of some mg/L. However, the odour transmitted is slightly intense and seems not to play an important role on the sensory characteristics of wines.

According to Cutzach *et al.* (1998) sotolon is mainly responsible for the dry plum aroma in wines, when *I*<30) and is responsible for the dried fig or burnt sugar, when *I* > 30 and I < 60.

Table I.2.9. Sensory properties of lactones

Lactones	Aroma descriptor	SPL (mg/L) [a]
γ-Butyrolactone	Sweety, buttery, acre, fetid, rubber	50 in wine [f]
Pantolactone	---	1.60 in water [b]
4-Ethoxycarbonyl-γ-butyrolactone	Red fruits, cherry	0.40 in water [b]
4-(1-Hydroxyethyl)-γ-butyrolactone	Red fruits	1.60 in water [b]
5-Oxo-γ-hexalactone	Alcoholic	1.60 in water[b]
β-Methyl-γ-octalactone	Wood, dry coconut	0.02 in water; 0.120-0.125 in wines [c]
γ-Nonalactone	Coconut, fruity, almond-like	0.065 in water [b]
Solerone	Bottle	1.60 in water[b]
Dihydrosolerone	Sweet	1.60 in water[b]
Sotolon	Dry fruits, nuts, toasty, rancid ('nutty' at low concentrations and 'curry' at higher levels)	0.010 in wine [d,e]

[a]- SPL- sensory perception limit ; [b]- Dufossé *et al.*, 1994 ; [c]- Boidron *et al.*, 1988 ; [d]- Cutzach *et al.*, 1998, [e]- Pham *et al.*, 1995, [f]- Aznar *et al.*, 2003.

The two 3-methyl-γ-octalactone isomers have coconut odour in the pure state. However, when diluted, their aromas resemble the wines aged in wood. Their concentrations in wines are some tens of mg/L and their SPL is a few tens of μg/L, therefore they are usually over their SPL (Ribéreau-Gayon *et al.*, 1998).

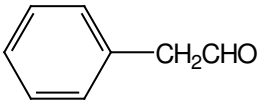
The wine lactone (3a,4,5,7a-tetrahydro-3,6-dimethylbenzofuran-2(3H)-one) exhibit an intense coconut, woody and sweet odour (Guth, 1997b).

I.2.4.7. Carbonylated compounds: aldehydes and ketones

Aldehydes and ketones are compounds also named carbonylic compounds due to the presence, in both groups, of a carbonyl group (C=O). The main compounds and those that can have an influence on the aroma of wines are the ethanal (acetaldehyde), 3-hydroxy-2-butanone (acetoin or acethylmethyl carbinol), 2,3-butanedione (diacethyl), 3-hydroxy-2-pentanone, 2,3-pentanedione and phenylethanal (Bayonove *et al.*, 1998b).

Table I.2.10 shows some carbonylic compounds identified in wines. These compounds can be formed by yeast, from the decarboxylation of α-ketoacids (Ribéreau-Gayon et al., 2000).

Table I.2.10. Aldehydes and ketones identified in wine

Formula	Compound name
CH_3CHO	Ethanal
CH_3-CHOH-CO-CH_3	3-Hydroxy-2-butanone
CH_3-CO-CO-CH_3	2,3-Butanedione
$CH_3COCHOHCH_2CH_3$	3-Hydroxy-2-pentanone
$CH_3COCOHCH_2CH_3$	2,3-Pentanedione
	Phenylethanal
CH_3-CH_2-CH_2-CH_2-CH_2-CHO	Hexanal
CH_3-$(CH_2)_6$-CHO	Octanal
CH_3-CH_2-CO-CH_3	Butanone

Formation and modifications during winemaking

- Aldehydes

Similarly to C_6 alcohols, the C_6 aldehydes are mainly formed during pre-fermentative operations (section I.2.4.4). They contribute for the bouquet of certain wines but the action of sulfur dioxide may have a neutralizing effect due to the combination with the aldehyde fraction, like hexanal - a compound in C_6 (Figure I.2.16; Joslin and Ough, 1978). Their amount in musts can be different depending on the variety. During the alcoholic fermentation, they can be reduced to their correspondent alcohol.

Ethanal is the most abundant aldehyde in wine. Its presence is closely linked to the oxidation-reduction phenomena, being produced by the oxidation of ethanol. This compound is involved in the alcoholic fermentation mechanism and plays an active role in the evolution of the colour of red wines during conservation and aging, reacting with sulfites and facilitating the copolymerization of phenolic compounds (Ribéreau-Gayon *et al.*, 2000). The ethanal can also be produced by the decarboxylation of pyruvate during alcoholic fermentation. In white winemaking, SO_2 is generally added before or during

fermentation and ethanal is preserved as an ethanal bound complex (Frivik and Ebeler, 2003). Ethanal levels in young wines are typically less than 100 mg/L. However, if a finished white wine has unacceptably high levels of aldehydes, possibly arising from oxidative reactions, the addition of SO_2 may turn the wine less aldehydic by reacting with them. In wines preserved with sulfur, it is usually present as CH_3-CHOH-SO_3H (ethanal combined with sulfite), a compound stable in an acid medium that promotes a decrease in the amount of free ethanal in wines (Figure I.2.23).

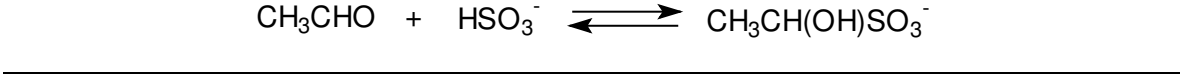


Figure I.2.23. Reaction of ethanal with sulfite

Factors such as yeast strain, temperature, pH of must, O_2 levels in juice, added SO_2 levels, and nutrient availability can influence the production of ethanal during fermentation.

The multiple origins, the high reactivity (-CHO is one of the most reactive chemical groups) at low temperatures with sulfur dioxide, and the organoleptic properties make ethanal one of the most important compounds in wine.

Other aldehydes exist generally in wines in small amounts. Benzaldehyde is an aromatic aldehyde that can come from grapes and is also possible to find in wines. It can be produced from shikimic acid, having phenylalanine as intermediate (Figure I.2.24).

- Ketones

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), present in concentrations around 10 mg/L and 0.3 mg/L, respectively. The diacethyl can have the pyruvic acid as its precursor (Revel *et al.*, 1989; Hayasaka and Bartowsky, 1999). It can also result from the oxidation of the acetoin and can be formed during excessive maturation of the fruits by the decarboxylation and oxidation of α-acetolactate.

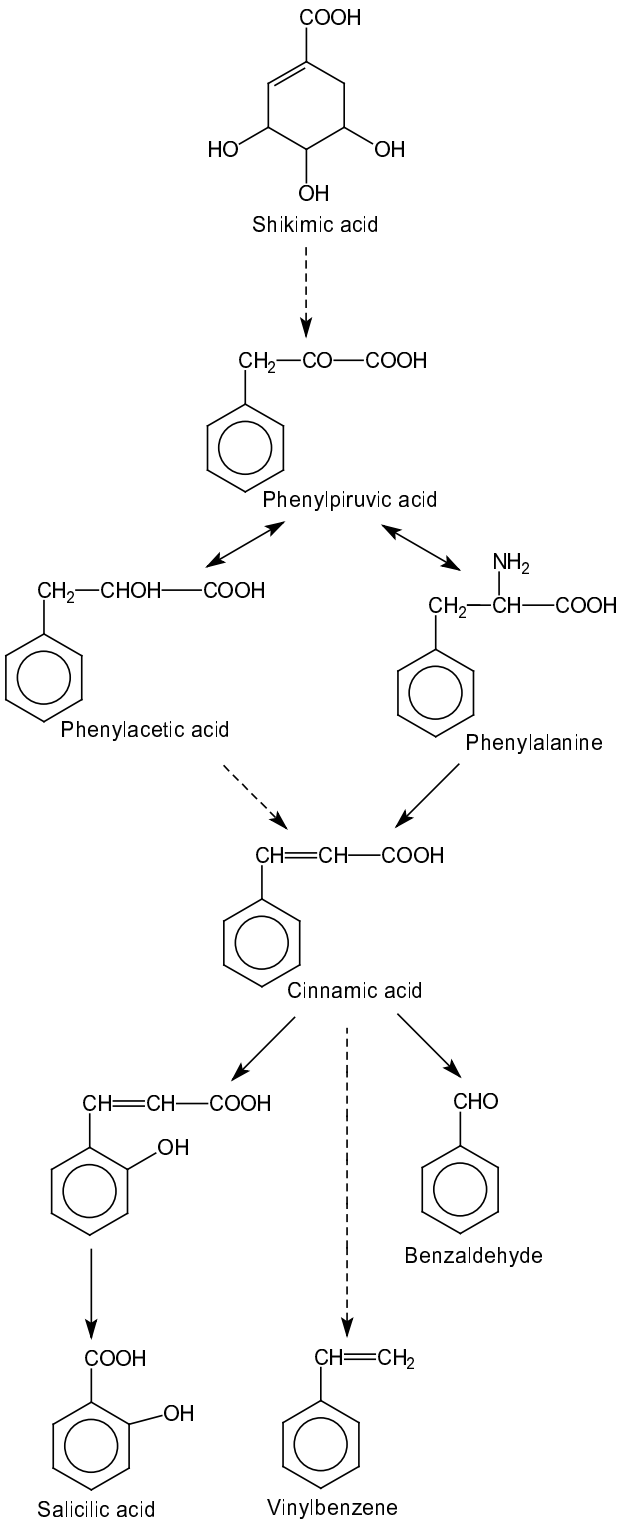


Figure I.2.24. Benzaldehyde formation through shikimic acid

Sensory properties

The aromatic ketones and aldehydes can be responsible for very intense odours, such as bitter almonds from benzaldehyde. The saturated short-chain aldehydes contribute favourably to many alcoholic beverages, including nutty, bruised apples, herbaceous, grassy, green, fruity, and pungent (Frivik and Ebeler, 2003). Table I.2.11 presents some ketones and aldehydes identified in wines with their respective aroma descriptors and sensory perception limits.

Table I.2.11. Sensory properties of ketones and aldehydes

Ketones and aldeydes	Aroma descriptors	SPL (mg/L) [a]
Ethanal	Overripe bruised apple, nutty, cherry	100-125 in wine [c]
Hexanal	Green, grassy, fruity	---
Benzaldehyde	Bitter almond, cherry	3 in wine [b]
Diacethyl	Butter, fermented cabbage, hazelnut	0.015 in water, 0.01 in in hydro alcoholic solution, 2.0-12.0 in wines [d,e]
Acetoin	Butter	152.6 in wine[e]

[a]- SPL- sensory perception limit; [b]- Frivik and Ebeler, 2003; [c]- Ribéreau-Gayon *et al.*, 2000; [d]- Revel *et al.*, 1989; [e]- Escudero *et al.*, 2004.

I.2.4.8. Sulfur compounds

Sulfur compounds have in their structure, at least, one sulfur atom. In wine, a considerable number of sulfur volatile compounds with several origins have been identified. Some come from grapes, having an important contribution to the varietal aroma of certain wines (thiols). In musts, some of these compounds, such as 3-mercaptohexen-1-ol, 4-methyl-4-mercaptopentan-2-one and 4-methyl-4-mercaptopentan-2-ol, were identified as S-conjugates to cysteine (Tominaga *et al.*, 1996, 1998a, Murat *et al.*, 2001). Aromas associated to these compounds were revealed during alcoholic fermentation, probably by the intervention of a specific β-lyase. Besides that, it seems that the enzymes present in the human oral cavity can release the volatile thiols from the glycosidically-linked forms.

Formation during winemaking

Most sulfur compounds are formed during winemaking (hydrogen sulfide, methionol, 2-methylthioethanol, etc) (Figure I.2.25). When in considerable amounts, some of these compounds are known to confer unpleasant odours that depreciate the wine quality (Dubois, 1994b). Others, even in small concentrations, are considered very important for the aroma of some wines, for example, the mercaptans (or thiols). They are usually present in concentrations lower than 1 μg/L. The most abundant compounds are present in concentrations lower then 100 μg/L and below the SPL. In concentrations near their SPL, many of sulfur compounds can contribute to the global wine aroma without depreciate it.

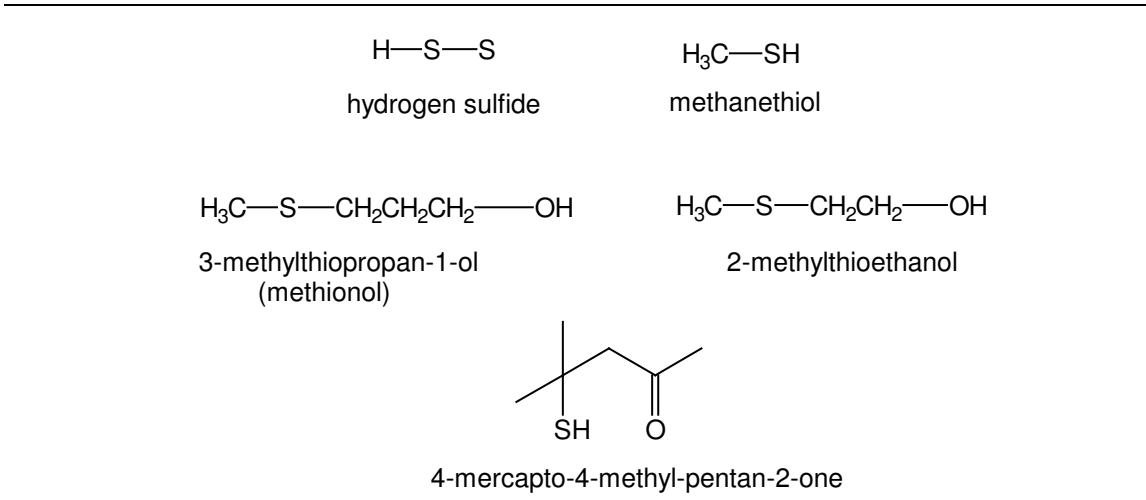


Figure I.2.25. Some sulfur compounds identified in wines

The intensity of the odour perception of sulfur compounds seems to be related with the molecular weight. The sulfur 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.*, 2000). The first group (for example, hydrogen sulfide, carbonyl sulfide, dimethyl sulfide, etc) is usually associated to defects in wines and the second group (2-methylthioethanol, 2-mercaptoethanol, methionol, etc) may contribute to the global white wine aroma. The second group is more abundant and is related with cysteine, methionine, thymine and homomethionine metabolism (Rapp *et al.*, 1985, Tominaga *et al.*, 1996, 1998a, Murat *et al.*, 2001). Among them, only a few play a significant role in reduction defects. The most important is

methionol (3-methylthiopropan-1-ol, which is produced by yeast from the methionine in the must, via deamination, followed by decarboxylation (Erlich reaction) (Figure I.2.15). The aldehyde thus formed (methional) is then reduced by an enzyme reaction into an alcohol (methionol) (Belitz *et al.*, 2004).

Several studies concerning thiols have been performed in the last years (Tominaga *et al.,* 1998b; Tominaga *et al.*, 2000; Tominaga and Dubourdieu, 2000; Blanchard *et al.*, 2001; Tominaga *et al.*, 2003; Schneider *et al.* (2006).

Benzothiazole is a heterocyclic compound, which contain a benzene ring fused with a thiazole ring (Figure 1.2.26). It was found in several Italian white and red wines (Bellavia *et al.*, 2000). However, the source of this molecule is still unclear. It may be formed during the fermentation process. Furthermore, benzothiazole can enter the environment by routes associated with synthesis of xenobiotics, thus being considered a food contaminant.

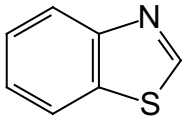


Figure I.2.26. Structural formula of benzothiazole

Sensory properties

Of all sulfur compounds, the thiols have been considered the most relevant to the aroma of some wines (Table I.2.12). Thiols are very odourant compounds and can confer aromas from herbaceous to fruity, resembling green pepper, boxwood, broom, grapefruit, granadilla or smoky. The 4-mercapto-4-methylpentan-2-one, for example, has a very low SPL and its amount in *Sauvignon* wine is 40 ng/L. Thus, this compound contributes to this wine aroma variety. Benzothiazole has an SPL of 50 μg/L but its amount in wines is usually inferior.

Table I.2.12. Sensory properties of sulfur compounds

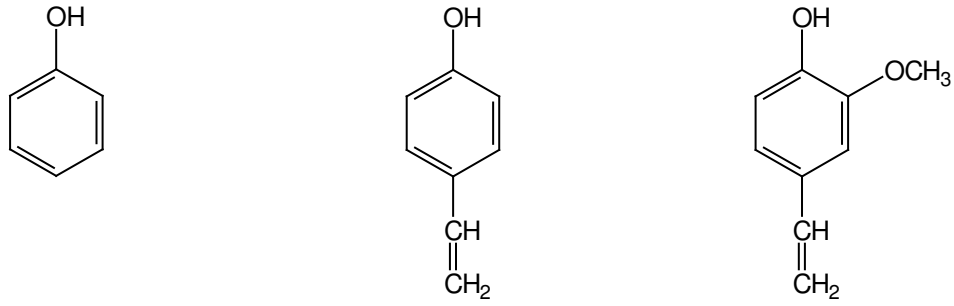
Sulfur compounds	Aroma descriptor	SPL [a]
Methionol	Cooked cabbage	1200 μg/L in wine [b]
2-Methylthioethanol	Cauliflower	250 μg/L in wine [b]
4-Mercapto-4-methylpentan-2-one	Boxwood, broom	0.066-0.165 ng/L in water [g], 0.8 ng/L in hydro alcoholic solution [d], 3 ng/L in wine [h]
3-Mercaptohexanol acetate	Wood, granadilla, boxwood, grapefruit	2.3-4.2 ng/L in hydro alcoholic solution [d,e]
3-Mercaptohexan-1-ol	Grapefruit, granadilla	12-15 ng/L in hydro alcoholic solution [d]; 60 ng/L in wine [d]
4-Mercapto-4-methylpentan-2-ol	Citric	20 ng/L in water [h], 55 ng/L in wine [d]
5-(2-Hydroxyethyl)-4-methyltiazole	Medicinal, peanut	0.1-1.0 mg/L in hydro alcoholic solution [c]
Benzothiazole	Rubber	50 μg/L in wine [i]
Benzenemethanethiol	Smoky	0.3 ng/L in hydro alcoholic solution [f]

[a]- SPL- sensory perception limit; [b]- Ribéreau-Gayon *et al.*, 2000; [c]- Rapp *el al.*, 1985; [d]- Tominaga *et al.*, 1998ab; [e]- Tominaga *et al.*, 1996; [f]- Tominaga *et al.*, 2003; [g]-Vermeulen et al., 2001; [h]- Bouchilloux *et al.*, 1998 ; [i]- Bellavia *et al.*, 2000

I.2.4.9. Phenolic compounds

Phenolic compounds are composed by several complex molecules: some polyphenols, are responsible for the wine colour; and simple molecules, the volatile phenols, may contribute to the wine aroma.

Volatile phenols are composed basically by a hydroxyl group and methyl/ethyl groups linked to an aromatic ring group (Figure I.2.27). The most widely represented compounds in white wines are 4-vinylphenol and 4-vinylguaiacol (Ribéreau-Gayon *et al.*, 2000). These compounds can, in some conditions, be responsible for certain defects in wines, but this subject will not be developed here.



phenol | 4-vinylphenol | 4-vinyl-2-methoxyphenol

Figure I.2.27. Some volatile phenols identified in wines

Formation during winemaking

In spite of being found only vestigial amounts in musts, in wines the concentration of volatile phenols has been reported to be from few tens to several hundreds of μg/L. The vinylphenols are mainly formed during fermentation by the yeasts metabolism (Dubois, 1994a). Other compounds can result from hydrolysis of their precursor glycosides but in small amounts.

In white wines, the vinylphenols are formed due to the enzymatic decarboxylation, promoted by yeast, of two cinnamic acids (*p*-coumaric acid and ferulic acid) of must, producing 4-vinylphenol and 4-vinyl-2-methoxyphenol (4-vinylguaiacol), respectively (Ribéreau-Gayon *et al.*, 2000) (Figure I.2.28). This reaction is catalysed by cinnamate decarboxylase (CD) from *Saccharomyces cerevisiae* that is highly specific for phenylpropenoic acids, but incapable of converting benzoic acids in volatile phenols. Among the cinnamic acids in grapes only ferulic and *p*-cumaric acids are affected by the CD activity. The CD of *S. cerevisiae* is activated only during alcoholic fermentation.

The vinylphenols can also be formed in clarified musts using a pectinase preparation with cinnamate esterase activity. The concentration of these compounds present in wine depends on the time of contact and must clarification, oxidation degree and use of certain pectolytic enzyme preparations (Ribéreau-Gayon *et al.*, 2000). In white wines, it is possible to observe a decrease on the vinylphenols amounts due to the polymerization phenomena of these compounds during the bottle aging.

Other phenolic compounds, such as vanillin (4-hydroxy-3-methoxybenzaldehyde) (Figure I.2.29) can be found, in grapes, in small amounts and in the glycosidically-linked

form. During winemaking, it can emerge from hydrolysis of its grape glycosides. Its origin is related with shikimic acid, having ferulic acid as intermediate (Boidron *et al.*, 1988; Spillman *et al.*, 1997).

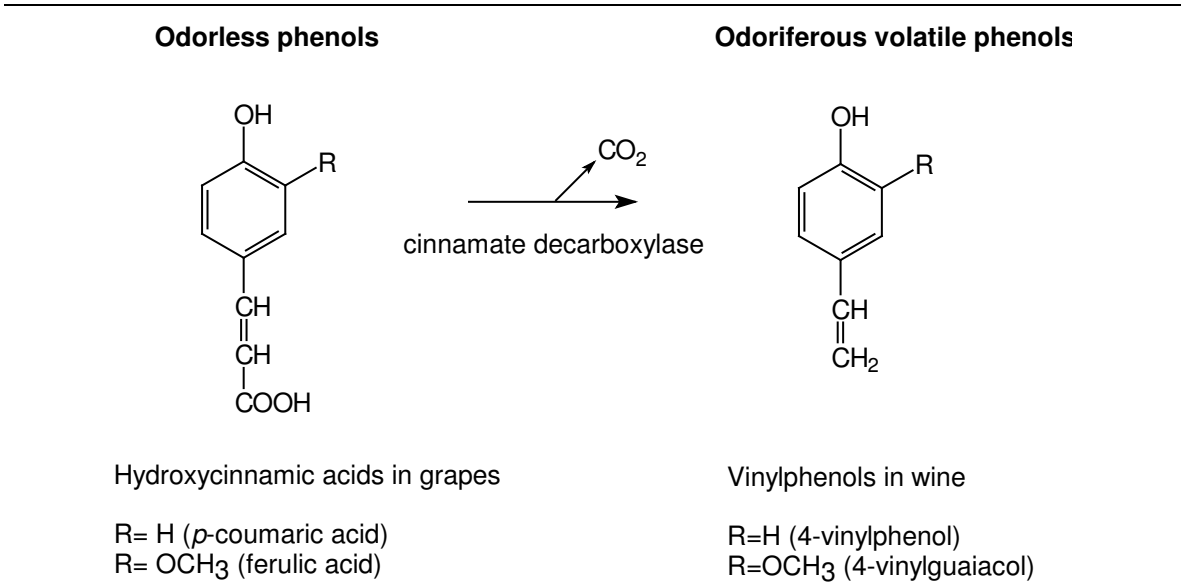


Figure I.2.28. Decarboxylation of phenolic acids by *Saccharomyces cerevisiae* action during alcoholic fermentation

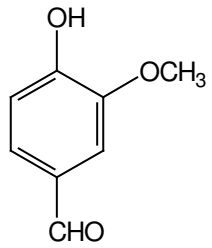


Figure I.2.29. Structural formula of vanillin

Sensory properties

Volatile phenols can confer an accentuated odour to the wines, like spices (4-vinylguaiacol, eugenol), colour (phenol) and vanilla (vanillin) (Table I.2.13). For *I*< 1, 4-vinylphenol and 4-vinyl-2-methoxyphenol may positively contribute to the global aroma of wine.

Table I.2.13. Sensory properties of volatile phenols

Phenols	Aroma descriptors	SPL (mg/L) [a, b]
Phenol	Phenolic, colour	5.5 in water; 15 in hydro alcoholic solution; 25-35 in wines
4-Vinylphenol	Dature	0.085 in water; 0.18 in hydro alcoholic solution; 0.77-1.5 in wines
4-Vinyl-2-methoxyphenol (4-Vinylguaiacol)	Black pepper, clove-tree	0.032 in water; 0.13 hydro alcoholic solution; 0.38 -0.44 in wines
Eugenol	Strong, spices, cinnamon, clove-tree	0.007 in water; 0.015 in hydro alcoholic solution; 0.1-0.5 in wines
Vanillin	vanilla, sweet, chocolate, caramel	0.105 in water; 0.065 in hydro alcoholic solution; 0.32-0.4 in wines [c]

[a]- SPL- sensory perception limit; [b]- Boidron *et al.*, 1988 ; [c]- Spillman *et al.*, 1997.

I.2.4.10. Other compounds

- Nitrogen compounds

The acetamides from primary amines are volatiles. In the fermentative medium the primary amines are acetylated by yeast to acetamides. The most abundant are the *N*-(2-methylbutyl)-acetamide, *N*-(3-methylbutyl)-acetamide, *N*-(2-phenylethyl)-acetamide, *N*-(3-methylpropyl)-acetamide, *N*-pentylacetamide and *N*-ethylacetamide (Dubois, 1994a; Bayonove *et al.*, 1998b). The *N*-(3-methylbutyl)-acetamide, for example, can appear in concentrations over 1 mg/L. However, it seems do not have any contribution in the wine aroma.

- Dioxolanes

The information about these compounds in wines is almost inexistent. They seem to result from the condensation reaction between an alcohol and an aldehyde under acid conditions (at wine pH), that leads to the formation of dioxolanes and dioxanes. However, these compounds seem to be mainly related with wine aging (Muller *et al.*, 1978; Ferreira *et al.*, 2002a; Câmara *et al.*, 2003).

- *Furans*

The 2-ethoxyfurfural, ethyl 2-furanoate and 2-furfural are the furfural derivates that can increase during the aging of wine, coming from de sugar degradation. However, these compounds do not seem to participate in the wine aroma (Bayonove *et al.*, 1998b). They can also appear as a result of high temperature exposure.

- *Pyrazines*

Pyrazines are heterocyclic compounds that can appear in some white wines. These compounds were first identified in grapes of the *Cabernet Sauvignon* variety but, since then, they have been also found in other varieties (*Cabernet Franc* and *Sauvignon Blanc*, etc). In these cases, they can be considered as varietal aromas. In particular, the methoxypyrazines have been reported to be produced by the metabolism of amino acids (Ribéreau-Gayon *et al.*, 2000). The majority of methoxypirazines were found in the skin of grapes (Bayonove *et al.*, 1975) and their amounts depend on the degree of maturation. These compounds passed on to the wines, where they were found in similar amounts. Some studies suggest that some methoxypyrazines have a microbiologic origin in wine (Allen *et al.*, 1995). Pyrazines can also be formed by Maillard reactions (Belitz *et al.*, 2004).

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 (Ribéreau-Gayon *et al.*, 2000). From these, the 2-methoxy-3-isobutylpyrazine has the greater contribution for the earth and vegetable notes. These compounds are strongly odourant and can be detected in water even if in very small amounts (1-2 ng/L) (Allen *el at.*, 1995). However, the aroma intensity of pyrazines seems to depend on their state and degree of ionization. The cationic form is the dominant one at normal pH values of wines but have a less intense aroma.

I.2.5. Effect of winemaking technologies on wine volatile composition

The wine is a beverage obtained by full or partial alcoholic fermentation of fresh, crushed grapes or grape juice (must) (Belitz *et al.*, 2004).

During winemaking, several steps of the technological processes may contribute to the aroma quality of the white wines: harvest, the time between the grape harvest and it

crushing/pressing, the pressing that may promote non-controled skin maceration, must clarification and alcoholic fermentation, etc.

The production of white wine is performed with the general following steps (Figure I.2.30):

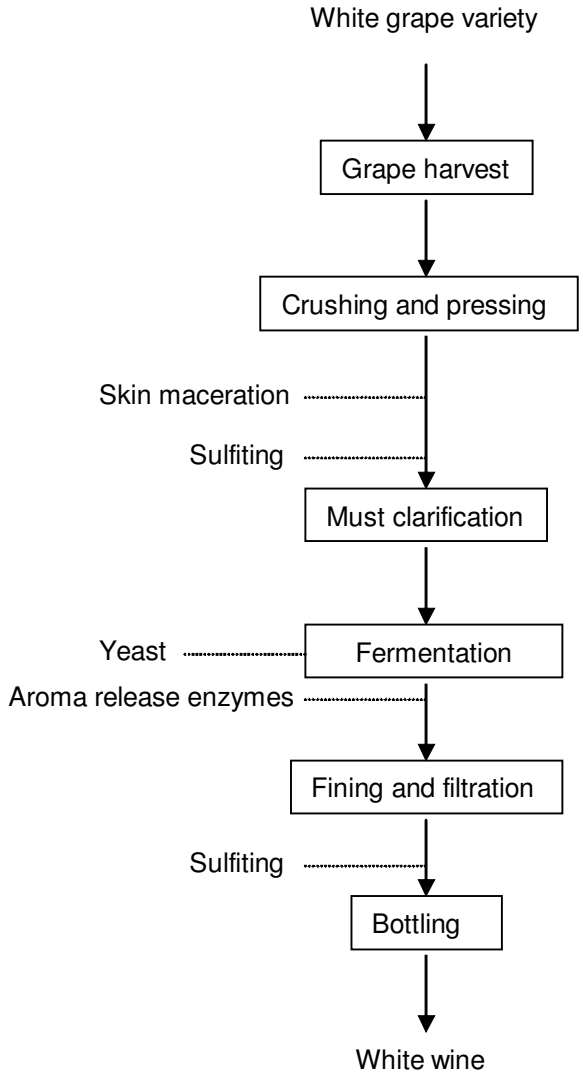


Figure I.2.30. Production of white wine

Harvest. The grapes picked must be healthy and their oenological maturity (sugar, acidity and aroma concentration) must be as uniform as possible. From the harvest to their arrival at the winery, the grapes must be as intact as possible to limit must oxidation and stem maceration. They must be transported in containers that minimize berry

crushing. The volatile composition of wine depends on grape maturation and healthy-state (Girard *et al.*, 2002). Sugar concentration and acidity are usually used to define the maturity of grapes destined to produce wine. However, several studies report that the volatile composition (mainly for terpenoids and C_{13} norisoprenoids) as well as their precursors is also determinating factors (Salinas *et al.*, 2004; Palomo *et al.*, 2006). Recent studies show that, for Fernão-Pires variety, the maximum amount of varietal volatile compounds was coincident with the harvesting day for white table wine production defined by the ratio sugar/acid content (Coelho *et al.*, 2007). This study also shows that the screening of linalool, α-terpineol, and geraniol during ripening can be used to define the evolution profile of the varietal volatile compounds.

Crushing and pressing. These operations, performed mechanically, promote the extraction of must in a relatively limited gap of time, while minimizing must loss. Some times, crushing is not performed; the whole grape clusters are directly placed into the press. In other cases, the destemming operation (separation of the berries from the stem) is performed. Crushing and destemming can be related operations, combined in a single peace of machinery. The diffusion of certain grape fruity/floral aromas and aroma precursors (mainly from the solid parts of grape) into the must may occur during these operations. The levels of monoterpenoids may increase in musts and in wines (Ribéreau-Gayon *et al.*, 2000; Kinzer and Schreier, 2004). These techniques can also increase the levels of C_6 alcohols (1-hexanol and *cis*-2-hexen-1-ol) in musts. However, the dissolution of herbaceous-odour and bitter-tasting compounds, associated with the solid parts of the berry, must simultaneously be limited.

Skin maceration. This step, when applied in white winemaking, consists of permitting a contact phase between the skins and the must in controlled conditions (such as very low temperatures), before alcoholic fermentation. An adapted tank is filled with moderately crushed, destemmed grapes (Ribérau-Gayon *et al.*, 2000). Several hours later, the drained juice is collected and the pomace is pressed. This process can be sought for the better extraction of grape skin components that participate in the aroma. During that time, grape temperature must be maintained below 15 ºC and the absence of oxygen must be guaranteed. Studies clearly demonstrate that skin contact increase the amount of volatile compounds in wines, particularly the free and glycosidically-linked forms of varietal aroma compounds (Cabaroglu and Canbas, 2002; Selli *et al.*, 2006; Esti and Tamborra, 2006). This operation promotes also the dissolution of C_6 alcohols but this may be limited by the absence of oxygen and at controlled temperatures/period of time.

Sulfiting. This technique is used with many purposes, mainly for protecting must from oxidation and from microbiological activity. The oxidation of substances and microbiological activity in white wine can occur at any time during winemaking but must be avoided. It seems to have also a protective effect on fruity aromas (Ribérau-Gayon *et al.*, 2000). Sulfiting is the most simple and effective method of protecting must from oxidation. For example, to destroy tyrosinase (oxidase involved in the enzymatic oxidation of phenolic compounds), 50-60 mg/L of sulfur dioxide (SO_2) must be added to the must. The addition of SO_2 may influence some volatile compounds (section I.2.4.7). The levels of SO_2 applied to the must have an influence on yeast production of higher sulfur compounds.

Clarification. Freshly extracted grape must is more or less turbid. It contains suspended solids of diverse origin: earth, skin and stem fragments, cellular debris from grape pulp, insoluble residues from vineyard treatments products, etc. Macromolecules in solution or in the course of precipitating are also involved in must turbidity. Among them, grape pectic substances play an essential role. Clarification consists of separating the clear must from the lees before alcoholic fermentation. The quantity of lees formed during juice extraction and the speed of sedimentation depend on variety, grape disease status, maturity and especially winemaking methods (crushing, draining, pressing, etc) (Ribérau-Gayon *et al.*, 2000). Clarifying musts improve wine quality when compared with the unclarifyed musts. Clarification can be performed, for example, by sedimentation, filtration, tangential microfiltration and pectolytic preparations. The amount of volatile compounds changes with clarification. Wines made from suitable clarified musts have lower concentrations of heavy-odour higher alcohols and higher concentrations of ethyl esters of fatty acids and higher-alcohols acetates, which have more pleasant aromas (Ribéreau-Gayon *et al.*, 2000). It also limits the concentration of C_6 alcohols in wines. However, extended clarifications are deleterious. The filtration by vacuum may promote higher losses of aroma compounds than sedimentation. Moio *et al.* (1991) observed a significantly decrease of glycosylated precursors of some monoterpenoids and aromatic alcohols with must clarification, but the concentration of free terpenols was not affected.

The operations described before are known as pre-fermentation operations. They comprise grape and must handling and treatments, which are deciding keys for the wine quality.

Fermentation. Fermentors are filled with must. At this point, active dry yeast may be added. Approximately 30 active dry yeast stains belonging to *Saccharomyces cerevisiae* are available to use in white winemaking (Ribérau-Gayon *et al.*, 2000). During alcoholic fermentation, temperature of fermenting tanks must be controlled (16-20ºC). The time of white wine fermentation depends on several parameters: conditions of juice extraction, sugar, concentrations of assimilable nitrogen, turbidity, yeast stain, aeration and fermentation temperature. After that period of time, the wine is sulfited. The type of yeast applied in winemaking influences the volatile composition (Antonelli *et al.*, 1999; Nurgel *et al.*, 2002). As mentioned before, it may contribute to the formation of higher alcohols and esters (Torrea *et al.*, 2003; Mateos *et al.*, 2006). It can also influence the terpenoid composition by transforming some terpenoids into others and by the capacity of some types of *Saccharomyces* to biosynthesize this group of compounds (Zoecklein *et al.*, 1997; Fernández-González *et al.*, 2003; Gil *et al.*, 2007). Other stains, like VL1, were selected also for their low vinyl-phenol production (Ribérau-Gayon *et al.*, 2000) as these compounds process rather unpleasant pharmaceutical aromas. During the alcoholic fermentation it is possible the occurrence of some loss of aroma compounds, by the produced gases.

Aroma release enzymes. In order to increase the white wine aroma, appropriated commercial enzymes can be used after fermentation or in some cases during fermentation. These enzymatic preparations have mainly *β*-glucosidase activity, but can also have pectinase, arabinosidase and rhamnosidase activities. They are capable of hydrolyzing terpenic and C_{13} norisoprenic glycosides and may also act on other aroma compounds present in the form of odourless precursors. However, they should be used on the basis of knowledge of the activity of the commercial enzymes, as well as knowledge of the aroma potential of the grape varieties. As mentioned before (section I.2.2.2), *β*-glucosidase activity was reported to have different specificities for different aglycones and the amount of volatile compounds released varies with the enzymatic preparation. Also, the action of aroma release enzymes in wines varies according to the wine's sugar content. Several aroma release commercial enzyme preparations are available to use in white winemaking. Higher concentrations of glucose inhibit their action but they are stable at must and wine pH. Several estudies indicate that the wines submitted to enzymatic treatment with exogenous β-glucosidases have a more intense and complex aroma, producing more fruity and floral wines (Shoseyov *et al.*, 1988; Dubourdieu *et al.*, 1988; Cordonnier *et al.*, 1989; Cordonnier, 1989; Gunata *et al.*, 1990a; Gunata *et al.*, 1990b;

Bayonove *et al.*, 1992; Vasserot *et al.*, 1993; Gueguen *et al.*, 1996; Cabaroglu *et al.*, 2003).

Fining and filtration. After the alcoholic fermentation, the white wines usually require fining with varying amounts of bentonite, in order to achieve stability with regard to heat-sensitive proteins. However, other fining agents can also be used. The filtration of white wine is also common, allowing to eliminate a slightly turbidity that occur in wines before bottling. Before bottling, and in order to avoid posterior precipitation of tartrate salts, the white wines are usually submitted to stabilization treatments. The amount of volatile compounds can change with fining. Cabaroglu *et al.* (2003) report a decrease in the amount of several volatile compounds after the application of fining treatments, such as bentonite, in some white wines. The glycosidically-linked fraction seems to be more affected by those treatments. As mentioned before, the filtration (a clarification method) usually promotes losses of aroma compounds from wines.

I.2.6. Interactions and/or retention of volatiles by wine macromolecules

The wine is a complex product composed by several constituents. The main ones are water and ethanol. Among the other compounds present in wine, there are also non-volatile wine constituents, namely polysaccharides, proteins and some polymerized polyphenols, usually known as wine macromolecules. The wine macromolecules influence the equilibrium of the volatile compounds between the liquid and vapour phases, ruled by physico-chemical properties such as volatility and solubility (Dufour and Bayonove, 1999a). The interactions between the volatile compounds and the polymeric fraction have been firstly investigated due to the observation that the processes of filtering and clarifying the wines, modifying the macromolecular balance, also affected the aroma quality of wines. During these winemaking processes a significant loss of aroma compounds was observed (Moio *et al.*, 1991). Thus, the macromolecules should have an important role on the volatile composition.

The interactions between volatiles and macromolecules have been studied using the dynamic exponential dilution method, which allows the determination of infinite dilution activity coefficients γ_i^{∞} of volatile components in an aqueous medium (Langourieux and Crouzet, 1994; Athès *et al.*, 2004). This coefficient varies with the polymeric fraction concentration and seems to be related with the nature and the strength of the interactions. Solid phase-microextraction (SPME) was also applied to study the disulfide interchange reactions between ovalbumin and volatile disulfides (Adams *et al.*, 2001).

Several studies report the interactions between wine macromolecules and volatiles, as well as the ability of each type of macromolecules on the retention of volatiles (Dufour and Bayonove, 1999ab; Voilley *et al.*, 1991; Lubbers *et al.*, 1993; Lubbers *et al.*, 1994ab; Ramirez-Ramirez *et al.*, 1994; Langourieux and Crouzet, 1994, 1997; Dufour and Bayonove, 1999b; Dufour and Sauvaitre, 2000; Lubbers *et al.*, 2004). These phenomena are dependent on the nature and concentration of both, macromolecules and volatiles. In wines, a few studies concerning this subject were performed with polysaccharides (Voilley *et al.*, 1990; Dufour and Bayonove, 1999a; Charlier *et al.*, 2007) and with glycopeptides (Langourieux and Crouzet, 1997). For example, Voilley *et al.* (1990) suggested that isoamyl acetate and ethyl hexanoate suffer an inclusion phenomenon with a particular polysaccharide fraction, the mannans. Dufour and Bayonove (1999a) studied the effect of the different polysaccharide fractions on the volatility of various aroma substances. Once more, higher levels of mannoproteins seem to retain the isoamyl acetate and ethyl hexanoate but strongly salted out 1-hexanol. Recent studies concerning mannoproteins show evidences of interaction between aroma compounds and mannoproteins produced by yeasts at the concentrations that these macromolecules occur in wine (Charlier *et al.*, 2007). This study also suggests the role of the conformational and compositional structure of these macromolecules on the interaction with aroma compounds.

Some other studies have been performed concerning polyphenols/flavour interactions (Aronson and Ebeler, 1999). Dufour and Bayonove (1999b) studied the influence of (+)-catechin and a wine highly condensed tannin fraction on the volatility of aroma substances. Dufour and Sauvaitre (2000) studied the interactions between anthocyanins and some aroma substances and Escalona *et al.* (2001) studied the effect of (+)-catechin on the volatility of ethyl hexanoate and octanal in ethanol/water solutions. They found that (+)-catechin caused a small reduction in the activity coefficient of ethyl hexanoate, while octanal volatility diminishes, suggesting that these components interact with aldehydes. Jung and Ebeler (2003) show that catechin decreases in 10-20% the volatility of ethyl hexanoate and hexanal, in water.

The retention between macromolecules and volatiles seem to occur by sorption or inclusion phenomena dependent on the macromolecule structure and on the characteristics of volatile compounds such as molecular weight, chemical groups, polarity and relative volatility (Goubet *et al.*, 1998). Generally, the sorption of the volatiles to the macromolecules depends on their ability to form hydrophobic interactions or hydrogen bonds (Voilley *et al.*, 1991). The retention of aroma compounds increases with the polymeric fraction molecular weight but decreases when polymerization degrees are high.

It can also increase when the viscosity of macromolecules is enhanced. The volatile compounds with higher molecular weight are usually more retained (Goubet *et al.*, 1998). In general, the alcohols are the chemical group more retained, followed by ketones, esters and acids. A highly polar compound will be less retained.

I.3. Methodologies for analysis of volatile compounds

As mentioned before the volatile compounds present in grapes, musts and wines represent several chemical groups, some of them being reactive and present in low concentrations. The difficulties usually encountered in qualitative and quantitative analysis of aroma compounds are based on these features. Other difficulties are associated with identification of aroma compounds, elucidation of their chemical structure and characterization of sensory properties.

The analysis of volatile compounds usually includes two steps: *i)* extraction, where the compounds are removed from the material, and *ii)* separation and identification, by chromatographic methods.

I.3.1. Methodologies for extraction of volatile compounds

The procedures to extract the volatile compounds of wine are mainly based on their physico-chemical properties, such as volatility and solubility in distinct organic immiscible phases with the aqueous matrix, and the capacity of being selectively sorbed by certain materials. Generally, the compounds, most of them present in small amounts, need to be extracted and concentrated before their determination. In the volatile compounds analysis, there are several factors that difficult their study: the trace amounts of some compounds and the different physico-chemical characteristics (such as high volatility/higher boiling points). They have also distinct characteristics in terms of polarity and solubility.

The handling of these compounds demands additional care, otherwise the reproducibility may be low and, due to the occurrence of modification on the volatile fraction, the aroma fraction is no longer representative of the sample. Thus, the more reliable samples are those who suffered the minimal manipulation possible and where the compounds that define the volatile composition were obtained with higher reproducibility.

From the available methods of extraction performed today for the wine matrix, it is possible to highlight: liquid-liquid extraction (Mamede and Pastore, 2006), liquid-liquid continuous extraction (Zhou *et al.*, 1996), ultrasounds (Vila *et al.*, 1999), microwaves (Ranzungles *et al.*, 1994), simultaneous distillation and extraction (SDE) (Blanch *et al.*, 1992, 1996); supercritical fluids extraction (SFE) (Blanch *et al.*, 1995); solid-phase extraction (SPE) (Piñeiro *at al.*, 2004; Ferreira *et al.*, 2004; Cabaroglu *et al.*, 2002, Selli *et*

al., 2004), solid-phase microextraction (SPME) (De la Calle García *et al*., 1998ab; Pozo-Bayón *et al*., 2001; Rocha *et al*., 2001; Mestres *et al*., 2002; Demyttenaere *et al*., 2003ab; Coelho *et al*., 2006 ; Neto *et al*., 2007), dynamic headspace extraction (Rosillo *et al*., 1999; Ortega-Heras *et al.* 2002) and stir bar sorptive extraction (SBSE) (Ortega-Heras *et al*., 2002; Hayasaka *et al*., 2003; Demyttenaere *et al*., 2003b; Díez *et al*., 2004; Salinas *et al*., 2004).

From the previously mentioned methods, only the ones used during this work will be reported.

To select the most adequated extraction method, it must take into account the different matrices, *i.e.* solid and/or liquid samples. The liquid samples (musts and wines) were analysed by liquid-liquid continuous extraction. However, the extractor used for liquid-liquid continuous extraction is not adequated to solid samples such as grapes. Thus, a different methodology was applied for grape volatile analysis (liquid-solid extraction by amberlite XAD-2 resin).

I.3.1.1. Liquid-liquid continuous extraction

The liquid-liquid extraction (LLE) is a transference process of one or more substances from a liquid phase to another that is immiscible, based upon the difference in solubility of a compound in the two phases. The basic principle behind extraction involves the contact of a solution with another solvent that is immiscible with the original. Usually, this process has an organic phase (allowing the separation of the compounds) and an aqueous phase (the one that initially contains the compounds for separation). A specific solute contained in the solution is soluble with the solvent. Two phases are formed after the addition of the solvent, due to the differences in densities. The solvent is chosen so that the solute in the solution has more affinity toward the added solvent. Therefore mass transfer of the solute from the solution to the solvent occurs.

For a given compound, the solubility differences between solvents are quantified as the “distribution coefficient” (K). At a certain temperature, the ratio (distribution coefficient, K) of concentration of a solute in each solvent is always constant.

Thus, the distribution coefficient (K) is given by:

K= (concentration in solvent$_2$) / (concentration in solvent$_1$);

where solvent 1 (solution) and solvent 2 (solvent) are immiscible liquids.

In liquid-liquid continuous extraction, an extraction glass apparatus and appropriate organic solvents are needed. Figure I.3.1 represents a model of an extractor for solvents denser than samples, which is the case of dichloromethane/wine. In this case, the solvent is added to the wine from the top, passing thought it drop by drop (1). Then, the solvent is laterally recovered through a lateral tube (2) where it is heated to boiling point. The time required for this process must be tested in order to allow extracting the volatile compounds that represent the sample.

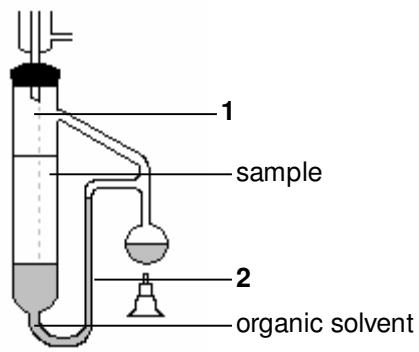


Figure I.3.1. Extractor for continuous liquid-liquid extraction of volatile compounds, with solvents denser than the sample (such as dichloromethane/wine)

To achieve a satisfactory process, the selection of the operation conditions (such as temperature) and the composition of the solvents used have an important role. In this method, all volatile compounds (low, medium and high volatility) have a high partition coefficient to the organic phase (K > 1) (Ortega-Heras *et al.*, 2002). However, it must take into account that separation of more polar compounds, which are readily soluble in water, is incomplete, and that resulting thermal treatment of the solvent can lead to reactions that may change some the volatile compounds. Usually, the final solvent extract must be partially evaporated. Thus, the solvent low boiling points are usually used to make sub-

sequent concentration of the volatile compounds easier. This process is carried out at normal pressure or slightly reduced pressure.

The main advantage of liquid-liquid continuous extraction technique comparatively to usual LLE is that the solvent passes repeatedly through the aqueous phase that contains the volatile compounds, extracting them more times.

Some studies use the LLE as a method to extract volatile compounds from wines (Étievant, 1987; Zhou *et al*., 1996; Castro *et al*., 2004). Zhou *et al.* (1996), for example, compare the Amberlite XAD-2 resin extraction with liquid-liquid extraction for wine flavour components. The results indicated that the two methods are comparable for most volatile of interest in wine. Both methods exhibited a high relative recovery for longer chain alcohols, esters, aldehydes, ketones and monoterpenes, but both showed low extraction efficiency for low molecular weight alcohols and organic acids. Castro *et al.* (2004) compare the liquid-liquid continuous extraction with solid-phase microextraction, concluding that both methodologies show adequate detection and quantification limits, and linear ranges for correctly analysing volatile compounds. The LLE procedure is a method with high repeatability and has the possibility of simultaneous extraction of several samples.

I.3.1.2. Liquid-solid extraction

The liquid-solid extraction of volatile compounds can be performed by using, for example, an Amberlite XAD-2 resin. Several studies use this method for the extraction of volatile compounds from grapes, musts and/or wines (Gunata *et al.,* 1985ab; Edwards and Beelman, 1990; Razungles *et al*., 1993; Gómez *et al*., 1994; Versini *et al*., 1995; Zhou *et al.,* 1996; López-Tamames *et al*., 1997; Cabaroglu *et al*., 2002; Selli *et al*., 2004). More recently, Bohlscheid *et al.* (2006) compared headspace solid-phase microextraction and liquid-solid extraction by XAD-2 resin for volatile compounds analysis. Both extraction procedures yielded high relative recoveries and reproducibilities for the different higher alcohols, esters and medium-chain fatty acids.

The Amberlite XAD-2 resin is a polymeric adsorbent hydrophobic cross linked polystyrene copolymer material, visually composed by 20-60 mesh size white insoluble beads. It is widely used to adsorb soluble organic compounds from aqueous streams and organic solvents. This resin is characterized by its macroreticular porosity, broad pore size distribution and large surface area, and a chemically homogeneous non-ionic structure

that differentiates it from most other adsorbents. It has unusually good physical durability and is stable at temperatures as high as 200 ºC.

A single bead of Amberlite XAD-2 resin is shown schematically in Figure I.3.2. Each bead consists of an agglomeration of many very small microspheres, giving a continuous gel phase and a continuous pore phase. The open-cell porous structure allows water to penetrate easily in the pores. The compounds were adsorbed to the resin.

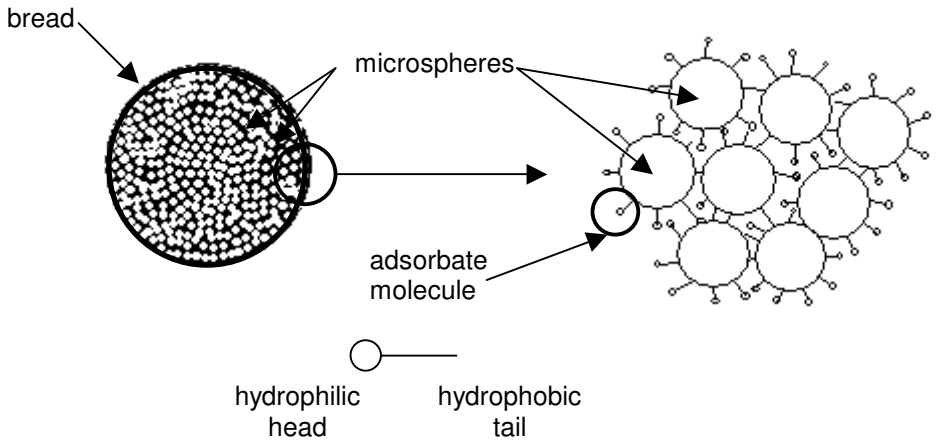


Figure I.3.2. Structure of an hydrophobic, macroreticular Amberlite XAD-2 resin bread (Adapted from Supelco)

In the adsorption process, the hydrophobic portion of the adsorbate molecule is preferentially adsorbed at the hydrophobic polystyrene surface of the resin, while the hydrophilic section of the adsorbate remains oriented in the aqueous phase (information provided by Supelco, Inc. -Bellefont, PA). The compounds being adsorbed ordinarily do not penetrate substantially into the microsphere phase, but remain adsorbed at the surface. Therefore, with proper elution or regeneration techniques, the adsorbed compounds can be rapidly eluted, because of the high rate of diffusion of the elution mobile phase through the porous structure of each bead. Since the compounds are bound to the outer and inner surfaces of the beads, penetration or solvation of the microspheres by the eluting agent is neither involved nor necessary. The selectivity and extent of adsorption of soluble organic compounds by Amberlite XAD-2 resin increases with the increase of the hydrophobicity of the adsorbate molecule. The adsorption forces are primarily van der Waals type. Thus, it is possible to change the extent of adsorption of a compound by changing its hydrophobic/ hydrophilic balance.

Gunata *et al.* (1985b) and Zhou *et al.* (1996) exposed the advantages of this methodology. The main one is that it allows the extraction of both free volatile compounds and glycosidically-linked compounds separately in a short period of time, using only two different solvents with different polarities. After that, the addition of an adequate enzymatic preparation, chosen as the best method to hydrolyse the aglycones, to the glycosidically-linked fraction allows to obtain the released bound compounds. During this methodology, it is possible to eliminate interfering substances, such as sugars, by a simple washing with water, without loss of volatiles and glycosides. This is an important step because it is known that sugars can interfere with enzymatic activity and in this procedure an enzymatic preparation is added in order to release the bound compounds. It is also possible to use low temperatures, which is important in the volatile compounds manipulation. The compounds, mainly terpenoids and their glycosides, are found to be fixed entirely on the column and the elution is then performed by using appropriate solvents.

The selection of the solvents used to elute the compounds is very important. The dichloromethane, for example, gives a better elution of the free compounds but removes also the glycosidically-linked fraction. The pentane does not elute the glycosidically-linked fraction and gives a good recovery of the free terpenols (depending on the compound, 7 to 16% remain attached to the column) (Gunata *et al.*, 1985b). The ethyl acetate, as well as methanol, elute glycosides but is more selective. The use of a mixture pentane/dichloromethane (2:1 v/v) to elute the free fraction has been chosen taking into account that pentane is not able to elute glycosides and, at the same time, it does not enable full recovery of free terpenols. So, to choose the solvent for free compounds extraction it is necessary a compromise. According to Gunata *et al.* (1985b) it is possible to achieve recovering of 90-100%. Furthermore, a small volume of resin (10 mL) is sufficient to adsorb the free and linked fraction from at least 300 mL of juice, so there is no danger of saturating the adsorbent.

I.3.1.3. Solid-phase microextraction (SPME)

SPME is a sample preparation technique based on sorption (absorption and/or adsorption), depending on the fibre coating, which is useful for extraction and concentration of analytes either by submersion into a liquid phase or by exposure to a gaseous phase (Arthur *et al.*, 1992). Following exposure of the fibre to the sample, sorbed analytes can be thermally desorbed in a conventional gas chromatography (GC) injection port.

Developed in 1990 by Pawliszyn *et al.*, it has been commercially available since 1993 and now is available with various sorbent materials and various coating thicknesses for a large number of purposes. SPME has been used in a range of fields including studies of flavours and taints, especially for quick screening of the volatile composition of a wide range of products (Yang and Peppard, 1994; Field *et al.*, 1996; Penton, 1996; De la Calle García *et al.*, 1996, 1997, 1998ab; Nishikawa *et al.*, 1997; Lee *et al.*, 1997; Song *et al.*, 1997; Fischer and Fischer, 1997; Wada and Shibamoto, 1997; Jia *et al.*, 1998; Ruiz *et al.*, 1998; Vas *et al.*, 1998; Jelén *et al.*, 1998; Deibler *et al.*, 1999; Marsili, 1999a; Namera *et al.*, 1999; Hayasaka and Bartowsky, 1999; Ong and Acree, 1999; Cardinali *et al.*, 2000). The application of this technique to flavour analysis of foods and beverages, namely wine, still requires knowledge of the fibre affinity for the specific volatile compounds under study as well as optimisation of experimental parameters to improve the reproducibility and sensitivity of the method. Many studies of wine aroma compounds aim to characterize or distinguish between different varieties and to follow specific steps involved in winemaking, where variations of the matrix composition occur (De la Calle García *et al.*, 1997; Francioli *et al.*, 1999; Rossilo *et al.*, 1999; Mestres *et al.*, 2000; Demyttenaere *et al.*, 2003ab; Mejías *et al.*, 2003). SPME combined with GC-MS is a methodology highly used to study the volatile composition of wines (De la Calle García *et al.*, 1997; Vas *et al.*, 1998; Mestres *et al.*, 2000, 2002; Marengo *et al.*, 2001; Pozo-Bayón *et al.*, 2001; Rocha *et al.*, 2001; Begala *et al.*, 2002; Bonino *et al.*, 2003).

Principle of headspace-SPME. The principle of HS-SPME for liquid matrices is the partition process of the flavour compounds between the two or three phases (Zhang and Pawliszyn, 1993). A two phase system is considered when a liquid sample occupies the total volume of the vial. The phases involved are the liquid (matrix) and the coating fibre. When the liquid matrix does not occupy the total vial volume, the system is composed by three phases: liquid (matrix), gas (headspace of the sample), and a coating fibre (Figure I.3.3). In this case, the analysis can be performed in two different ways: immerging the fibre coating in the solution to analyse or placing it in the headspace. This means that in this system the amount of analite extracted by the coating fibre after achieving the equilibrium does not depend on the location of the fibre coating, as long as the volume of the liquid phase and headspace are the same for both situations (Górecki and Pawliszyn, 1997). In the studies performed in this work, we are in the presence of a three system phases, but where the equilibrium state is not achieved.

$K_1 = C_F/C_H$

$K_2 = C_H/C_L$

$K_3 = C_F/C_L$

$C_0 V_L = C_H V_H + C_L V_L + C_F V_F$

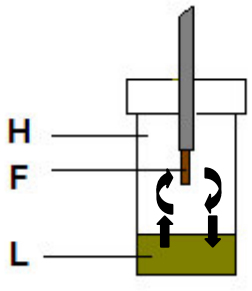


Figure I.3.3. Equilibrium composed by three phases from fibre in liquid samples. (L)- liquid (wine), (F)- coating fibre and (H)- headspace; K_1, K_2 and K_3 are the equilibrium constants between the different phases; C_0 is the initial concentration of the analyte; C_L, C_F and C_H are the concentrations of the analyte in liquid phase, fibre and headspace, respectively; V_L, V_F and V_H are the volumes of the liquid phase, fibre and headspace, respectively.

Types of coating fibres. The fibre is composed by polymers held into a support with a protective coating. It is inserted in a syringe designed with a piston which allows one to introduce and retract. It is also possible to reutilize and replace. Figure I.3.4 shows the fibre inserted on the support. There are several commercial types of coating fibres and their use depends on the selected compounds to be analysed. The affinity of the coating fibre to the analyte depends, for example, on the polarity of both. The polymers can be divided in denser and less dense polymers. With denser polymers, the process is an adsorption process as the analytes remain in the superficial layer of the polymer. In the case of a less dense polymer, the analytes penetrate also in its pores, conducing to a simultaneous process of adsorption and absorption. Commercially, the fibres available are, for example, polydimethylsiloxane (PDMS), polyacrylate (PA), carbowen/ polydimethylsiloxane (CAR-PDMS), divinylbenzene/ carboxen/ polydimethylsiloxane (DVB-CAR-PDMS), polydimethylsiloxane/ divinylbenzene (PDMS-DVB), and carbowax/ divinylbenzene (CW-DVB) (Pillonel *et al.*, 2002). The polar analytes attracted by polar phases are, for instance, PA or CW. The fibres with thicker layer suitable for volatile compounds can also be used for less volatile compounds, requiring more extraction times. The fibres with less thick layers, such as PDMS 7 and 30 μm, are suitable for bigger molecules. On the other hand, the porous fibres with CAR or DVB layers can also retain small analytes and are appropriate for C_2-C_6 analytes.

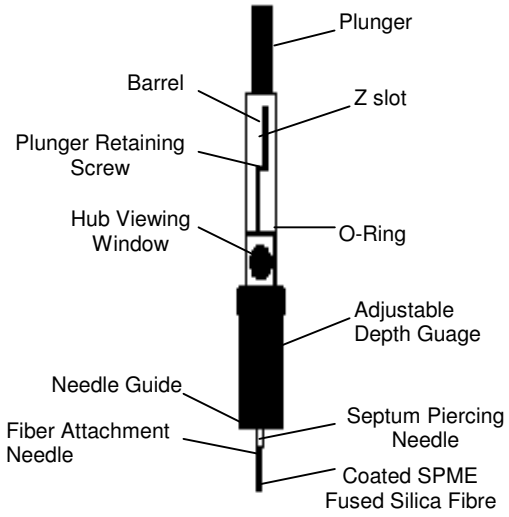


Figure I.3.4. SPME fibre with support

Adsorption vs. Absorption. The transport of analytes from the matrix into the coating begins as soon as the coated fibre is placed in contact with the sample. The performance between the liquid and the solid coatings is substantially different (Figure I.3.5.) (Pawliszyn, 2000). It is quite useful to compare the adsorptive and the absorptive performing modes. In both cases, the extraction process begins with the adsorption of analytes at the extraction phase-matrix interface, and then diffusion of analytes into the bulk of the extraction-phase follows. If the diffusion coefficients of the analytes in the extraction phase are high, then the analytes partition between the two phases and adsorption extraction is fully accomplished. This process is aided by thin extraction phase coatings or the convection of the samples matrix (if flowing liquid). However, if the diffusion coefficient is low, the analyte remains at the interface and adsorption results. The main advantage of absorption extraction (partitioning) is a linear isotherm over a wide range of analyte and interferences concentrations, because the property of the extraction phase does not change substantially until the extracted amount is approximately 1% of the extraction phase weight. However, in adsorption extraction, the isotherm has highly nonlinear concentrations when the surface coverage is substantial. This causes a particular problem in the equilibrium methods because the response of the fibre to the analyte at high sample concentrations depends on the concentration of both analytes and interferences. The advantages of the solid sorbents include higher selectivity and capacity for polar and volatile analytes.

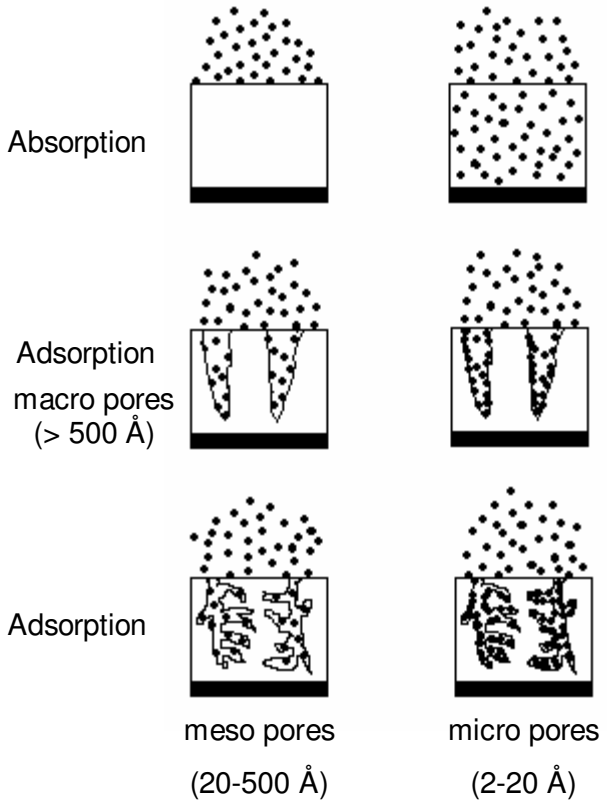


Figure I.3.5. Schematic representation of absorptive *versus* adsorptive extraction and adsorption in small *versus* large pores (Adapted from Pawliszyn, 2000)

Modes of extraction. From the different modes of SPME sampling available at the moment, only headspace extraction (HS) is described because it is the only mode used in this work. In the headspace mode (Figure I.3.3), the analytes are extracted from the gas phase around the sample. In this case, the fibre is protected from adverse effects caused by non-volatile, high-molecular-weight substances present in the sample matrix. The headspace mode also allows matrix modifications (including pH adjustment) without affecting the fibre. The amount of an analyte sorbed on the fibre, and the resulting sensitivity, are determined both by sorption kinetics and distribution coefficient of the compound between the coating fibre (Zhang *et al.*, 1994), the headspace and the sample. SPME is, however, sensitive to experimental conditions. Any change in experimental parameters, which affects the distribution coefficient and the sorption rate, will influence the amount sorbed and the corresponding reproducibility and sensitivity of the method (Yang and Peppard, 1994). The partition equilibrium of the volatile compounds between the headspace of the sample and the SPME coating depends on the exposure time,

temperature, volume of sample, concentration of salts, and type and uniformity of the matrix (Yang and Peppard, 1994; De la Calle García *et al.*, 1996; Fischer and Fischer, 1997; Jia et al., 1998; Rocha *et al.*, 2001). The ratio of the volume of the liquid phase to the headspace volume (1/β) may be considered, where β= headspace volume / sample volume (De la Calle García *et al.*, 1998b).

SPME provides many advantages over conventional sample preparation techniques. This methodology is simple to use, relatively fast, does not require solvent extraction, and allows characterization of the headspace composition in contact with the sample (Arthur and Pawliszyn, 1990; Arthur *et al.*, 1992; Zhang and Pawliszyn, 1993). Conventional methods such as steam distillation or direct solvent extraction produce extracts with a flavour composition that is representative of the liquid matrix and not of the headspace. The molecules present in the headspace are indeed responsible for the smell that is perceived by the olfactory system if they are in concentrations above their SPL. Another drawback of the conventional methods is that the extracts have to be concentrated prior to analysis, resulting in loss of low-boiling volatiles. Also, the solvent required by successive dilutions will mask the first eluting peaks.

I.3.2. Methodologies for characterization of wine volatile composition and wine aroma

The extraction/concentration step is usually followed by capillary one-dimensional gas chromatography, coupled with several kinds of detectors, namely flame ionization detector (FID) and mass spectrometry (MS) detector (ion trap or quadrupole). In spite of the great separation power of conventional one-dimensional modern chromatographic techniques, the complex nature of the samples, including different kinds of chemical classes requires extended GC runs. Furthermore, deep analyses of the chromatograms frequently indicate that some peaks are the result of two or more co-eluting compounds. As a consequence of chromatographic co-elution, reliable MS identification is not possible.

One-dimensional chromatographic processes are widely applied in the analysis of wines. Although such methods often provide rewarding analytical results, the complexity of many naturally occurring matrices exceeds the capacity of any single separation system. As a consequence, very recently a considerable research has been dedicated to the combination of independent techniques with the aim of strengthening resolving power

(Tranchida *et al.*, 2004). Comprehensive two-dimensional Gas Chromatography (GCxGC) employs two orthogonal mechanisms to separate the constituents of the sample within a single analysis. The technique is based on the application of two GC columns coated with different stationary phases, such as one apolar and one polar column, connected in series through a special interface (modulator). The separation potential is greatly enhanced when compared to the one-dimensional GC. The GCxGC has recently been used for wines analysis (Ryan *et al.*, 2005). The two-dimensional Gas Chromatography coupled with a Time-of-Flight Mass Spectrometer (GCxGC-ToFMS) brings advantages such as full mass spectra acquisition at trace level sensitivity and mass spectral continuity, which allows for deconvolution of spectra of co-eluted peaks (Beens *et al.*, 1998; Philips, 2001).

Most studies concerning the analysis of the volatile compounds from wine use one-dimensional gas chromatography with a mass spectrometer detector (GC-MS) (Falqué *et al.*, 2002; Fernández-González *et al.*, 2003). Some recent studies use GC-multidimensional (GC-MD) (Ryan *et al.*, 2005). Other methods are used for the sensory characterization of samples, such as gas chromatography coupled with an olfactometric detector (GC-O) (López *et al.*, 2003; Martí *et al.*, 2003; Lee *et al.*, 2003; Culleré *et al.*, 2004), sensory analysis (Escudero *et al.*, 2004) and electronic nose (Pinheiro *et al.*, 2002).

From the previously mentioned methods, only the gas chromatography (one-dimensional) coupled with FID or quadrupole-MS detectors, used during this work, will be reported.

I.3.2.1. Gas chromatography (GC)

The GC is an analytical technique of separation, where a mixture of compounds (solutes) is detached into separate components (Sekomburg, 1990; Cazes, 2005). The improvement of instrumentation (capillary columns, software for integration, temperature system, new detectors, etc.) offers a good resolution for volatile compounds using gas chromatography technique. The main limitation is the lability of solutes as the compounds need to be stable within the temperature required for their volatilization, *i.e.* to be suitable for GC analysis, a compound must have sufficient volatility and thermal stability.

Figure I.3.6 shows the main parts of a basic GC system. A gas chromatograph is composed by the following components:

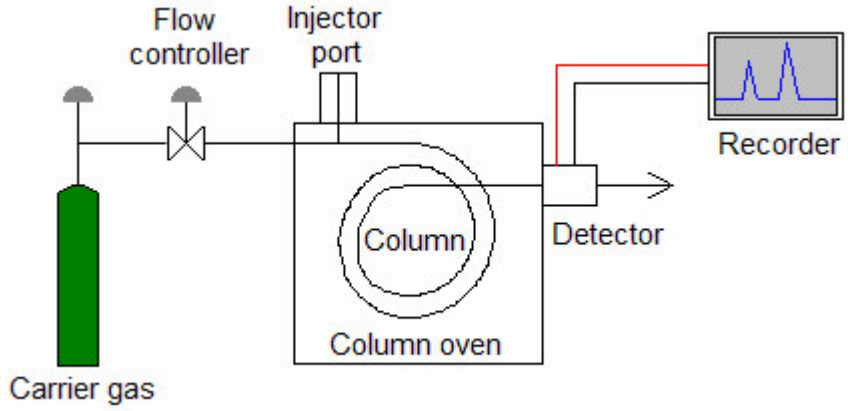


Figure I.3.6. Basic components of a GC system

a) ***Gas system***, which is composed by one or more high purity gases. The gases to be used must be chemically inert and do not react with both column and/or components of the sample. One of the gases (called the carrier gas) allows the transportation of the sample, from its introduction in the chromatographic system to the detection system, passing though the column where the separation of compounds is performed. Commonly used gases include nitrogen, helium and hydrogen. The choice of the carrier gas is often dependent upon the type of detector which is used. The carrier gas linear velocity or flow rate directly influences the retention time of the compounds and the efficiency of the separation (Sekombur, 1990; Cazes, 2005). It is controlled by adjusting the carrier gas pressure at the front of the column (commonly called the head pressure). The pressure setting is dependent on the type of carrier gas, the column length and diameter, column temperature, and the desired linear velocity or flow rate. For capillary columns, the average linear velocity (ū) is better and more meaningful measure of the carrier gas then the flow rate (F). The average linear velocity can be thought as the average 'speed' of the carrier gas in cm/s (*i.e.* centimetres of column travelled per second by a carrier gas molecule). The average linear velocity is calculated using the equation as follow: ū (cm/s) = L / t_M, where L is the column length (cm) and t_M is the retention time of an unretained peak (s).

The effect of carrier gas average linear velocity on efficiency is best illustrated using a van Deemter curve or plot, which shows that there is an average linear velocity that provides maximum efficiency. This curve is different for each carrier gas.

b) ***Temperature control system*****,** composed by three zones where the temperature must be controlled: injection port, column and detector. The success of the separation depends on the adequate temperatures choice for each zone.

c) ***Injector***; that allows the introduction of the sample in the system, putting it in contact with the carrier gas (Sekomburg, 1990). Usually, the injectors are equipped with split and splitless types of injection, called split/splitless injectors. Split injectors are used for more concentrated samples since only a small fraction of the injected sample is introduced into the column and splitless injections are used for lower concentration or trace level samples. The carrier gas flows through the liner and into the column. The sample is introduced into the injector usually with a syringe (that pierce the septa) or an exterior sampling device. Since the injector is heated (usually to 150-250ºC), the volatile solutes of the sample vaporize. The resulting sample vapour and microscopic liquid droplets are mixed with the carrier gas, and transported into the column. This vaporization and transportation process is responsible for introducing the sample into the column.

d) ***Column***, where the separation of the compounds is performed. A capillary GC column is comprised of two major parts: tubing (fused silica and stainless steel materials) and stationary phase (liquid or solid) with different sizes (internal diameter i.d. and film thickness) according to its application (Cazes, 2005). There are numerous stationary phases (solid adsorbent, liquid retained on a solid support or impregnated liquid on the internal wall of capillary column). Most are high molecular weight, thermally stable polymers that are liquids or gums. The most common stationary phases of this type are the polysiloxanes and polyethylene glycols. A thin film (0.1-10.0 μm) of a high molecular weight, thermally stable polymer is coated onto the inner wall of small diameter (0.05-0.53 mm i.d.) tubing. The column is maintained in a temperature controlled oven. Upon introduction into the column, solutes travel through the column at different rates primarily determined by their physical properties (structure of the compounds), the column and chemical structure of the stationary phase. The solute molecules were distributed between

the stationary and mobile phases. The molecules in the mobile phase are carried down the column; the molecules in the stationary phase are temporarily immobile and do not move down the column.

Following, some important terms and definitions concerning the chromatographic methods:

Retention time (t_r) is the time it takes a solute to travel through the column. The retention time is assigned to the corresponding solute peak. The retention time is a measure of the amount of time a solute spends in a column. It is the sum of the time spent in the stationary phase and the mobile phase.

Retention time of an unretained time compound (t_m) or hold-up time is the time for an unretained compound to travel through the column. The unretained molecules of the solute do not enter the stationary phase as they travel down the column at the same rate as the carrier gas. This is equivalent to the time a compound spends in the mobile phase.

Retention factor (k) is another measure of retention. It is the ratio of the amount of time a solute spends in the stationary and mobile phases (carrier gas). It is calculated using the following equation:

$$k = \frac{t_r - t_m}{t_m} = \frac{t'_r}{t_m}$$

where t_r is the retention time, t'_r is the adjusted retention time, and t_m is the retention time of unretained compounds.

Separation factor (α) is a measure of the time or distance between the maxima of two peaks. It is calculated using the following equation: $\alpha = k_2/k_1$, where k_1 is the retention factor of the first peak and k_2 is the retention factor of the second peak.

The column efficiency is expressed by the *number of theoretical plates* (N). Theoretical plate's numbers are an indirect measure of peak width for a peak at a specific retention time. Columns with high plate numbers are considered to be more efficient (*i.e.* higher column efficiency) than column with lower plate numbers. Column efficiency is a function of the column dimensions (diameter, length and film thickness), the type of carrier gas and its flow rate or average linear velocity, and the compound and its retention.

Another measure of column efficiency is the *height equivalent to a theoretical plate* (H). It is calculated using the equation: H= L / N (mm), where L is the column length (mm) and

N is the number of theoretical plates. The shorter each theoretical plate, the more plates "contained" in a given length of column.

Resolution. The higher is the resolution the less the overlap between two peaks. Separation is only the distance or time between two peak maxima (α). Resolution takes both α and the width of the peaks into account.

As each solute elutes from the column, it enters the heated detector.

e) ***Detection system***, located at the end of the column, produces an electrical signal for each compound separately, which is amplified adequately and send to the data system. Most detectors require one or more gases to function properly (combustion, auxiliary and makeup gases). Through the detector pass the gases and the separated compounds.

From the several detectors available, only FID and quadrupole-MS are described. In the FID, the compounds are burned in a hydrogen-air flame (Sekomburg, 1990). Carbon containing compounds produce ions that are attracted to the collector. The number of ions hitting the collector is measured and a signal is generated. The gases used are: hydrogen and air for combustion and helium or nitrogen as makeup. In the quadrupole-MS, which operates under vacuum, the ionization of the molecules is mainly carried out by electron impact (EI), where the molecules fragment into characteristic charged ions or fragments (Kitson *et al.*, 1991). The resulting ions are focused and accelerated into a mass filter, usually a quadrupole mass analyser. It is responsible for filtering sample ions based on their mass-to-charge ratio (*m/z*), *i.e.* it selectively allows all ions of a specific mass to pass through to the electron multiplier. All the ions of the specific mass are detected. The mass filter scans stepwise through the designed range of masses several times per second and the total number of ions is counted for each scan. The abundance or number of ions per scan is plotted versus time to obtain the mass spectrum. A mass spectrum is obtained for each scan which plots the various ion masses versus their abundance or number. It can operate in full scan (inclusive range of masses) or SIM (only select ions). The GC-MS allows the separation and identification of the compounds by comparing the retention time and the mass spectrum of each compound with that of the data library system.

f) ***Recorder***, where the chromatogram is carried out. The signals of detector are registered as peaks, which together comprise the chromatogram. The chromatogram gives information that allows performing a quantitative and qualitative analysis. The

magnitude of the signal is recorded by the data system and is plotted versus time (from the time of injection) and a chromatogram is generated. The detector sensivity is measured as a signal-to-noise (S/N) ratio. The signal is the height of the peak, and the noise is the height of the peak-to-peak signal. For accurate quantitative chromatography, it is best to operate within the linear range of the detector. This is the range where the peak area is directly proportional to the solute amount, allowing the quantification of unknown compounds.

g) ***Computer/ integrator***, is the instrument's incorporated informatics system that obtains the results and carries out the data treatment. It performs automatically the peaks integration, retention time, etc, and prints the final information of the analysis.

I.3.3. Methodologies for data treatment

The analysis of volatile compounds by GC-FID and GC-MS allows obtaining a chromatogram (Figure I.3.7), which provides quantitative and qualitative information. The quantitative information is obtained by peak area or height and the qualitative information is obtained by the position of the peaks (retention time) and/or by mass spectrum. With these informations it is possible to identify and quantify volatile compounds using pure standards, mass spectrum, retention time and kovats index. The peaks areas can be used to estimate the concentration of each compound by an internal standard or an external calibration curve, or can be use for multivariate analysis.

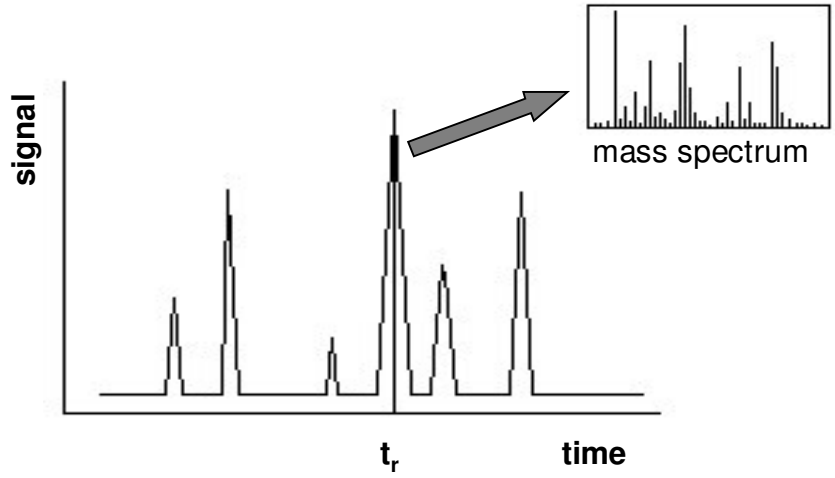


Figure I.3.7. Simplified scheme of a chromatogram (each peak corresponds to a mass spectrum obtained by GC-MS analysis); t_r- retention time

I.3.4. Methodologies for rapid distinction of wines based on the global volatile signature

The technique using solid-phase microextraction-mass spectrometry-multivariate analysis (SPME-MS-MVA) was developed in 1999 for the rapid characterization of foods (Marsili, 1999a), as an alterative approach to current used commercial *e*-nose instruments. This methodology has been used for food applications such as the detection of defects in milk (Marsili, 1999ab; Marsili, 2000; Marsili, 2001) and for the classification of cheeses (Pérès *et al.*, 2003). SPME-MS-MVA, where the analysis of the samples is performed by direct injection of its volatile fraction into the mass spectrometer ionization chamber, allows to obtain in a few minutes a characteristic spectrum called the “signature” or “spectral fingerprint” (Figure I.3.8). This signature can then be used for the classification of samples, prediction of sensory properties, estimation of technological parameters and for the detection of compounds directly responsible for the odours (Marsili, 1999a; Marsili, 2000; Goodner and Rouseff, 2001; Pérès *et al.*, 2001, 2003;). It also has the advantage to provide specific detailed chemical information that is not possible with current e-nose instruments. Furthermore, these methodologies based on the analysis of the “spectral fingerprint” are faster than the conventional gas chromatography-mass spectrometry (GC-MS) analysis. The GC-MS analyses are time-consuming due to the time necessary for obtaining an adequate chromatographic resolution, and especially due to the time required for data interpretation.

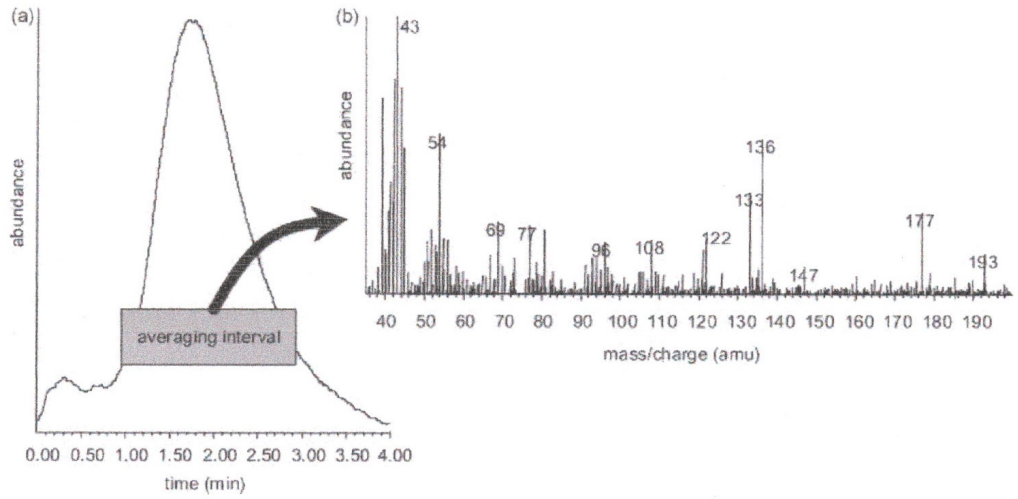


Figure I.3.8. Signal produced by an HS-MS system (Pérès *et al.*, 2003)

The general principle of headspace solid-phase microextraction-mass spectrometry (HS-SPME-MS) systems consists of introducing volatile components present in the headspace of a sample, that have been extracted by solid phase microextraction, without prior chromatographic separation, into the ionization chamber of a mass spectrometer (Pérès *et al.*, 2003). The spectrum resulting from simultaneous ionization and fragmentation of the mixture of molecules introduced constitutes a fingerprint that characterizes the product being analysed. When this spectral information is analysed it is possible to determine the composition of the sample. The HS-SPME-MS comprises a module for extraction and injection of the volatile components, directly linked through a transfer line to a mass spectrometer (Pérès *et al.*, 2003).

Figure I.3.9 summarizes the main components of a HS-SPME-GC-MS. In this system, the extraction of the volatile components is given by SPME, followed by its introduction on a GC-MS injector. The transfer line ensures coupling of the extraction-injection module to the mass spectrometer. This line may allow rapid transfer of the extracted molecules between the extraction-injection modules to the mass spectrometer. The length is usually in the range of 0.5-5 m, but is also possible to use a normal column, using appropriate temperatures in order to allow an unresolved profile.

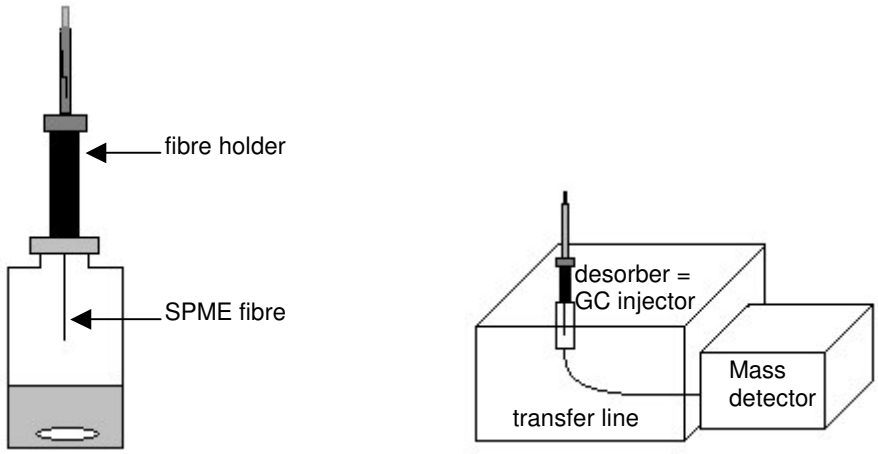


Figure I.3.9. HS-SPME-GC-MS system (adapted from Pérès *et al.*, 2003)

I.4. Objectives

The Bairrada Appellation represents an important role in the Portuguese wine production. As mentioned earlier, from the recommended white varieties used in this region, the most important is Fernão-Pires, known in this region as Maria-Gomes. It represents 70% of the white grape vineyard. Bical, Cerceal and Arinto are other important white varieties also used, which represent 10%, 10% and 5% respectively. The study of the volatile composition for these varieties and their potentialities renders possible to enhance the knowledge about their behaviour during the technological processes, allowing to find ways to increase the quality of sensory properties of wines. Nevertheless, the aroma composition of these varieties was not yet characterized.

Hence, the main goals of this work are:

1. To characterize the main white variety from Bairrada Appellation, *Vitis vinifera* L. Fernão-Pires variety, by studing its volatile composition, and comparing it with the other major white varieties (Bical, Cercial and Arinto).

2. To evaluate the effectiveness of a broad-spectrum enzyme preparation, normally used in the Bairrada Appellation, on the volatile composition and aroma quality of Fernão-Pires wine.

3. To propose the use of the Fernão-Pires musts varietal composition to predict the quality of wine aroma.

4. To develop a methodology for the rapid distinction of wines based on their global volatile signature of the wines headspace obtained by headspace solid-phase microextraction-gas chromatography-mass spectrometry-principal component analysis (HS-SPME-GC-MS-PCA).

5. To propose an approach to study, in a wine model solution, the interactions between wine polymeric fraction and three ethyl esters based on headspace-solid phase-microextraction followed by gas chromatographic analysis (HS-SPME-GC).

II

Samples and Methodologies

Summary

This chapter describes in detail the experimental procedures concerning the developed work (section III – Results and Discussion).

II.1. Samples ……..…….…… 85

II.2. Methodologies for characterization of volatile compounds …..…….... 86

II.3. Developed method for rapid distinction of wines based on the global volatile signature ……..... 95

II.4. Developed method to study the interactions between the wine polymeric fraction and ethyl esters ……...…….. 100

II.1. Samples

II.1.1. Grapes

Healthy-state *Vitis vinifera* L. Fernão-Pires grapes from the 2002 harvest were collected in Bairrada Appellation, in the maturity state. The grapes were manually divided in two fractions: skin and pulp. Skins were stored in plastic bags in vacuum and pulp was transferred to 1.5 L bottles. They are stored at -20 ºC until use in the following days of their preparation.

II.1.2. Musts

The monovarietal musts used in these studies were produced from Fernão-Pires grapes (1998, 1999, 2000 and 2002) and, Bical, Cerceal and Arinto grapes (1998, 1999). Their production (microvinification) was carried out in Estação Vitivinícola da Bairrada (EBV), Anadia, Portugal, from healthy-state grapes collected in the Bairrada Appellation in the maturity state. The musts were submitted to an SO_2 (60 mg/L) treatment, followed by a skin contact step for 6h at room temperature, and clarification by centrifugation. All samples were transferred to 1.5 L bottles and stored at -20 ºC until use in the following days of their preparation.

II.1.3. Wines

The monovarietal wines used in these studies were produced from Fernão-Pires and Bical grapes (1997). Their production was carried out in EBV, Anadia, Portugal, from the healthy-state grapes collected in the Bairrada Appellation in the maturity state. The grapes were racked, pressed and sulfited (60 mg/L). The musts were inoculated with active dry yeast VL1, and the fermentation occurred at 20 ºC in 5 L glass vessels with a headspace of 250 mL. After fermentation and deposition of the suspended solids, the liquids were transferred to 5 L glass vessels, and separated into two groups. A commercial enzyme preparation normally used in Bairrada white winemaking, Lallzyme de Lalvin, obtained from Lallemand/Proenol (Vila Nova de Gaia, Portugal), was added to one group of wines (1 g/hL), and the second group was left as reference. This enzyme preparation is reported by the producer to have activity of *β*-glucosidase, pectinase, arabinosidase and rhamnosidase. After one month, the wines were decanted and transferred to 0.75 L

bottles and sulfited. The bottles were stoppered and stored at 10ºC until use. The analyses were carried out one year after bottling.

The wines produced were sensory evaluated in the tasting room of EVB by five official tasters. All the tasters distinguished between Fernão-Pires wines used as reference and enzyme-treated wines, and all recognized that the enzymatic treatment clearly benefited the overall wine aroma. The sensory analysis concerning the Bical variety indicated that four of the five judges distinguished between the reference and enzyme-treated wines. Furthermore, only two of the tasters preferred the enzyme-treated wines. These results clearly indicate that the tasters considered that the enzymatic treatment benefited the global aroma of Fernão-Pires wine but did not promote significant modifications in the Bical variety.

II.2. Methodologies for characterization of volatile compounds

II.2.1. Methodology for grapes extraction – Use of Amberlite XAD-2 resin

The volatile compounds from grapes were extracted using an Amberlite XAD-2 resin. This method allows obtaining both free volatile and glycosidically-linked fractions. Previously to the extraction, some procedures were executed:

i) Grape preparation

The skinned berries were centrifuged at 10000 rpm during 25 min at 4 ºC, in order to separate the solid from the liquid pulp. The supernatant represented the liquid pulp (juice) fraction. The seeds were separated manually from the solid pulp fraction.

ii) Amberlite XAD-2 resin preparation

The resin Amberlite XAD-2 (20-60 mesh), obtained from Supelco, Inc. (Bellefont, PA), was used to separate and extract the free volatile compounds and the glycosidically-linked compound fraction. The resin was submitted to a pre-treatment before use. It was sequentially washed with methanol and pentane/dichloromethane (2:1 v/v) by Soxhlet (each one for 8h), and then dried and stored in methanol. Before the extraction procedure the resin, suspended in methanol, was poured into a glass column (50 x 1 cm i.d.). The

packed column contained about 12 cm of resin. Methanol (25 mL) and pentane/dichloromethane (2:1 v/v) (25 mL) were passed through it and finally water (50 mL). The column was then ready for use. All the solvents used for the extraction of volatile compounds were analytical grade and were redistilled before use: pentane (boiling point: 35-36ºC; d=0.63); dichloromethane (boiling point: 40ºC, d=1.36) and methanol (boiling point: 64-65ºC, d=0.89).

iii) Internal standard preparation

The internal standard used, 3-octanol (≥99.0 %) from Aldrich Chemical Co (Milwaukee, WI, USA), was prepared in absolute ethanol (purity ≥ 99.8%) from Riedel-de-Haën (Seelze, Germany). Different concentrations of 3-octanol were prepared taking into account the different samples examined (grapes, musts and wines), as is indicated in this section.

iv) Buffer solutions

Two different buffer solutions were prepared: phosphate buffer pH 7.0 and phosphate-citrate buffer pH 5.0. The compounds used to prepare the buffers were phosphate potassium monobasic (KH_2PO_4) (Merk), phosphate sodium dibasic (Na_2HPO_4) (Merk), and citric monohydrate acid ($C_6H_8O_7.H_2O$) (Riedal-De-Haën). The first buffer was prepared by adding 39.0 mL of a KH_2PO_4 solution (0.5M) to 53.6 mL of a Na_2HPO_4 solution (0.5M), filling with water until 1L. The second buffer was prepared by adding 100 mL of a Na_2HPO_4 solution (0.1M) to 100 mL of $C_6H_8O_7.H_2O$ solution (0.05M), filling with water until 250 mL.

The extraction methodology was performed in 2 steps. In the first step, the free volatile compounds were extracted from the different grape fractions (skin, solid pulp and liquid pulp), and in the second, the glycosidically-linked compounds were extracted from the same fractions.

Procedure for the free volatile compounds extraction:

1. The solid fractions (100.0 g of skins and 50.0g solid pulp) were independently homogenized in 250 mL of phosphate buffer pH 7.0 (0.1M) and after 24h, were centrifuged in order to obtain the skin and solid pulp fractions.
2. Each fraction: skins, solid pulp and liquid pulp (100 mL) was passed through the resin XAD-2.
3. The free volatile compounds were eluted with pentane/dichloromethane (2:1 v/v) (50 mL) and 3-octanol (81.90 µg for skins, 12.29 µg for pulp, 8.19 µg for juice, respectively) was added as internal standard.
4. The column was rinsed with 15 mL of water to eliminate interfering substances, such as sugars.
5. The pentane/dichloromethane (2:1 v/v) extractions were dried with anhydrous sodium sulfate (Merk) and then the excess of low-boiling solvent was removed by distillation at low pressure using a trap with liquid nitrogen to a final volume of 500 µL.
6. The free fractions obtained from skin, solid pulp and liquid pulp were then analysed by gas chromatography- mass spectrometry (GC-MS).

Procedure for the glycosidically-linked volatile compounds extraction:

1. The glycosidically-linked fraction was eluted with methanol (75 mL). The methanolic extract was evaporated under vacuum until dryness.
2. The residue was then dissolved with a phosphate-citrate buffer pH 5 (10 mL) and an extraction with pentane/dichloromethane 2:1 v/v (50 mL), for 3h, was made to ensure that no free volatile compounds were present.
3. To eliminate the polyphenolic compounds that could interfere with the process, all samples were passed through polyvinylpirrolidone (PVP) (Sigma-Aldrich). The PVP, an insoluble compound with high molecular weight, was used to improve the stability of the enzymes by removing phenolic compounds.
4. To release the aglycones from the glycosidically-linked compounds, a commercial enzyme (Novoferm 12G, obtained from Novo Nordisk Ferment Ltd, Dittingen) was used. This enzyme preparation, reported by the producer to have

activity of *β*-glucosidase, pectinase, arabinosidase and rhamnosidase, was chosen for hydrolysis of the aglycones. The enzyme was added and allowed to act for 24h at 35 ºC.

5. The generated free volatile compounds from the glycosidically-linked fractions of skin, solid pulp and liquid pulp were then extracted with pentane/dichloromethane 2:1 (v/v) (75 mL) and 3-octanol (according to step 3 from the procedure for the free volatile compounds extraction) was used as internal standard.
6. Those fractions were then concentrated to a final volume of 500 μL, stored in a screw-top vial at -20 ºC until analysis by GC-MS.

Two independent extractions were done for each of the fractions (skin, solid pulp, liquid pulp) in a total of 12 and each extract was injected into the GC unit three times.

II.2.2. Methodologies for musts extraction - Use of liquid-liquid continuous extraction

The Figure II.2.1 represents the general procedure for obtaining the musts volatile composition by liquid-liquid continuous extraction. This methodology comprises two approaches: *i)* one corresponds to the direct liquid-liquid extraction of the musts which allows to obtain the free volatile compounds (F) – exemplified in a); *ii)* the other approache involves the heat treatment of the musts followed by an enzymatic treatment and liquid-liquid extraction, which allowed to obtain the potential volatile compounds (PVC) – exemplified in b).

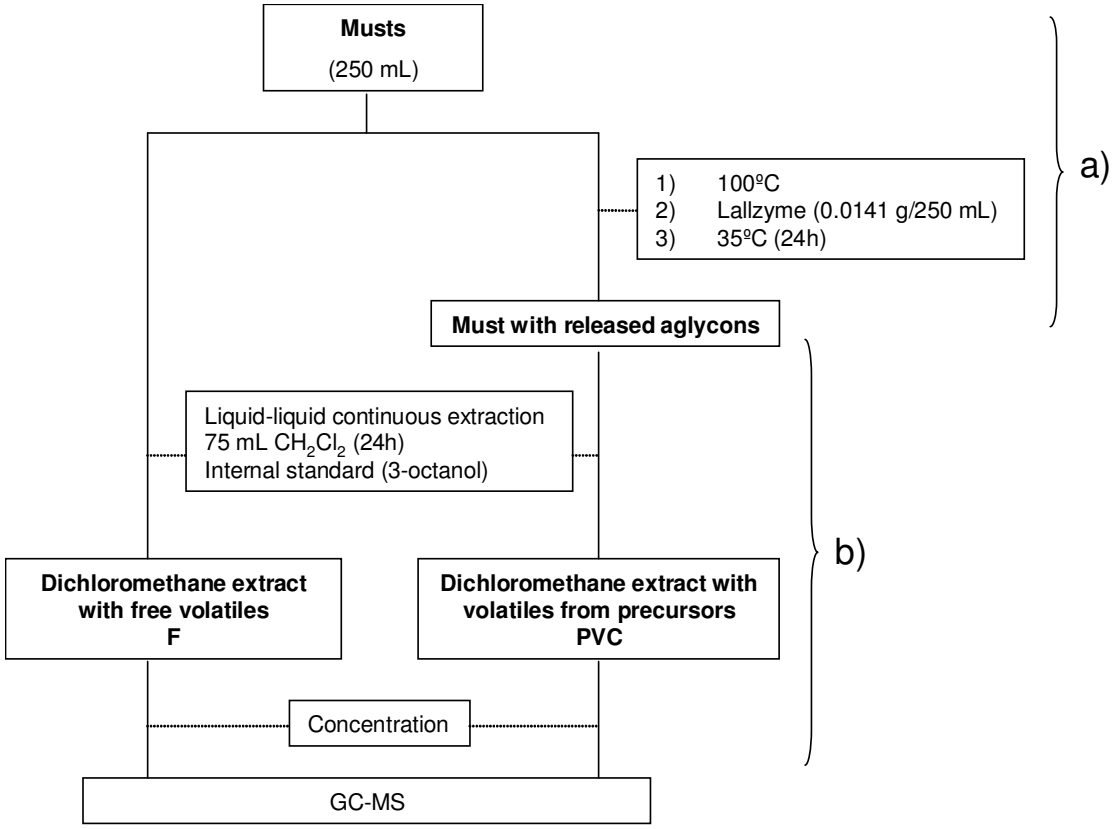


Figure II.2.1. Diagram of treatments leading to free volatile components (F) and the precursors volatile compounds (PVC) of the white varieties musts used in this work.

a) Heat treatment and enzyme hydrolysis of musts

Procedure:

1. The musts of each variety were heated under stirring for 15 min at 100 ºC (Cordonnier and Bayonove, 1974), in order to inactivate the endogenous enzymes and to eliminate the free volatile components.
2. A commercial enzymatic preparation usually used in white winemaking, Lallzyme de Lalvin, obtained from Lallemand/Proenol (Vila Nova de Gaia, Portugal) (14.1 mg), was added to 250 mL of must. This enzyme preparation is reported by the producer to have activity of *β*-glucosidase, pectinase, arabinosidase and rhamnosidase.
3. The mixture was incubated at 35 ºC for 24 h.
4. After incubation, the released aglycones were extracted as described below.

b) Extraction method

The extraction procedure was a modification of the method described by Etiévant (1987). The musts without and with heat and enzymatic treatments, which contained, respectively, the free (F) and the potential volatile components (PVC) were submitted to a process of liquid-liquid continuous extraction with dichloromethane.

Six independent extractions were done for each one of the four must samples, in a total of 24 extractions (section III.1). For each harvest, six different bottles of each variety were used, three to obtain the F fraction and the other three to obtain the PVC fraction (section III.3). Two independent extractions were done for each bottle. Six independent extractions were done for each one of the eight must samples, in a total of 48 extractions (section III.4).

Procedure:

1. The must (250 mL), supplemented with internal standard (130 μg of 3-octanol to Fernão-Pires variety and 65 μg to Bical, Arinto and Cerceal varieties), and 75 mL of dichloromethane were placed in the extractor, constructed in the Glass Laboratory of the Department of Chemistry the University of Aveiro, according to Etiévant (1987). (Figure II.2.2).
2. Extractions were carried out for 24 h at *ca.* 50 ºC.
3. The dichloromethane extracts were cooled to -20 ºC to separate the frozen water from the organic phase by decantation and then dried over anhydrous sodium sulfate.
4. The excess of low-boiling point solvent was removed by distillation at low pressure using a trap with liquid nitrogen.
5. The concentrate (about 1 mL) was stored in a glass screw-top vial at -20 ºC.
6. The musts extracts were analysed by GC-MS.

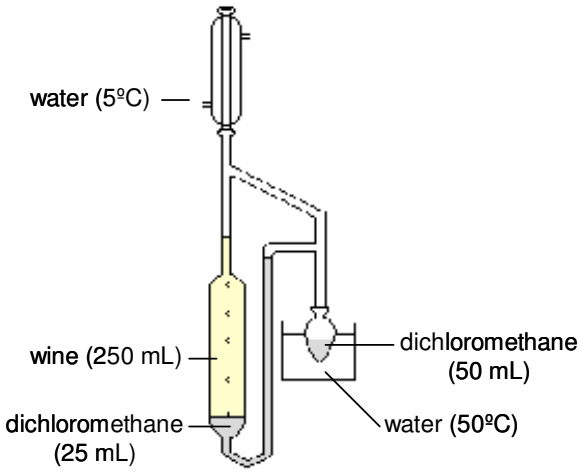


Figure II.2.2. Extractor used in liquid-liquid continuous extraction procedure.

II.2.3. Methodologies for wines extraction – use of liquid-liquid continuous extraction

The Figure II.2.3 represents the general procedure concerning the analysis of the wines volatile compounds by liquid-liquid continuous extraction.

The wines (250 mL) supplemented with internal standard (1 mg of 3-octanol in each extraction) were submitted to a process of liquid–liquid continuous extraction with dichloromethane, as described in II.2.2.b for the musts. Four independent extractions were done for each of the four wine samples, obtained from two independent fermentation vessels, in a total of 16 extractions. Each extract was injected twice into the GC unit.

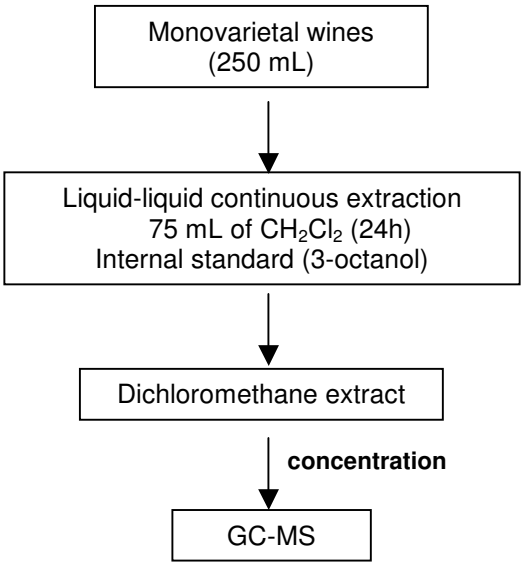
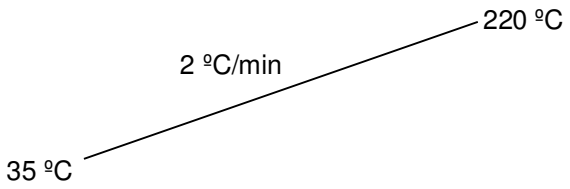


Figure II.2.3. Diagram representing the general methodology used for wine volatile compounds extraction.

II.2.4. Chromatographic conditions for analysis of grapes, musts and wines

The volatile compounds extracts of grapes, musts and wines were analysed by GC-MS, on a Hewlett-Packard (Palo Alto, CA, USA) 5890 series II gas chromatograph, equipped with a 30m × 0.32mm (i.d.), 0.25 µm film thickness DB-FFAP fused silica capillary column (J&W Scientific Inc., Folsom, CA, USA) and connected to a Hewlett-Packard quadrupole mass selective detector, according to the method described by Rocha *et al.* (1996). Splitless injections were used. Chromatographic conditions are described in Table II.2.1. The DB-FFAP column is a high polarity column and is recommended for the analysis of volatile fatty acids and phenols. It is recommended for operation limits temperature between 40 and 250 ºC.

Table II.2.1– Chromatographic conditions for grapes, must and wine analysis

Parameter	Conditions
Injector temperature	255 ºC
Carrier gas	Helium (12 psi) (1.7 mL/min)
Column	DB-FFAP (30 m x 0.32 mm i.d., 0.25 μm film thickness)
Temperature program	 Total time= 92.5 min
Transfer line temperature	250 ºC
Detector	MS (impact mode: 70 eV, scanning range *m/z* 30-300 1 s cycle)

II.2.5. Identification and quantification of volatile compounds

In order to compare and relate the information obtained from grape and must analysis, all results were displayed taking into account: the skin, solid pulp and liquid pulp proportion in grape (19, 10 and 68%, respectively).

Identification of volatile compounds was achieved by comparison of the GC retention times and mass spectra with those, when available of the pure standard compounds. All mass spectra were also compared with those of the data system library (Wiley 275 software from Hewlett-Packard) and other published spectra (*Eight Peak Index of Mass Spectra,* 1974). Estimated concentrations for all compounds were made by peak area comparisons with the area of the known amount of internal standard. Chromatographic resolution adequate for peak quantification was obtained. Peak integrations were performed when the signal-to-noise ratio was higher than 5. The reproducibility of the extracts analysed was expressed as coefficient of variation (CV) in the tables and as error bars in the figures.

The data obtained during the studies were treated using Principal Component Analysis (PCA) or Contrast-PCA, which were gently performed and provided by Dr. António Barros, from Departamento de Química, Universidade de Aveiro.

II.3. Developed method for rapid distinction of wines based on the global volatile signature

The headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry, followed by principal component analysis of the data (HS-SPME-GC-MS-PCA), was the methodology developed for the rapid distinction of wines.

Eight bottles from each one of two white *V. vinifera* L. monovarietal wines were used: Fernão-Pires (FP) and Arinto (Ar), from 1999 harvest, provided by Estação Vitivinícola da Bairrada, Portugal.

The headspace SPME was used to extract and concentrate the volatile and semi-volatile fractions. A SPME holder from Supelco Inc. (Bellefonte, PA, USA) was used to perform headspace manually. Two SPME fibres, a Polyacrylate (PA) 85 μm film thickness (absorbent or liquid-phase coated fibre) and a Carbowax-divinylbenzene (CW-DVB) 65 μm film thickness (mixed coating that contains a liquid polymer and solid particles), both from Supelco, were tested. PA is an absorbent or liquid-phase coated fibre. The absorptive fibres are indicated to extract volatile and semi-volatile compounds from the headspace, having a greater capacity and linear concentration ranges than adsorptive ones, and utilise partitioning for the extraction (Pawliszyn, 2000; Shirey, 2000). It is recommended for polar organic compounds. CW-DVB is a mixed coating that contains a liquid polymer and solid particles (Pillonel *et al.*, 2002). This type of coating combines the absorption properties of the liquid polymer with the adsorption properties of porous articles. The mutually synergetic effect of adsorption and absorption to the stationary phase promotes a high retention capacity and, consequently, a higher sensitivity than the absorption fibres. It is recommended for alcohols and polar compounds. The PA fibre was conditioned at 300 ºC for 2 h in the GC injector and the CW-DVB was conditioned at 220 ºC for 1 h, according to the manufacturer's recommendations.

Figure II.3.1 represents the scheme of the general experimental procedure. It is composed by 2 main steps: *i)* optimisation of the SPME parameters, given by resolved chromatograms, and *ii)* establishment of the global volatile signature, given by unresolved chromatograms "fingerprint". For a better understanding, the procedure of the general conditions for headspace volatile compounds extraction will be described in three phases: *A)* optimization of SPME parameter: coating fibre and temperature of extraction, *B)* SPME

analysis to study the volatile components of wines and *C)* SPME analysis to establish the global volatile signature of the wine headspace.

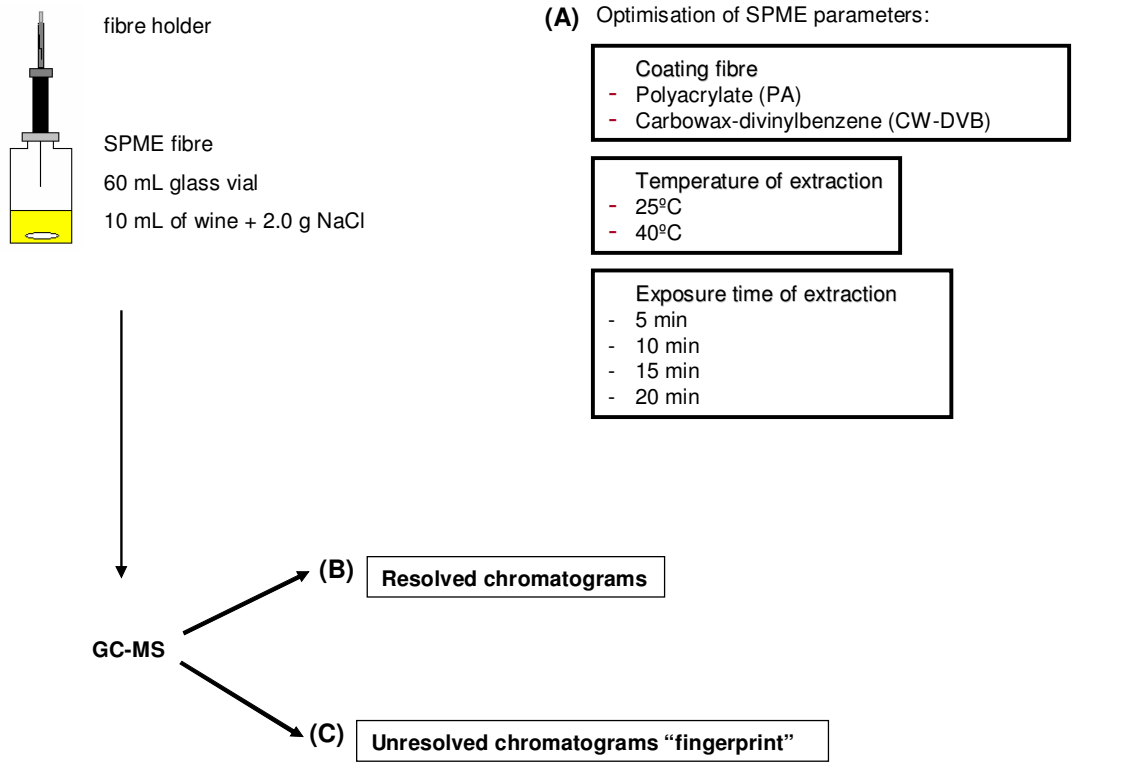


Figure II.3.1. Scheme of the experimental procedure of the developed method for distinction of wines

II.3.1. General conditions for headspace SPME volatile compounds extraction

The methodology developed by Rocha *et al.* (2001) for the volatile compounds extraction using SPME was used with modifications required by the analysis to establish the global volatile signature of the wine headspace described in *C)*.

Procedure:

1. Aliquots of 10.0 mL of wine were placed in a 60 mL glass vial, containing 2.0 g of NaCl and a stirring bar (2.0 cm x 0.5 cm) at 200 rpm.
2. A PTFE septum and an aluminium cap sealed hermetically the vial.
3. The coating fibre was manually inserted into the headspace of the vial for a total time period of 10 min.

Since headspace volume can be a critical factor determining the precision of the results in three-phase systems (liquid-phase, headspace phase and coating fibre phase), vials from the same producer and lot were used. Blanks were run between a set of three analyses.

A) Optimisation of SPME parameter: coating fibre and temperature of extraction

Procedure:

1. The performance of the two fibres, PA 85 μm film thickness and CW-DVB 65 μm film thickness was tested using the FP wine.
2. For each type of coating fibre, two different temperatures of extraction were tested (25.0 and 40.0±0.1 ºC). At least four replicates were done for each pair coating fibre/temperature of extraction.
3. To optimise these two parameters the GC conditions were selected in order to obtain adequate chromatographic resolution (section II.3.2.).

B) SPME analysis to study the volatile components of wines

Procedure:

1. The volatile composition of wines was carried out according to the general SPME conditions described in section II.3.1, using the CW-DVB coating fibre and the temperature of extraction of 40±0.1 ºC.
2. At least four replicates were done for each monovarietal wine.

C) SPME analysis to establish the global volatile signature of the wine headspace

Procedure:

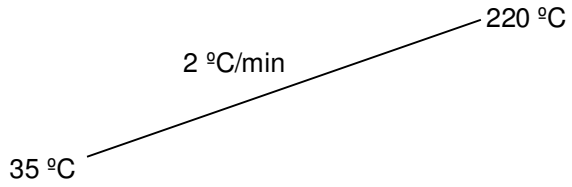
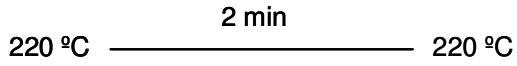
1. The global volatile signature of the wine headspace was carried out according to the general SPME conditions described in section II.3.1, using CW-DVB coating fibre and the temperature of extraction of 40.0 ±0.1 ºC. Four different extractions time were tested: 5, 10, 15 and 20 min, using FP and Ar wines.
2. At least four replicates were done for each monovarietal wine.
3. To have a total ion current in MS detector that did not saturate the MS source, a ratio of 0.2 was chosen from the different ratios of volume of sample/headspace assayed.

II.3.2. Chromatographic conditions in the developed methodology for distinction of wines

The SPME fibre containing the headspace volatile and semi-volatile compounds was introduced into the GC injection port of an Agilent Technologies 6890N Network gas chromatograph. The injection port was lined with a 0.75 mm i.d. splitless glass liner. The GC was equipped with a 30 m x 0.32 mm (i.d.) DB-FFAP fused silica capillary column (J&W Scientific Inc., Folsom, CA, USA), 0.25 μm film thickness, connected to an Agilent 5973 quadrupole mass selective detector, according to the method described in II.2.4. Splitless injections were used (1 min). Chromatographic conditions are described in Table II.3.1.

The identification of volatile compounds was achieved by comparison of the GC retention times and mass spectra with those, when available, of the pure standard compounds. All mass spectra were also compared with the library data system of the GC–MS equipment (Wiley 275 software from Hewlett-Packard) and other published spectra (*Eight Peak Index of Mass Spectra,* 1974).

Table II.3.1 Chromatographic conditions for wine analysis

Parameter	**Conditions**
Injector temperature	250 ºC
Carrier gas	Helium (12 psi) (1.7 mL/min)
Column	DB-FFAP
	(30m x 0.32 mm i.d., 0.25 µm film thickness)
Temperature program	*i)* for selecting the coating fibre and temperature, and study the volatile components of the wines:
	
	Total time= 92.5 min
	ii) for establishing the global volatile signature
	
	Total time= 2 min
Transfer line	230 ºC
Detector	MS (impact mode: 70 eV, scanning range *m/z* 30-300 1s cycle)

II.3.3. Data treatment

In the developed methodology for distinction of wines, a PCA was applied to the data. The experimental plan consisted of two wine varieties (FP and Ar) subjected to four different extraction times (5, 10, 15 and 20 min.), each one with four replicates, given a total of (2 x 4 x 4) 32 samples (objects). The GC-MS run for each sample was set to 2 min given an original signal with 667 points, and at each one of these chromatographic points the correspondent mass spectra was recovered from 30 to 300 *m/z*, in a total of 21 points. Hence, for each sample, the chromatographic signal consisted of (667 x 271) 180757 points (variables). For multivariate analysis purposes, the chromatographic domain used was set between 0.7 and 1.5 min (249 points) because no structured information was obtained before 0.7 min and after 1.5 min. The correspondent mass spectra domain used was set between 33 and 300 *m/z* (268 points), given a total of 66732 points to be

analysed (no structural information was obtained at *m/z* 30-32). This GC and MS data is inherently 2D, one related to the chromatographic dimension and the other to the mass spectra dimension and the other to the mass spectra dimension. Therefore, each sample can be viewed as a matrix (two dimensions) as opposed to a vector (as usually seen in many chromatographic and spectroscopy studies). Therefore, each one of the 32 acquired samples consisted of a matrix $\mathbf{S}_{i(249,\ 268)}$, where i={1, …, 32}. Then, each one of these 32 matrices were unfolded to give a $\mathbf{S}_{i(1,\ 66732)}$ vector, where i={1, …, 32}, and then all of them were row concatenated to give a matrix **Q**(32, 66732). This **Q** matrix was then used to perform the PCA. The PCA consists in the decomposition of this Q matrix into: $\mathbf{Q}_{(32,66732)} = \mathbf{T}_{(32,k)}\mathbf{P}^{T}_{(66732,\ k)}$ where T represents the scores matrix, **P** represents the loadings matrix and k corresponds to the number of principal components. The scores matrix (**T**) gives e relationships between the samples; the loadings matrix (**P**) gives the importance of each variable (*i.e.* retention times and their correspondent mass spectra). Finally, and in order to facilitate the interpretation of the loadings (**P**), each one of its columns (principal components) were folded back to give a matrix, which was easily depicted as a 2D map (or surface): $\mathbf{P}_{i(66732,\ 1)} \rightarrow \mathbf{P}_{i(249,268)}$, where i={1, …, k} principal components.

II.4. Developed method to study the interactions between the wine polymeric fraction and ethyl esters

A headspace-solid phase microextraction followed by gas chromatographic analysis (HS-SPME-GC) was developed to be applied in the study of the interactions between the Fernão-Pires wine polymeric fraction and ethyl esters. Three ethyl esters were used: ethyl hexanoate (≥99.0 %), ethyl octanoate (≥99.0 %) from Aldrich Chemical Co (Milwaukee, WI, USA), and ethyl decanoate (≥99.0 %) from Fluka (Buchs, Switzerland). A SPME holder from Supelco Inc. (Bellefonte, PA, USA) was used to perform headspace manually. The SPME fibre coated with 85 μm polyacrylate (PA) was also purchased from Supelco. Polyacrylate is an absorbent liquid-phase coated fibre, which is recommended for polar organic compounds. However, according to its performance in the analysis of the esters, namely the esters used in the present study, and considering the future applicability of the present methodology towards other volatiles of wine, polyacrylate coating fibre was chosen. The absorptive fibres are used to extract semi-volatile compounds from the

headspace. Absorptive fibres have greater capacity and linear concentration ranges than adsorptive, and utilise partitioning for the extraction (Pawliszyn, 2000; Shirey, 2000). The SPME fibre was conditioned at 300 ◦C for 2 h in the GC injector, according to the manufacturer's recommendations. All fibres used were from the same lot.

II.4.1. Wine polymeric origin and preparation

Vitis vinifera L. var. Fernão-Pires monovarietal wines from the Portuguese Bairrada Appellation were used in order to obtain the wine polymeric fraction (WP).

Procedure:

1. The wine (9 L) was rotary-evaporated under reduced pressure at 35 ºC to eliminate the ethanol and concentrate the total solids present to a final volume of 300 mL.
2. The solid material was then dialysed (12-14 kDa cut off) to remove the tartaric acid and other small molecules.
3. The dialysate was concentrated, frozen, and freeze-dried to give the wine polymeric fraction (WP) with a fluffy dry appearance.

II.4.2. Preparation of the standard solutions

Since the main objective of this study was to obtain information that may be helpful to understand the retention process of volatile compounds on the wine and considering future applicability of the proposed methodology towards of different chemical families, the pH and the ethanol concentration of the work solution were chosen according to the wine reference parameters.

Procedure:

1. Individual standard stock solutions of ethyl hexanoate (7.68 g/L), ethyl octanoate (8.78 g/L) and ethyl decanoate (1.12 g/L) were prepared in absolute ethanol.
2. All standard solutions were stored in glass flasks in the dark at 5 ºC ±1 until use.

3. A solution containing 10% (v/v) aqueous ethanol at pH 3.5, adjusted with tartaric acid (3%), was used to prepare the different wine models (WM) utilized in this work. The WM without polymeric fraction was used as reference wine model (RWM).

4. Three polymeric concentrations were used: 1.0 g/L (PWM_1), a polymeric concentration approaching the real one in wine (Coimbra *et al.*, 1998; Vidal *et al.*, 2004), 10.0 g/L (PWM_{10}) and 30.0 g/L (PWM_{30}), consisting the later one in a WM saturated with polymeric fraction.

5. The PWM_1, PWM_{10} and PWM_{30} were obtained by dissolution of the appropriate amount of WP in the wine model solution.

6. Each ester was added individually to the RWM and to the PWMs (PWM_1, PWM_{10} and PWM_{30}) in order to obtain a total volume of 40 mL, and the concentration of 4.0 mg/L for each standard. [a]

7. The RWM was also used to prepare the calibration curves of ethyl hexanoate (0.11 - 6.0 mg/L), ethyl octanoate (0.59 - 7.0 mg/L) and ethyl decanoate (0.053 - 8.4 mg/L). At least three replicates of each concentration were carried out for all experiments.

[a]- the concentration ranges of the esters in wine were very dependent on the grape variety and winemaking technology. The concentration ranges used were chosen taking in account *i)* that they should be included in the concentration range corresponding to a linear isotherm for the SPME fibre used and *ii*) the values accounted for these compounds in wine. The concentration used was included in the concentration range reported for some red varieties (Perestrelo *et al.*, 2006), although above the concentration reported for other white (Rocha *et al.*, 2001) and red varieties (Ugliano and Moio, 2005).

II.4.3. Chemical characterization of the wine polymeric fraction

II.4.3.1. Sugar analysis

The sugar analysis was performed as followed. Neutral sugars were obtained by sulfuric acid hydrolysis (Selvendran *et al.*, 1979) and analysed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (Coimbra *et al.*, 1996). Uronic acids (UA) were quantified by the 3-phenylphenol colorimetric method, as described by Coimbra *et al.* (1996).

A) Hydrolysis of the cell wall polymers

Saeman hydrolysis (Selvendran *et al.*, 1979)

Procedure:

1. The polymer (2-3) mg was placed into a Sovirel tube, recording the exact mass.
2. Sulfuric acid at 72% (w/w) (200 µL) was added and the mixture was incubated for 3 h, at room temperature, stirring frequently.
3. Distilled water (2.2 mL) was added, and the mixture was stirred and incubated at 100 ºC, for 2h:30min. After 1 h, 0.5 mL were removed for uronic acid analysis.

B) Reduction and acetylation of the monosaccharides to their alditol acetates

(Blakeney *et al.*, 1983; Harris *et al.*, 1984)

Procedure:

1. The tube containing the hydrolyzate was placed in a crushed ice bath, 200 µL of 2-deoxy-D-glucose (1 mg/mL) were added as internal standard and the mixture was stirred in a Vortex mixer.
2. 1 mL from the previous solution was removed into a new Sovirel tube, and 200 µL NH_3 at 25% and 100 µL NH_3 3M containing 150 mg/mL $NaBH_4$ (to reduce the hemiacetal groups) were added.
3. The mixture was stirred and incubated for 1h at 30ºC.
4. The mixture was cooled in an ice bath. Following, 2 x 50 µL of acetic acid (AcOH) were added, to destroy the excess of $NaBH_4$, and stirred.
5. 0.3 mL of the previous solution was removed into a new tube, cooled on iced and 0.45 mL of 1-methylimidazole (as a catalyst for the acetylation) and 3 mL of acetic anhydride were added.
6. The mixture was stirred and incubated at 30 ºC for 30 min.
7. The mixture was cooled in a crushed ice bath and 4.5 mL of distilled water (to decompose excess acetic anhydride and to aid phase separation) and 3.0 mL of dichloromethane were added.
8. The mixture was stirred vigorously and centrifuged at 1000 rpm, for 1-2 min.

9. The aqueous phase (upper phase) was removed.
10. To the organic phase, 3 mL of distilled water and 2 mL of dichloromethane were added.
11. The mixture was stirred, centrifuged and the aqueous phase removed.
12. To the organic phase, 3 mL of distilled water were added, and the mixture was stirred, centrifuged. The aqueous phase was removed. This step was repeated twice.
13. The organic phase was transferred into a new Sovirel tube.
14. The dichloromethane was evaporated completely, under a stream of N_2, at 40ºC.
15. 1 mL of acetone was added and the evaporation process repeated. This step was repeated twice.

A mixture of monosaccharides was prepared as calibrations standards: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose. Treatment of the standards was done repeating the above procedure (B).

C) Determination of the uronic acid content

Uronic acids (UA) were determined colorimetrically by modification of the method of Blumenkrantz and Asboe-Hansen (1973) according to Coimbra *et al.* (1996).

Procedure:

1. 0.5 mL of the sample previously removed from the hydrolizate was diluted with 3.0 mL of distilled water. The mixture was homogenized.
2. 0.5 mL of the solution was placed into 3 tubes (refrigerated in crushed ice), containing 3 mL of boric acid solution (12.5 mM) in concentrated sulfuric acid. The mixture was homogenized in a Vortex mixer.
3. The samples were boiled for 10 min in a water bath and cooled to room temperature.
4. To 2 of the 3 tubes, 100 µL 3-phenylphenol solution (0.15% in NaOH at 0.5%) were added. The mixture was homogenized and allowed to react in the dark for 30

min. The absorbance at 520 nm was determined with a 6405 Jenway UV/Vis spectrophotometer (U.K.). To the other tube, 100 µL 0.5% NaOH were added.

Galacturonic acid (at various concentrations), as a calibration standard, was prepared in the same manner as described above (B).

II.4.3.2. Determination of total phenolic compounds

Total phenolic composition was determined by the Folin-Ciocalteu colorimetric method (Ferreira *et al.*, 2002b).

Procedure:

1. The WP was dissolved in 2.5 % (v/v) aqueous solution of acetic acid (1.82 g/L) and 0.5 mL of this solution was mixed with of 250 μL of Folin-Ciocalteu reagent.
2. After homogenization with a vortex followed by a pause of 3 min allowed for reaction, 1 mL of Na_2CO_3 (200 g/L) and 3.25 mL of ultra-pure water were added, giving a total volume of 5 mL.
3. The mixture was homogenized in a vortex and was thermostatted for 10 min at 70 ℃, and then for 30 min at room temperature.
4. The absorbance was measured at 700 nm with a 6405 Jenway UV/Vis spectrophotometer (U.K.) against a blank, using gallic acid as standard in the concentration range 0.01 - 0.1 g/L. At least three replicates of each concentration were carried out for all experiments.

II.4.3.3. Protein analysis

Total protein content was estimated according to the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as standard (Smith *et al.*, 1985), using the Bicinchoninic Acid Protein Assay Kit from Sigma (Aldrich-Chemie, Steinheim, Germany).

Procedure:

1. The BCA (a) was mixed with a copper sulfate penta-hydrate solution (b), in a 50:1 proportion, giving a (c) solution. 50 µL of the aqueous WP solution (0.70 mg/L) was diluted in 1 mL of (c) solution, in eppendorfs.
2. The samples were incubated in a water bath at 60 ºC during 15 min.

3. The absorbance was measured at 562 nm with a 6405 Jenway UV/Vis spectrophotometer (U.K.) against a blank in the reference cell, where the 50 μL of the aqueous WP solution was replaced by 50 μL distilled water.
4. The data were correlated with the calibration curve of BSA standard (concentration range of 0.05 to 0.40 mg/L), also analysed in the same conditions of the samples.
5. At least three replicates of each concentration were carried out for all experiments.

II.4.4. Conditions for extraction of volatile compounds by headspace-SPME

Procedure (according to Figure II.4.1)**:**

1. 40 mL of each wine model solution (RWM and PWM) was transferred into a 120 mL glass vial, which corresponds to a ratio of the volume of the liquid phase to the headspace volume (1/β) of 0.5 (Rocha *et al*., 2001).
2. A Teflon septum and an aluminium cap sealed the vial that was subsequently placed in a thermostatted bath adjusted to 25.0±0.1 ºC, under continuous stirring (containing a 2.0 cm stirring bar, 300 rpm).
3. The SPME fibre was manually inserted into the headspace of the sample vial 15 min after sealing.
4. The samples were placed inside the vial for a period of 60 min, and the SPME fibre was kept in the flask for the last 45 min of this period.
5. The first SPME sampling occurred 15 min after addition of the ester to the RWM or PWM sealing the glass vial. Along 17 days with, at least, 24 h delay between each extraction, the volatile compounds present in the headspace of each WM were extracted 11 times, until the concentration of the esters was near to zero or similar in the RWM and PWM.
6. Four independent assays were done for each paired ester/wine model in a total of 24 independent experiments = 2 [3 esters x (1 RWM + 3 PWM_c)], where *c* correspond to the amount of polymeric material in solution (1.0, 10.0 or 30 g/L).

Since headspace volume can be a critical factor determining the precision of the results in three-phase systems (liquid-phase, headspace phase, and coating fibre phase), vials from the same producer and lot were used. Blanks, corresponding to the analysis of the coating fibre not submitted to any extraction procedure, were run between a set of three analyses.

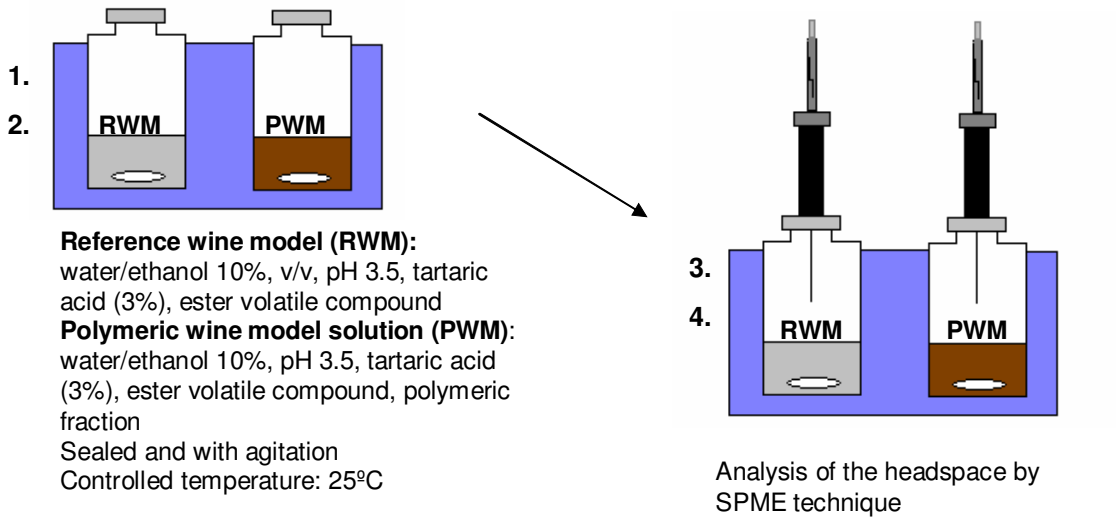


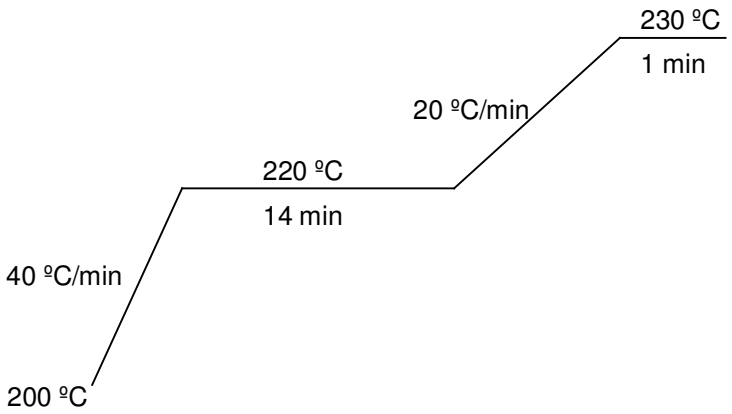
Figure II.4.1. Developed methodology for the study of the interactions between the Fernão-Pires wine polymeric fraction and ethyl esters.

II.4.5. Chromatographic analysis

II.4.5.1. Chromatographic conditions used for analysis of neutral sugars

A Carlo Erba GC 6000 GC apparatus, with split injector and a flame ionisation detector (FID) was used to perform the sugar analysis. A 30 m DB-225 column (J&W Scientific) was used. Table II.4.1 shows the chromatographic conditions used for the study neutral sugar analysis concerning model wines by HS-SPME-GC.

Table II.4.1. Chromatographic conditions for neutral sugar analysis

Parameter	Conditions
Injector temperature	220 ºC
Carrier gas	Hydrogen (1 mL/min)
Column	DB-225
	(30 m x 0.25 mm i.d., 0.15 μm film thickness)
Temperature program	
	Total time= 16 min
Detector	FID
Detector temperature	230 ºC

Reference alditol acetates eluted in the following order: rhamnitol pentaacetate, fucitol pentaacetate, arabinitol pentaacetate, xylitol pentaacetate, mannitol hexaacetate, galactitol hexaacetate and glucitol hexaacetate.

The quantification of neutral sugars was achieved by comparison of the GC retention times of the pure standard compounds. Estimated concentrations for all compounds were made by peak area comparisons with the area of the known amount of internal standard (2-deoxyglucose).

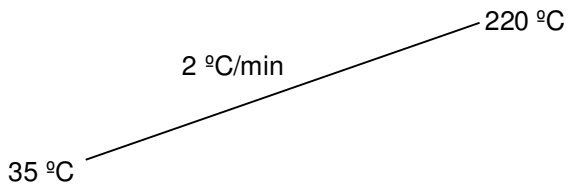
II.4.5.2. Chromatographic conditions used for analysis of the three esters

A Hewlett Packard 5890 gas chromatograph (Agilent, Wilmington, USA) equipped with a split/splitless injector and a flame ionisation detector (FID) was used to perform all GC analyses. The SPME coating fibre containing the headspace volatile compounds was introduced into the GC injection port at 250 °C and kept for 15 min for the desorption. The injection port was lined with a 0.75 mm i.d. splitless glass liner. The desorbed volatile

compounds were separated in a GC equipped with a 30 m x 0.32 mm (i.d.), 0.25 μm film thickness DB-FFAP fused silica capillary column (J&W Scientific Inc., Folsom, CA, USA). Splitless injections were used (5 min). The reproducibility, expressed as standard deviation, was shown as error bars in figures (section III.7).

The Table II.4.2 shows the chromatographic conditions used for analysis of volatile compounds.

Table II.4.2. Chromatographic conditions for the analysis of the three esters

Parameter	**Conditions**
Injector temperature	250 ºC
Carrier gas	Hydrogen (35 cm/s)
Column	DB-FFAP (30m x 0.32 mm i.d.), 0.25 μm film thickness
Temperature program	 Total time= 92.5 min
Detector	FID
Detector temperature	255 ºC

III

Results and Discussion

Summary

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III.1. Aroma potential of two white grape varieties: Fernão-Pires and Bical

To estimate the aroma potentialities of Fernão-Pires (FP) variety, the free (F) and potential volatile components (PVC) from the musts were examined using liquid-liquid continuous extraction and gas chromatography coupled with mass spectrometry (GC-MS) (section II.2.2.1 and II.2.4). Concurrently, the aroma potentialities of Bical (Bic) variety were also studied as a comparative approach.

Table III.1.1 shows the volatile composition of the FP and Bic musts, and its different distribution in the free form (F) and in potential (PVC). The PVC were determined after elimination of the free components by heat treatment followed by enzymatic treatment. The PVC fraction contains the glycosidically-linked components released by the enzymes plus the compounds produced by the heat treatment (70 ºC, 15 min) at the must pH (3.2) and also those compounds that arise from the thermal degradation of sugars. Some of these compounds are known to be developed during the winemaking and aging, contributing to the final wine aroma (Cordonnier and Bayonove, 1978; Williams *et al.*, 1980; Strauss *et al.*, 1987). This fact is particularly interesting to the neutral varieties such as these under study.

The free volatile compounds isolated from FP accounted for 3.84 mg/L, a value higher than that obtained for Bic (2.04 mg/L). These values do not include the furan-derived compounds because the majority of furans are products of sugar degradation (Tu *et al.*, 1992) due to the thermal treatment. These compounds are higher in FP than in Bic, which is consistent with the higher amount of sugars present in former musts. For these reasons these compounds were not considered. Both varieties contain aliphatic and aromatic alcohols, ketones, terpenoid compounds, aliphatic acids, and C_{13} norisoprenoids. Because of the considerable significance of volatile monoterpenes to flavour and varietal character of *V. vinifera* varieties (Strauss *et al.*, 1986), particular attention was devoted to these compounds. Alcohols were also the object of particular considerations because, quantitatively, the alcohol fraction was the main chemical group present in the musts.

Table III.1.1. Free and potential volatile components identified in dichloromethane extracts of Fernão-Pires and Bical musts, grouped by chemical classes

peak	Compound	Ident.[a]	Concentration[b] (µg/L) FP_F		FP_{PVC}		Bic_F		Bic_{PVC}	
	Terpenoids									
6	*trans*-linalool oxide	A,B,C	---		25.7	(7)	---		----	
8	*cis*-linalool oxide	A,B,C	---		36.2	(4)	16.2	(17)	---	
15	linalool	A,B,C	64.2	(3)	133.0	(9)	6.7	(19)	17.3	(27)
20	hotrienol	B,C	56.6	(12)	152.9	(8)	9.9	(10)	20.9	(8)
23	α-terpineol	A,B,C	22.4	(5)	148.6	(3)	3.2	(6)	5.5	(4)
25	linalool (*E*)-pyranic oxide	B,C	23.9	(5)	23.6	(6)	3.8	(9)	1.6	(8)
27	linalool (*Z*)-pyranic oxide	B,C	10.1	(3)	13.3	(8)	---		---	
31	nerol	A,B,C	---	---	18.1	(2)	---		---	
33	geraniol	A,B,C	53.6	(3)	66.8	(4)	---		---	
38	3,7-dimethylocta-1,5-dien-3,7-diol	B,C	621.8	(3)	249.3	(6)	123.4	(4)	41.8	(3)
39	3,7-dimethylocta-1-en-3,7-diol	B,C	94.4	(3)	234.3	(5)	---		---	
43	3,7-dimethylocta-1,7-dien-3,6-diol	B,C	23.5	(5)	53.9	(7)	---		---	
50	(*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol	B,C	95.2	(8)	93.5	(6)	48.8	(8)	55.0	(8)
52	farnesol	A,B,C	35.5	(7)	85.2	(9)	17.0	(6)	Tr.	
	Subtotal (µg/L)		**1101.2**		**1334.4**		**229.0**		**142.1**	
	Subtotal (%) [c]		**28.7**		**17.4**		**11.3**		**6.1**	
	Alcohols									
2	1-hexanol	A,B,C	447.7	(1)	190.5	(5)	203.9	(1)	11.4	(5)
3	*trans*-3-hexen-1-ol	A,B,C	15.5	(4)	9.5	(4)	10.7	(3)	Tr.	
4	*cis*-3-hexen-1-ol	A,B,C	16.7	(2)	12.5	(6)	13.6	(1)	2.4	(7)
5	*trans*-2-hexen-1-ol	A,B,C	245.6	(2)	160.2	(3)	154.8	(1)	28.6	(2)
12	2-(methylthio)ethanol	B	---		---		1.2	(9)	1.3	(9)
14	(*R,R*) + (*S,S*)-2,3-butanediol	A,B,C	29.9	(9)	414.2	(6)	15.8	(14)	38.3	(13)
18	(*R,S*)-2,3-butanediol	A,B,C	73.6	(6)	477.0	(6)	52.9	(9)	102.7	(4)
24	methionol	A,B,C	13.3	(8)	21.4	(4)	26.7	(6)	30.6	(5)
29	2-(2-butoxyethoxy)ethanol	B	27.0	(5)	34.7	(10)	5.3	(3)	4.0	(7)
34	benzyl alcohol	A,B,C	78.0	(3)	142.2	(8)	153.4	(4)	149.7	(6)
35	2-phenylethanol	A,B,C	191.2	(3)	267.8	(5)	298.2	(5)	255.3	(7)
45	4-vinyl-2-methoxyphenol	A,B,C	---		---		128.6	(8)	392.6	(5)
48	4-methyl-5-thiazoethanol	B	577.6	(9)	586.0	(6)	51.7	(5)	122.1	(11)
	Subtotal (mg/L)		**1716.1**		**2316.0**		**1112.8**		**1135.0**	
	Subtotal (%) [c]		**44.7**		**30.2**		**54.6**		**48.3**	
	Acids									
7	acetic acid	A,B,C	99.3	(8)	467.3	(6)	114.6	(8)	201.8	(5)
13	propanoic acid	A.B.C	---				2.2	(7)	3.6	(5)
16	isobutyric acid	A.B.C	22.0	(6)	39.4	(5)	16.2	(9)	20.3	(9)
21	butyric acid	A,B,C	---		---		3.2	(9)	7.8	(8)
32	hexanoic acid	A,B,C	115.0	(3)	157.2	(4)	50.4	(5)	43.1	(9)
37	*trans*-2-hexanoic acid	A,B,C	---		128.8	(9)	---		48.8	(4)
42	octanoic acid	A,B,C	40.9	(4)	43.4	(4)	31.5	(5)	22.9	(7)
44	nonanoic acid	A,B,C	---		---		18.4	(6)	17.1	(6)
47	decanoic acid	A,B,C	---		---		51.1	(8)	18.9	(3)
55	dodecanoic acid	A,B,C	49.7	(7)	134.6	(9)	32.7	(9)	20.5	(9)
	Subtotal (µg/L)		**326.9**		**970.7**		**320.3**		**404.8**	
	Subtotal (%) [c]		**8.5**		**12.7**		**15.7**		**17.2**	
	Ketones									
1	3-hydroxy-2-butanone	A,B,C	155.7	(2)	389.1	(7)	148.9	(2)	152.4	(6)
19	γ-butyrolactone	A,B,C	100.1	(4)	365.1	(3	71.8	(5)	136.6	(5)
28	tetrahydro-2H-pyran-2-one	B	7.4	(3)	19.9	(4)	7.4	(8)	11.4	(6)
40	3-hydroxy-2-methyl-4H-pyran-4-one	B,C	tr.[d]		25.4	(7)	---		---	
41	2-pyrrolidinone	B	32.8	(6)	60.6	(6)	16.6	(8)	29.1	(9)
46	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	B,C	31.4	(8)	1252.2	(2)	---		208.3	(9)
49	4-(1-hydroxyethyl)-γ-butanolactone	B,C	152.9	(2)	345.2	(4)	122.6	(3)	113.7	(11)
57	tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one	B,C	77.0	(6)	242.4	(7)	---		---	
	Subtotal (µg/L)		**557.3**		**2699.9**		**367.3**		**651.5**	
	Subtotal (%) [c]		**14.5**		**35.2**		**18.0**		**27.7**	
	C_{13} norisoprenoids									
10	vitispyrane	A,B,C	---		12.1	(5)	---		5.4	(11)
30	β-damascenone	A,B,C	94.6	(2)	17.1	(8)	6.7	(8)	5.9	(7)

Table III.1.1. (*Continued*) Free and potential volatile components identified in dichloromethane extracts of Fernão-Pires and Bical musts, grouped by chemical classes

			Concentration (μg/L)							
peak	**compound**	**Ident.[a]**	**FP_F**		**FP_{PVC}**		**Bic_F**		**Bic_{PVC}**	
	C_{13} norisoprenoids (cont.)									
59	dihydro-β-ionone	B,C	23.9	(7)	93.4	(4)	---		---	
	Subtotal (μg/L)		**188.5**		**122.6**		**6.7**		**11.3**	
	Subtotal (%) [c]		**3.1**		**1.6**		**0.3**		**0.5**	
	Furans									
9	furfural	A,B,C	tr.		159.9	(2)	---		40.1	(3)
17	5-methylfurfural	A,B,C	tr.		62.2	(5)	---		---	
22	furfuryl alcohol	A,B,C	13.7	(5)	53.8	(9)	15.0	(8)	23.5	(7)
26	2-(5H)-furanone	B,C	11.7	(5)	40.1	(4)	10.1	(6)	17.6	(7)
51	1 (3H)-isobenzofuranone	B	69.5	(6)	213.6	(6)	---		---	
53	2,3-dihydrobenzofuranone	B,C	66.0	(9)	596.9	(8)	38.0	(4)	383.2	(9)
54	2-furancarboxylic acid	B,C	tr.		85.3	(8)	---		---	
56	5-hydroxymethylfurfural	A,B,C	tr.		12350.5	(3)	38.7	(9)	3579.5	(9)
	Subtotal (μg/L)		**160.9**		**13562.3**		**101.8**		**4043.9**	
	Others									
11	benzaldehyde	A,B,C	10.6	(2)	19.4	(8)	2.2	(8)	3.9	(7)
36	benzothiazole	A,B,C	8.7	(6)	tr.		tr.		tr.	
58	vanillin	A,B,C	tr.		197.5	(8)	tr.		tr.	
	Subtotal (μg/L)		**19.3**		**229.0**		**2.2**		**9.3**	
	Subtotal (%) [c]		**0.5**		**2.9**		**0.1**		**0.2**	
	TOTAL (μg/L)		**4000.2**		**21222.8**		**2140.1**		**6392.5**	
	TOTAL without furans (μg/L)		**3839.3**		**7660.5**		**2038.3**		**2348.6**	

FP_F free volatile components of Fernão-Pires must; FP_{PVC} potential volatile components of Fernão-Pires must; Bic_F free volatile components of Bical must; Bic_{PVC} potential volatile components of Bical must. [a] The reliability of the identification or structural proposal is indicated by the following: A mass spectrum and retention time consistent with those of an authentic standard; B structural proposals are given on the basis of mass spectral data (Wiley 275); C mass spectrum consistent with spectra found in the literature. [b] Mean of six replicates; numbers in parentheses correspond to the CV (%). [c] Furans were not considered. [d] trace- concentration less than 0.02

III.1.1. Free alcohol and terpenoid volatile compounds

The composition of terpenoid fraction was different in the two varieties studied: the must of FP contains 11 terpenoids that represent 1.10 mg/L, and the must of Bic exhibits 8 terpenoids, corresponding to 0.23 mg/L. Quantitatively, the terpenols (3,7-dimethylocta-1,5-dien-3,7-diol, (*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol, 3,7-dimethylocta-1-en-3,7-diol, linalool, hotrienol, and geraniol) are the main terpenoids present in FP musts; the terpendiols (3,7-dimethylocta-1,5-dien-3,7-diol and (*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol) are the main terpenoids present in Bic. Many of these compounds, such as geraniol and linalool, are fragrant and are doubtless important to the general enhancement of the floral and fruity aromas (Marais, 1983). The hotrienol and linalool have been reported as having a determinant role in the wine aroma profile due to their aroma properties and low sensory perception limit (Simpson, 1979; Marais, 1983). The 3,7-dimethylocta-1,5-dien-3,7-diol was the dominant monoterpene of FP and Bic musts; although odourless, it can represent a major potential source of grape flavour as precursors of flavorants such as hotrienol

(Marais, 1983; Wilson *et al.*, 1984). For both varieties, all free terpenoid compounds determined are under the sensory perception limits, reported by Marais (1983).

The alcohol fraction is the major one, although its composition is different in the two varieties. This fraction is composed mainly by n-alcohols of C_6 chain length and aromatic compounds such as benzyl alcohol and 2-phenylethanol. The aliphatic alcohols were more abundant in the must from FP (84% of the alcohols extracted), whereas the aromatic alcohols were present in higher amount in the must from Bic (52% of the alcohols extracted). The presence of benzyl alcohol and 2-phenylethanol may cause the sweet and flowery notes (Belitz *et al.*, 2004), what could be considered as a positive characteristic for the Bic variety. C_6 alcohols have herbaceous and greasy odours, which seem related to deleterious effect in the wine (Baumes *et al.*, 1986; Cordonnier, 1989), although, in white wines, a small herbaceous perception is appreciated by some consumers (Dubois, 1994b). Their origin was reported as being related mainly to the lipooxygenase activity of the grape (Cordonnier, 1989) or must aeration (Cordonnier and Bayonove, 1978). C_6 alcohols accounts for 18% of total volatile compounds of FP and Bic, which represents 725 μg/L in FP musts and 383 μg/L in Bic musts. These values indicate, mainly for the FP variety, that attention should be paid to avoid the deleterious effect associated with the presence of these components. The more abundant thiol in the musts is 4-methyl-5-thiazoethanol. It accounts for 15% of total free aroma compounds in FP and 3% in Bic.

III.1.2. Alcohol and terpenoid volatile compounds in potential form

The PVC are 67% of the total volatile compounds in FP (7.66 mg/L) and 54% in Bic (2.35 mg/L). These values do not include the furan-derived compounds. The major classes of PVC in FP are ketones (35.2%), alcohols (30.2%), terpenoids (17.4%), and acids (12.7%); in Bic are alcohols (48.3%), ketones (27.7%), acids (17.2%), and terpenoids (6.1%). For the two varieties, the amount of PVC is higher than the corresponding free forms.

In FP musts, the amount of PVC terpenoids is 21% higher than the amount of terpenoids in F. Conversely, the level of PVC terpenoids of Bic is 61% lower. As observed in the free form, the PVC terpendiols are the major terpenoid component of the musts (26% of total terpenoids in FP and 68% in Bic). The terpendiol 3,7-dimethylocta-1-en-3,7-diol is significant in FP variety, although it is absent in Bic. The PVC levels of linalool, hotrienol, and α-terpeniol are higher than in the free forms. FP contains twice more

linalool, three times more hotrienol, and seven times more α-terpeniol. The *trans*-linalool oxide and *cis*-linalool oxide are present mainly in the PVC fractions. The terpenoid oxide exhibits a higher sensory perception limit compared with terpenols. The presence of a higher amount of terpenoids in the PVC fraction compared with the free one shows the potential properties of FP variety due to the significant importance of terpenoids in the aroma as well as their role as precursors of other aroma compounds (Williams *et al.*, 1980; Marais, 1983; Strauss *et al.*, 1988). In FP variety, the sum of the free and PVC forms of hotrienol (0.21 mg/L) and linalool (0.20 mg/L) are over the sensory perception limits for these compounds (0.11 and 0.10 mg/L, respectively- Marais, 1983), which may allow one to infer its contribution to the improvement of the wine aroma quality.

The amount of PVC alcohols are 35% higher than the free alcohols in FP; on the other hand, in Bic, the level of free and PVC alcohols is similar. However, it is observed that the level of C_6 PVC alcohols is lower than their free forms, 29 and 89% respectively for FP and Bic. The 2,3-butanediol *cis* and *trans* isomers are abundant in PVC form in FP musts, as they account for 38% of the total alcohols; in Bic, these compounds account only for 12%. However, they seem not to have influence in the sensory wine properties (Webb *et al.*, 1967; Radler and Zorg, 1986). The level of PVC aromatic alcohols (benzyl alcohol and 2-phenylethanol) is 52% higher in FP, but in Bic, these compounds are 10% lower. Voirin (1992b) indicates that the presence of aromatic alcohols is associated with the neutral cultivars. The 4-methyl-5-thiazoethanol accounts for 8% of total PVC in FP and 5% in Bic. This compound exists in similar amounts in F and PVC forms in FP, and in Bic, the amount of PVC doubles the amount of F. Thiazoles may occur naturally in foods such as tomato and wine or as a result of heat treatment (Belitz *et al.*, 2004). In the case of the musts of the varieties under study, especially FP, 4-methyl-5-thiazoethanol seems to be related to its varietal character, as it occurs in significant amounts in the F and as PVC.

The sensory perception limit for 1-hexanol, *trans*-2-hexen-1-ol, and *cis*-3-hexen-1-ol is estimated as 4, 15, and 13 mg/L in beer and 4.8, 6.7, and 0.07 mg/L in water, respectively (Dubois, 1994b). In FP and Bic musts the values are, respectively, 0.64 and 0.22 mg/L for 1-hexanol, 0.41 and 0.18 mg/L for *trans*-2-hexen-1-ol, and 0.03 and 0.02 mg/L for *cis*-3-hexen-1-ol. These concentrations are within the limits expected to occur in wines and are not expected to have any negative sensory contribution to the wine aroma; nevertheless the levels of these compounds need to be kept under control.

III.1.3. Other compounds

The sensory perception limit value for ketones is substantially higher than for other compounds studied; thus this chemical group was not the object of an exhaustive study. Considering the two varieties, the amount of free ketones was lower than their PVC amount, due mainly to 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, a product usually associated with thermal degradation (Belitz *et al.*, 2004). The major norisoprenoid present in the musts is β-damascenone (111.7 μg/L in FP and 12.6 μg/L in Bic). Because of its very low sensory perception limit of 0.002 mg/L in water (Belitz *et al.*, 2004), this compound seems to be important for these must aroma characteristics. In FP, β-damascenone is 85% in F; however, in Bic, the amount of F is comparable with the amount of the PVC. Vitispyrane is only found as a PVC, both in FP (12.1 μg/L) and Bic (5.4 μg/L). These values are lower than the sensory perception limit in water, estimated as 800 μg/L (Rapp and Pretorius 1990; Belitz *et al.*, 2004). Dihydro-β-ionone was only detected in FP, mainly as PVC (80%). C_{13} norisoprenoids are grape-derived compounds but are usually not present in the F and arise in juice and wines by hydrolytic degradation of precursor substances (Williams *et al.*, 1982b; Simpson and Miller, 1983; Strauss *et al.*, 1987). This is the reason for the higher amount of vitispyrane and dihidro-β-ionone as PVC.

III.1.4. Principal Component Analysis (PCA)

A PCA was applied to the normalized areas of the 59 compounds identified by GC-MS (FP and Bic varieties, both in F and as PVC, in a total of four samples, each with six extraction replicates). PCA, as an exploratory technique, allows one to study the main sources of variability present in the data sets, to detect clustering formation, and to establish relations between samples (objects) and compounds (variables) (Jolliffe, 1986). In this case, the PCA was used to study the main sources of variability between the different must varieties (in the F and PVC forms), and to establish relations between the varieties (in both forms) and volatile components. Figure III.1.1a shows the scores scatter plot of the two first principal components (which contains 93.7% of the total variability) that represents the distinction among the 24 samples. Figure III.1.1b represents the corresponding loadings plot that establishes the relative importance of each volatile component, and it is therefore useful for the study of relations among the volatile compounds and relations between volatile compounds and samples.

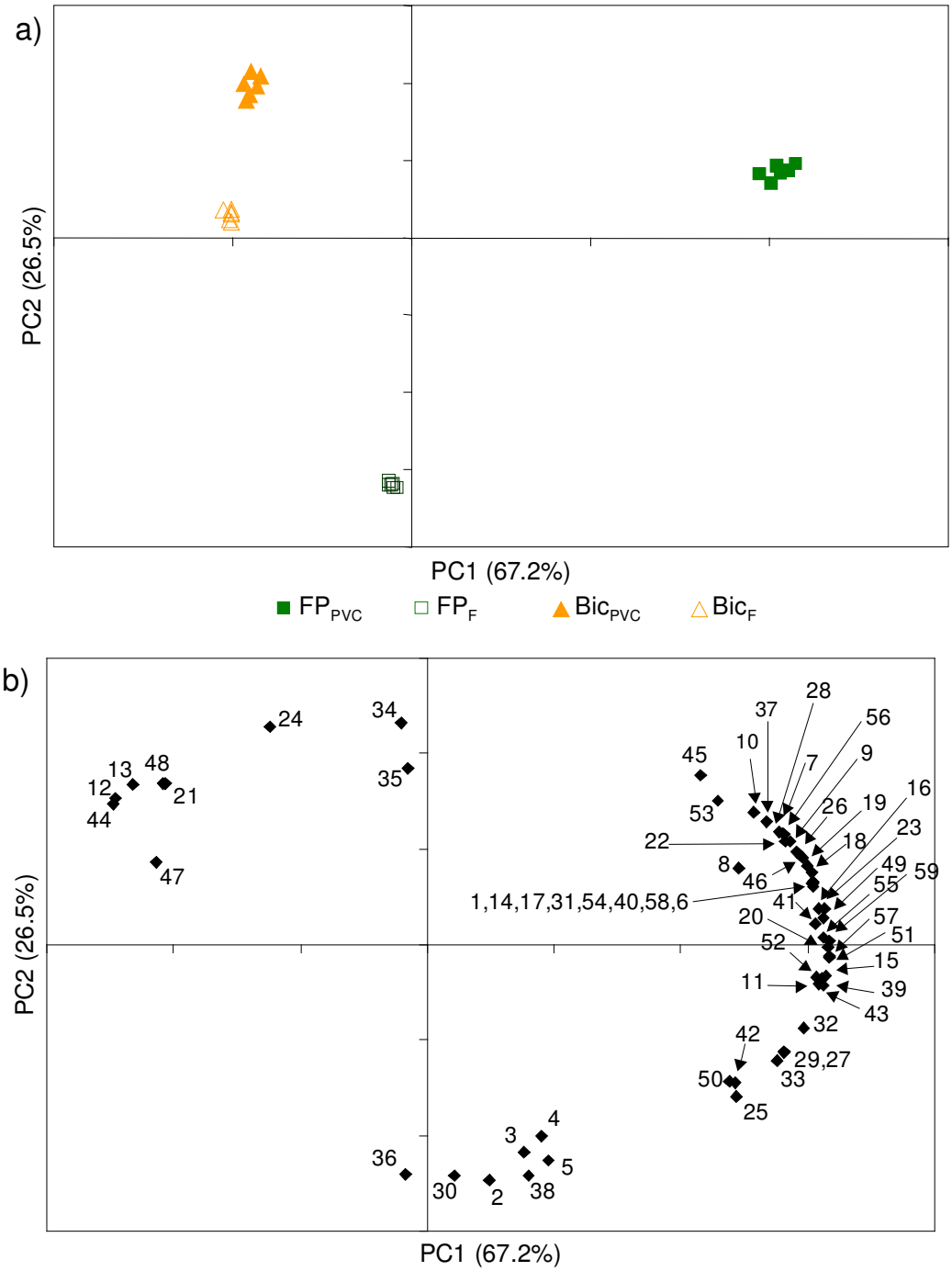


Figure III.1.1. PC1 X PC2 scatter plot of the main sources of variability between the Fernão-Pires and Bical musts. (a) Distinction between the samples FP$_F$, FP$_{PVC}$, Bic$_F$, and Bic$_{PVC}$ (Scores); (b) relation between the 59 volatile components (loadings).

The first quadrant contains the PVC of Fernão-Pires (FP_{PVC}). These samples are characterized by the furans and pyranones, products of thermal degradation (Table III.2.1, peaks 9, 10, 17, 22, 26, 28, 40, 41, 46, 51, 53, 54, 56-58), terpenoid compounds (6, 8, 15, 20, 23, 25, 27, 31, 33, 39, 43, 50, 52), acids (7, 16, 32, 37, 42, 55), alcohols (14, 18, 29, 45), ketones (1,19, 49), dihydro-β-ionone (59), and benzaldehyde (11). The free volatile components of Fernão-Pires (FP_F) are related to the negative PC2 side. These samples are characterized by C_6 alcohols (2-5), β-damascenone (30), 3,7-dimethylocta-1,5-dien-3,7-diol (38), and benzothiazole (36). Bical samples, both F and PVC, are represented in the second quadrant. These samples are characterized by the propanoic (13), butyric (21), nonanoic (44), and decanoic (47) acids, the thioalcohols 2-(methylthio) ethanol (12), methionol (24), and 4-methyl-5 thiazoethanol (48), and the aromatic alcohols (34, 35). Furthermore, the analysis of the PC1 x PC3 scores and loadings plots (data not shown) allows one to relate nonanoic and decanoic acids, 2-(methylthio)ethanol (12), and 2-phenylethanol (35) with the free volatile components of Bical (BIC_F) and 4-methyl-5-thiazoethanol and propanoic and butyric acids with the PVC of Bical (BIC_{PVC}).

III.1.5. Conclusions

The results of this work show that FP and Bic varieties have different aroma profiles. FP has a higher amount of volatile compounds and in higher concentration than Bic. The sum of the terpenoids in F and as PVC is beyond the sensory perception limit for hotrienol and linalool. Furthermore, the occurrence of odourless terpendiols allows us to infer that this variety might be an important one if new strategies of winemaking technology are used, such as enzymatic treatments to release the aroma components. The presence of aromatic alcohols, both in F and as PVC, may be an interesting characteristic of the musts from Bic. These properties may profit the development of an adequate winemaking technology to release these flowery and sweet compounds and increase the aroma quality. Furthermore, as a consequence of the fact that the FP and Bic varieties exhibit different volatile composition patterns, winemaking technologies should be developed specifically for each variety.

III.2. Effect of enzymatic aroma release on the volatile compounds of white wines presenting different aroma potentials

The work previously developed concerning the musts of Fernão-Pires (FP) and Bical (Bic) varieties showed that FP has a higher amount of monoterpenols in potential form and odourless terpendiols, and Bic has an important amount of aromatic alcohols (section III.1). These characteristics suggested that the quality of the monovarietal wines produced could be improved if appropriate strategies of winemaking technology, such as aroma release enzyme treatments, were used. Furthermore, considering that the volatiles composition patterns of FP and Bic varieties are different, winemaking technologies should be developed specifically for each variety. However, as *β*-glucosidase preparations were never developed for aroma release of the different Bairrada varieties, throughout the years commercial enzyme preparations, when used, have been applied indiscriminately in varieties presenting different aroma potentials.

The aim of this work was to evaluate the effectiveness of a broad-spectrum enzyme preparation, normally used in the Bairrada Appellation, on the volatile composition and aroma quality of two white wines with distinct aroma potential characteristics, FP and Bic. As the volatile composition of the wines from FP and Bic varieties has not yet been studied, attention was also focused in this research on the characterization of the volatile components of these monovarietal wines. The volatile composition of FP and Bic wines from 1997 harvest were analysed according to section II.2.3 and II.2.4. A commercial enzyme preparation (section II.1.3) was added to one group of wines (FP_E and Bic_E), and the second group was left as reference (FP_R and Bic_R).

Table III.2.1 shows the volatile composition of FP and Bic wines, and their different distribution between reference and enzyme-treated wines. Compounds extracted from FP_R wine accounted for 199 mg/L, a value 6% higher than that obtained for Bic_R *(*188 mg/L*)*. Enzymatic treatment showed an increase to 217 mg/L for Fernão-Pires, but for Bical no changes were observed in the total amount of volatile compounds. Both varieties contained aliphatic and aromatic alcohols, esters, terpenoid compounds, aliphatic acids and lactones. Owing to the considerable contribution of volatile terpenols to flavour and to varietal character of *Vitis vinifera* varieties (Strauss *et al.*, 1986), particular attention has been devoted in this work to these compounds. Particular consideration was also devoted to alcohols and esters since, quantitatively, these are the major chemical groups in wines.

Lactones were also considered since they have been reported as occupying a place of prominence in terms of their contribution to overall wine aroma (Muller *et al.*, 1973).

III.2.1. FP_R and Bic_R volatile composition

The composition of the terpenoid fraction was very different in the two wines studied: FP wine contained 10 terpenoids which totalled 5.65 mg/L, and in Bic only one terpenoid, geraniol, was quantified, corresponding to 0.88 mg/L. The concentration of geraniol was above its sensory perception limit (0.13 mg/L) in Bic_R, suggesting its individual contribution to floral notes. (*Z)*-2,6-dimethylocta-2,7-dien-1,6-diol, linalool and *α*-terpineol represented the major terpenol components of FP wine. For FP_R, linalool (1.59 mg/L), hotrienol (0.60 mg/L) and *α*-terpineol (0.53 mg/L) were above their sensory perception limits, respectively, 0.05–0.10, 0.11 and 0.4–0.5 mg/L (Simpson, 1979; Marais, 1983), which allowed us to infer their individual contributions to citrus and flowery notes. Linalool has characteristic citrus-like, sweet and flowery notes (Muller *et al.*, 1973; Steinhaus and Schieberle, 2000), and hotrienol and *α*-terpineol exhibit flowery and sweet aromas (Muller *et al.*, 1973). The (*Z)*-2,6-dimethylocta-2,7-dien-1,6-diol, which represented 360 mg/g of FP_R terpenoids, contrary to others diols, is odourant (Gunata *et al.*, 1990b) and may also contribute to the overall wine aroma. Terpenediols are odourless but represent a major potential source of monoterpenoid compounds by hydrolysis at wine pH (Muller *et al.*, 1973; Williams *et al.*, 1982c; Wilson *et al.*, 1984). The prior study of the volatile characterization of FP and Bic musts showed that, for both varieties, 3,7-dimethylocta-1,5-dien-3,7-diol was the major terpenoid component (section III.1). The values of this compound in FP_R and Bic_R (Table III.2.1) suggest that its hydrolysis may occur during the fermentative process releasing monoterpenoids, such as hotrienol, linalool and *α*-terpineol (Williams *et al.*, 1980; Voirin *et al.*, 2000). The amount of these monoterpenoids in FP_R was in accordance with this suggestion; however their absence from Bic_R wines does not confirm this conversion.

The ester and alcohol fractions were quantitatively the major wine components, although their composition was different between the two varieties. The alcohol fraction was composed mainly of *n*-alcohols of C_6 chain length and aromatic compounds such as benzyl alcohol and 2-phenylethanol. The aliphatic alcohols were more abundant in FP_R wines (600 mg/g of the alcohols), and the aromatic alcohols were present in similar amounts in FP_R and Bic_R wines, 21 and 23 mg/L, respectively. Benzyl alcohol and 2-phenylethanol are associated with sweet and flowery notes (Belitz *et al.*, 2004), which suggests their contribution to the aroma characteristics of these varieties. In both varieties,

2-phenylethanol was over its sensory perception limit (10 mg/L). Its contribution can be considered as a positive characteristic, especially for the Bic variety, which had a poor terpenic character. C_6 alcohols have herbaceous and greasy odours that have been related to deleterious effects in the wines (Cordonnier, 1989; Baumes *et al.*, 1986), although in white wines a small herbaceous perception is appreciated by some consumers (Dubois, 1994b). Their origin was reported as being related mainly to the lipooxygenase activity of the grape (Cordonnier, 1989) and/or must aeration (Cordonnier and Bayonove, 1978). The sensory perception limits for 1-hexanol, *trans*-2-hexen-1-ol, and *cis*-3-hexen-1-ol have been estimated as 4, 15 and 13 mg/L in beer and 4.8, 6.7 and 0.07 mg/L in water, respectively (Belitz *et al.*, 2004). According to Table III.5.1, only *cis*-3-hexen-1-ol was over its sensory perception limit in FP_R (0.17 mg/L) and a contribution to an herbaceous aroma cannot be excluded. This result indicates that, mainly for the FP variety, attention should be taken to avoid the deleterious effect associated with the presence of this compound (section III.1).

The ester fraction was composed of 14 compounds for FP_R, totalling 49.8 mg/L and 11 compounds for Bic_R, totalling 41.4 mg/L. Quantitatively, ethyl 2-hydroxypropanoate, diethyl butanodioate and ethyl octanoate were the major esters present in FP and Bic wines. Many of these compounds are fragrant and can contribute to floral and fruity notes. In both wines, ethyl octanoate was above its sensory perception limit (0.58 mg/L), which allows us to infer its individual contribution to fruity notes, such as ripe fruits, pear and sweet notes. Ethyl decanoate was also above its sensory perception limit (0.51 mg/L) and may have contributed to sweet and fruity notes.

The composition of the lactones fraction was very similar in the two varieties studied: FP wine contained seven lactones, totalling 8.1 mg/L, and in Bic wine six lactones were identified, totalling 7.4 mg/L. Among the many volatile components of wine, lactones, particularly γ-lactones, occupy a place of prominence in terms of their contribution to the overall aroma (Muller *et al.*, 1973). γ-Butyrolactone always occurs in wines (Muller *et al.*, 1973) and is associated with buttery (Muller *et al.*, 1973; Williams *et al.*, 1980; Dufossé *et al.*, 1994) and rubber descriptors (Etiévant, 1991). Quantitatively, γ-butyrolactone and 4-ethoxycarbonyl-γ-butyrolactone were the major lactones present in FP wine, and γ-butyrolactone and pantolactone were the main lactones present in BIC.

Table III.2.1. Volatile components identified in dichloromethane extracts of enzyme-treatment Fernão-Pires and Bical wines, grouped by chemical classes.

Peak number	Compound	Identity[a]	Concentration[b] (mg/L)							
			FP_R		FP_E		Bic_R		Bic_E	
			$\bar{x}$ (n = 8)		$\bar{x}$ (n = 8)		$\bar{x}$ (n = 8)		$\bar{x}$ (n = 8)	
Terpenoids										
19	linalool	A, B, C	1.59	(5)	1.23	(1)	—		—	
24	hotrienol	B, C	0.60	(4)	0.40	(3)	—		—	
31	α-terpineol	A, B, C	0.53	(3)	0.42	(2)	—		—	
33	linalool (*E*)-pyranic oxide	B, C	tr[c]		tr		—		—	
38	geraniol	A, B, C	0.09	(6)	0.15	(5)	0.88	(6)	0.47	(4)
44	3,7-dimethylocta-1,5-dien-3,7-diol	B,C	tr		tr		—		tr	
46	3,7-dimethylocta-1-en-3,7-diol	B,C	0.40	(7)	0.43	(2)	tr		tr	
52	3,7-dimethylocta-1,7-dien-3,6-diol	B,C	tr		tr		—		—	
58	(*E*)-2,6-dimethylocta-2,7-dien-1,6-diol	B,C	—		1.34	(8)	—		—	
61	(*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol	B,C	2.03	(6)	3.10	(4)	—		—	
62	geranic acid	B,C	0.42	(6)	0.41	(10)	—		—	
Sub-total (mg/L)			**5.66**		**7.48**		**0.88**		**0.47**	
Sub-total (%)[c]			**2.84**		**3.45**		**0.47**		**0.25**	
Alcohols										
3	4-methyl-1-pentanol	B,C	0.14	(3)	0.24	(5)	tr		0.16	(9)
6	1-hexanol	A,B,C	3.47	(6)	3.76	(8)	2.68	(8)	2.86	(3)
7	*trans*-3-hexen-1-ol	A,B,C	tr		0.53	(5)	—		0.37	(9)
8	3-ethoxy-1-propanol	B,C	0.62	(7)	0.71	(7)	tr		0.33	(6)
9	*cis*-3-hexen-1-ol	A,B,C	0.17	(7)	0.17	(2)	—		—	
10	*trans*-2-hexen-1-ol	A,B,C	0.15	(3)	0.13	(8)	tr		0.13	(9)
15	2-(methylthio)ethanol	B,C	0.11	(8)	0.11	(10)	tr		0.09	(5)
17	unknown alcohol (*m/z* 69, 43, 87, 45)	B	0.20	(4)	0.13	(7)	tr		0.12	(4)
18	(*R*,*R*) + (*S*,*S*)-2,3-butanediol	A,B,C	16.88	(2)	9.31	(6)	10.05	(8)	12.62	(8)
21	(*R*,*S*)-2,3-butanediol	A,B,C	7.56	(9)	5.46	(7)	3.19	(7)	5.10	(5)
22	propylene glycol	B,C	1.08	(9)	—		0.29	(7)	—	
32	methionol	A,B,C	1.19	(9)	0.76	(5)	1.08	(5)	1.46	(3)
40	benzyl alcohol	A,B,C	1.82	(5)	4.06	(2)	2.42	(6)	5.14	(3)
43	2-phenylethanol	A,B,C	19.19	(4)	21.17	(5)	20.28	(2)	24.15	(2)
47	phenol	A,B,C	0.14	(9)	0.25	(8)	tr		tr	
54	4-vinyl-2-methoxyphenol	A,B,C	0.34	(6)	0.91	(8)	0.20	(5)	0.19	(6)
Sub-total (mg/L)			**53.05**		**47.09**		**40.18**		**52.73**	
Sub-total (%)[c]			**26.64**		**22.03**		**21.36**		**28.03**	
Esters										
2	hexyl acetate	A,B,C	tr		0.09	(2)	—			
5	ethyl 2-hydroxypropanoate	B,C	35.95	(3)	53.70	(2)	29.74	(6)	35.90	(7)
11	ethyl octanoate	A,B,C	1.48	(5)	1.60	(7)	1.15	(6)	1.60	(4)
14	ethyl 3-hydroxybutanoate	B,C	0.36	(10)	0.29	(7)	0.22	(4)	0.33	(5)

Table III.2.1. (*continued)* Volatile components identified in dichloromethane extracts of enzyme-treatment Fernão-Pires and Bical wines, grouped by chemical classes.

Peak	Compound	Identity[a]	Concentration[b] (mg/L)							
			FP_R		FP_E		Bic_R		Bic_E	
			$\bar{x}$ (*n* = 8)		$\bar{x}$ (*n* = 8)		$\bar{x}$ (*n* = 8)		$\bar{x}$ (*n* = 8)	
Esters (cont.)										
26	ethyl decanoate	A,B,C	0.84	(10)	0.66	(6)	0.63	(8)	0.67	(5)
30	diethyl butanodioate	B,C	5.29	(1)	3.12	(8)	6.95	(5)	7.15	(2)
34	1,3-propanodiol diacetate	B,C	0.73	(6)	0.43	(6)	0.63	(5)	0.40	(3)
35	2-phenylethyl acetate	A,B,C	0.15	(2)	0.13	(7)	tr		0.10	(10)
36	ethyl 4-hydroxybutanoate	B,C	0.86	(8)	0.63	(2)	0.6	(7)	0.82	(5)
42	1,4-butanediol diacetate	B,C	0.29	(7)	0.35	(3)	—		—	
49	diethyl hydroxybutanedioate	B,C	1.19	(2)	1.39	(9)	0.46	(7)	0.43	(9)
53	diethyl 2-hydroxypentanodioate	B,C	0.90	(9)	1.15	(9)	0.60	(10)	0.49	(1)
56	ethyl 2-hydroxy-3-phenylpropanoate	B,C	0.97	(10)	1.42	(9)	0.36	(10)	0.66	(8)
63	methoxycarbonyl butanodioic acid	B,C	0.81	(11)	1.00	(13)	—		—	
Sub-total (mg/L)			**49.83**		**65.97**		**41.38**		**48.55**	
Sub-total (%)[c]			**25.03**		**30.46**		**22.00**		**25.81**	
Acids										
12	acetic acid	A,B,C	13.30	(6)	13.59	(6)	14.43	(6)	18.06	(8)
16	propanoic acid	A,B,C	—		0.07	(3)	—		0.10	(8)
20	2-methylbutanoic acid	A,B,C	1.92	(12)	1.07	(6)	1.19	(9)	1.97	(6)
25	butanoic acid	A,B,C	0.90	(8)	0.89	(8)	0.44	(8)	1.12	(8)
29	3-methylbutanoic acid	B,C	0.83	(7)	0.44	(7)	0.31	(9)	1.38	(3)
37	hexanoic acid	A,B,C	5.94	(4)	6.20	(4)	4.37	(8)	5.79	(1)
45	2-hexanoic acid	A,B,C	0.08	(9)	tr		—		—	
51	octanoic acid	A,B,C	11.58	(7)	11.71	(2)	10.30	(1)	10.35	(3)
57	decanoic acid	A,B,C	5.48	(2)	5.70	(3)	3.56	(9)	3.82	(9)
Sub-total (mg/L)			**40.05**		**39.67**		**34.61**		**42.58**	
Sub-total (%)[c]			**20.11**		**18.32**		**18.40**		**22.64**	
Lactones										
23	γ -butyrolactone	A,B,C	3.54	(11)	2.99	(3)	3.36	(5)	4.67	(7)
39	dihydro-2-methyl-3-(2H)-furanone	B,C	—		tr		—		—	
48	pantolactone	B,C	0.52	(9)	0.56	(6)	0.64	(4)	3.70	(6)
50	5-acetyldihydro-2-(3H)-furanone	B,C	tr		0.30	(7)	tr		0.09	(4)
55	4-ethoxycarbonyl-γ-butyrolactone	B,C	1.84	(6)	2.95	(3)	1.10	(9)	tr	
59	4-(1-hydroxyethyl)-γ-butanolactone	B,C	0.94	(10)	1.14	(10)	1.41	(8)	1.33	(14)
60	2-hydroxymethylbenzoic acid butanolactone	B,C	0.46	(8)	0.50	(3)	tr		tr	
64	4-(1-hydroxyethyl)-γ-butanolactone	B,C	0.79	(5)	1.04	(14)	0.90	(9)	0.77	(1)
Sub-total (mg/L)			**8.10**		**9.48**		**7.40**		**10.56**	
Sub-total (%) [c]			**4.07**		**4.38**		**3.93**		**5.62**	
Others										
1	3-hydroxy-2-butanone	A,B,C	2.49	(4)	3.53	(10)	12.46	(6)	1.57	(2)

Table III.2.1. (*continued)* Volatile components identified in dichloromethane extracts of enzyme-treatment Fernão-Pires and Bical wines, grouped by chemical classes.

Peak	Compound	Identity[a]	Concentration[b] (mg/L)							
			FP_R		FP_E		Bic_R		Bic_E	
			$\bar{x}$ (*n* = 8)		$\bar{x}$ (*n* = 8)		$\bar{x}$ (*n* = 8)		$\bar{x}$ (*n* = 8)	
Others (cont.)										
4	3-hydroxy-2-pentanone	B,C	0.27	(7)	0.34	(9)	tr		—	
13	benzaldehyde + unknown acid (*m*/*z* 60)	A,B,C	0.08	(7)	0.09	(9)	0.18	(6)	0.23	(5)
27	*N*-methylacetamide	B,C	0.29	(7)	0.16	(9)	0.12	(7)	0.16	(14)
28	2,2-dimethyl-1,3-dioxolane	B,C	0.30	(6)	0.16	(6)	0.11	(8)	0.16	(14)
41	*N*-(3-methylbutyl)acetamide	B,C	0.52	(4)	0.61	(3)	6.30	(6)	4.30	(1)
65	unknown (*m*/*z* 101, 45, 55, 73)	—	38.48	(1)	41.35	(11)	44.50	(1)	26.77	(3)
66	2,3-dihydrobenzofuran	B,C	—		tr		—		—	
Sub-total (mg/L)			**42.43**		**46.25**		**63.66**		**33.19**	
Sub-total (%) [c]			**21.31**		**21.36**		**33.84**		**17.65**	
TOTAL (mg/L)			**199.12**		**216.53**		**188.12**		**188.08**	

FP_R, Fernão-Pires wine not treated with aroma release enzymes, used as reference; FP_E, Fernão-Pires wine treated with aroma release enzymes; Bic_R, Bical wine not treated with aroma release enzymes, used as reference; and Bic_E, Bical wine treated with aroma release enzymes.

[a] The reliability of the identification or structural proposal is indicated by the following: A, mass spectrum and retention time consistent with those of an authentic standard; B, structural proposals are given on the basis of mass spectral data (Wiley 275); C, mass spectrum consistent with spectra found in the literature.

[b] Concentration—mean of four extraction replicates, numbers in parentheses correspond to the coefficient of variation (%).

[c] tr, trace = concentration less than 0.02 mg/L.

The 4-ethoxycarbonyl-γ-butyrolactone was above its sensory perception limit (0.4 mg/L) in both varieties and may have contributed a sherry-like aroma (Muller *et al.*, 1973; Dubois *et al.*, 1994a). The amount of γ-butyrolactone, and especially 3-hydroxy-2-butanone, may explain the buttery odour associated with the Bic variety.

III.2.2. Effect of aroma release enzymatic treatment

Enzymatic treatment resulted in a 9% increase in the total amount of volatile compounds in FP wine, but for Bic the total amount of volatile compounds remained unchanged.

The amount of terpenoids increased 32% in FP_E when compared with FP_R wines. This was due mainly to the 53% increase in (*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol to significant levels, as well as the appearance of its *E*-isomer which is also odourant (Gunata *et al.*, 1990b). The levels of linalool, hotrienol and *α*-terpineol decreased by 23, 33 and 21%, respectively; however, they still remained above their sensory perception limits. It is also important to note that the geraniol level increased 66% in FP_E and exceeded its sensory perception limit, which can be considered as a positive effect of the aroma release treatment. Conversely, the level of geraniol in Bic_E decreased by 47% relative to Bic_R, but still remained above its sensory perception limit.

The amount of alcohol in FP_E was 10% lower than in FP_R. This was due mainly to the decrease observed in 2,3-butanediol isomers; nevertheless these *cis* and *trans* isomers seem not to have influenced the sensory wine properties (Webb *et al.*, 1967; Radler and Zorg, 1986). However, a positive effect may be noticed as a consequence of the enzymatic treatment related to the 19% increase observed in the amount of aromatic alcohols. Similar effects were observed in the Bic variety. The enzymatic treatment did not promote significant modifications in the amount of C_6 alcohols in FP varieties; however in the Bic variety an increase in the amount and type of C_6 alcohols was observed. Considering that these compounds were below their sensory perception limits (with the exception of *cis*-3-hexen-1-ol in FP_R, for which the sensory perception limit considered as the reference was in water), the enzymatic treatment seemed not to promote a deleterious effect associated with the presence of these compounds. In FP, the enzymatic treatment increased the amount of 4-vinyl-2-methoxyphenol from 0.34 to 0.91 mg/L, a value higher than its sensory perception limit (0.44 mg/L), which may contribute black pepper and clove-like aromas (Williams *et al.*, 1980).

The enzymatic treatment promoted increases of 32 and 17% to the total amounts of esters in FP_E and Bic_E, respectively, which may contribute favourably to fruity and floral overall wine aroma. The main variation in the ester group was due to increase of ethyl 2-hydroxypropanoate, the major ester in both varieties.

The amount of lactones increased by 15 and 43% in FP_E and Bic_E, respectively, with the enzymatic treatment. This observed increment in FP_E was due mainly to 4-ethoxycarbonyl-γ-butyrolactone, which exhibits a sherry-like aroma descriptor (Muller, 1973; Dubois, 1994a). This effect may contribute favourably to the overall FP wine aroma. Conversely, this compound decreased drastically (detected as trace amount) in Bic_E compared with Bic_R. The enzymatic treatment promoted an increase in pantolactone in the Bic variety. Considering that no aroma descriptor was found in the literature for this compound, no effect can be attributed to this increment. The enzymatic treatment also promoted a drastic decrease of 3-hydroxy-2-butanone in Bic_E compared with Bic_R. As no explanation was found for this observation, the analytical data were exhaustively examined, confirming this result.

III.2.3. Conclusions

FP and Bic wines have different volatile compositions. The enzymatic aroma release treatment increased the amounts of volatile compounds of FP wine due, mainly, to the increase of monoterpenoids, terpendiols and aromatic alcohols. Conversely, no significant modifications were introduced in the volatile composition of Bic wine. These results are closely related to the sensory analysis, which indicated that the enzymatic treatment seemed to be effective for the improvement of the aroma of FP, but only represented a cost and did not promote any improvement in the Bic wine quality. The presence of aromatic alcohols in significant amounts, considered as an interesting characteristic of Bic wines, was not enhanced by this treatment. These results are in accordance with the results on the potential aroma of these varieties, which reported that the volatile composition patterns o FP and Bic varieties are different, so winemaking technologies should be developed specifically for each variety (section III.1). The effectiveness of the enzymatic treatment, even with a broad range of enzymatic activities, is closely dependent on the varietal aroma potential, knowledge of which is a key determinant for the full exploitations of a wine's qualities. Furthermore, only the establishment of the specific activities of *β*-glucosidase for the aglycones of each variety allows profitable use in winemaking technology of aroma release enzymes.

III.3. Establishment of the varietal volatile profile of musts from white *Vitis vinifera* L. varieties

The work previously developed made it possible to establish the aroma potential of Fernão-Pires and Bical varieties (section III.1 and III.2), however, the volatile composition of Arinto and Cerceal was not yet characterized.

The aim of this work was to define the varietal volatile profile characteristics of all four major Bairrada Appellation white varieties Fernão-Pires (FP), Bical (Bic), Cerceal (Cer) and Arinto (Ari), thus allowing the aroma potential of each variety to be established. To fulfil this objective, musts from the four varieties from two harvests (1998 and 1999), were analysed (section II.2.2.1 and II.2.4). In order to minimise the harvest effect in the characterization of must varieties, a contrast data pre-treatment (Wu *et al.*, 1999) was applied before the utilization of principal component analysis (Jolliffe, 1986).

III.3.1. Musts volatile composition

Figure III.3.1 shows the total concentrations of free (F) and potential volatile compounds (PVC) identified in dichloromethane extracts of FP, Bic, Cer and Ari musts from the 1998 and 1999 harvests. The total concentration was obtained as the sum of the individual concentrations of all compounds detected under the experimental conditions used. The 97 compounds detected include aliphatic and aromatic alcohols, ketones, terpenoids, aliphatic acids, phenols, lactones, sulfur compounds, and C_{13} norisoprenoids.

The PVC fraction contains not only the glycosidically-linked components released by the enzymes but also the compounds produced by the heat treatment (100 ºC, 15 min) at the must pH (3.2) as well as compounds arising from the thermal degradation of sugars. Some of these compounds, which are developed during the winemaking and wine ageing, contribute to the final wine aroma (Strauss *et al.*, 1986). This fact is particularly valuable for neutral varieties and/or varieties with poor floral and fruity aromas, such as those under study here. Therefore the free and potential volatile fractions, which include the varietal and the pre-fermentative volatiles, represent the volatile profile of each variety.

In 1998, the free volatile compounds (F 98) from FP accounted for 3.87 mg/L, a value higher than that obtained for Bic (2.67 mg/L), Cer (1.76 mg/L) and Ari (0.84 mg/L). In

1999, harvest, Cer (1.92 mg/L) showed the higher amount of free volatile compounds (F 99), followed by FP (1.84 mg/L), Bic (1.57 mg/L), and Ari (0.47 mg/L).

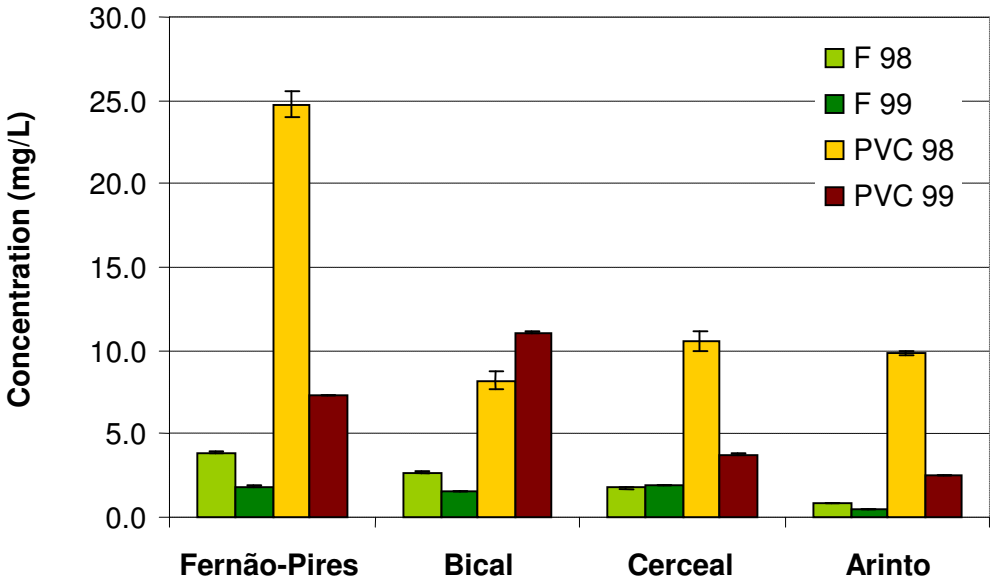


Figure III.3.1. Free (F) and potential volatile compounds (PVC) identified in dichloromethane extracts of Fernão-Pires, Bical, Cerceal and Arinto musts from 1998 and 1999 harvests

In 1998, the PVC (PVC 98) from FP accounted for 24.76 mg/L, a value higher than those obtained for Bic (8.20 mg/L), Cer (10.57 mg/L) and Ari (9.83 mg/L). In 1999, the higher amount of PVC (PVC 99) was found in Bic (11.06 mg/L), followed by FP (7.36 mg/L), Cer (3.75 mg/L) and Ari (2.53 mg/L).

For all four varieties, the amount of the free and potential volatile compounds was higher in 1998 harvest than in 1999. As the majority of furan-derived compounds are sugar degradation products of the thermal treatment done during extraction procedure, they were not included (section III.1).

Because of the considerable importance of volatile terpenoids (mainly monoterpenoids and terpendiols), C_{13} norisoprenoids (Strauss *et al.*, 1986) and aromatic alcohols (section III.1) in determining the flavour and varietal character of *V. vinifera* varieties, particular attention was focused on these three grouos of compounds. Furthermore, these chemical groups were identified as making a particular contribution to the aroma properties of some of the varieties under study, and their concentration in wine may be increased by

appropriated winemaking procedures, mainly by the use of aroma release enzymes (section III.1 and III.2). Concerning these three classes of varietal compounds, which represent the varietal volatile profile, a subset of 22 compounds was established from all volatile components identified in the four varieties. Tables III.3.1 and III.3.2 list the free and potential varietal volatile components of FP, Bic, Cer and Ari, respectively.

III.3.1.1. Free varietal volatile fraction

The composition of the free volatile fraction was different in the four varieties studied, and differences were also observed in the same variety between the two harvests (Table III.3.1). In 1998, the free varietal fraction from FP accounted for 1.50 mg/L, a value higher than those obtained for Bic (0.68 mg/L), Cer (0.38 mg/L) and Ari (0.21 mg/L). In 1999, the higher amount of free varietal fraction was also found in FP (0.82 mg/L), followed by Cer (0.14 mg/L), Bic (0.11 mg/L) and Ari (0.065 mg/L). For all four varieties, the amount of the free varietal volatile compounds was higher in 1998 harvest than in 1999.

In the terpenoid fraction in free form over the two harvests, seven terpenoids were identified in common in FP musts, with terpendiol I (3,7-dimethylocta-1,5-dien-3,7-diol) being the major one, followed by linalool, hotrienol and terpendiol II. Beyond the variability observed between the two harvests, the profile previously established for this variety in the 1998 harvest (section III.1) was maintained in 1999. Of these terpenoids, hotrienol and linalool have been reported as having a determinant role in the wine aroma profile owing to their aroma properties and low sensory perception limits (Marais, 1983). Musts from Bic had terpendiol I as the only terpenoid present in free form in common between the two harvests, confirming the low terpenic character of this variety (section III.1). Cer and Ari varieties had one and three terpenoids, respectively, in common between the musts obtained from the two harvests. Terpendiol I was the only compound common among all varieties and harvests. Although odourless, it can represent a major potential source of grape flavour as precursor of flavourants, such as hotrienol (Wilson *et al.*, 1986).

trans-β-Damascenone seems to be the C_{13} norisoprenoid that characterizes the FP variety, although it was also identified in Bic and Ari musts from only one harvest.

Table III.3.1. Free varietal volatile components identified in dichloromethane extracts of Fernão-Pires, Bical, Cerceal and Arinto musts from 1998 and 1999 harvests, grouped by chemical class

Peak nº	*Compound*	*Ident.*[a]	Concentration (µg/L)															
			Fernão-Pires				Bical				Cerceal				Arinto			
			1998 $\bar{x}$ (n= 6)[b]		1999 $\bar{x}$ (n= 6)		1998 $\bar{x}$ (n= 6)		1999 $\bar{x}$ (n= 6)		1998 $\bar{x}$ (n= 6)		1999 $\bar{x}$ (n= 6)		1998 $\bar{x}$ (n= 6)		1999 $\bar{x}$ (n= 6)	
	Terpenoids																	
1	*trans*-linalool oxide	B,C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	*cis*-linalool oxide	B,C	-	-	-	-	16.2	(29)	-	-	-	-	-	-	-	-	-	-
3	linalool	A,B,C	64.2	(4)	36.8	(3)	6.7	(39)	-	-	55.3	(3)	-	-	4.7	(2)	3.5	(8)
4	hotrienol	B,C	56.8	(13)	15.0	(13)	10.0	(10)	-	-	-	-	-	-	11.8	(5)	-	-
5	*(E)*-2,6-dimethylocta-5,7-dien-2-ol	A,B,C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	α-terpineol	B,C	22.4	(6)	7.9	(15)	3.3	(6)	-	-	4.6	(7)	-	-	2.7	(5)	-	-
7	linalool (*E*)-pyranic oxide	B,C	23.9	(5)	9.2	(13)	3.8	(9)	-	-	4.8	(7)	-	-	3.5	(5)	-	-
8	linalool (*Z*)-pyranic oxide	A,B,C	10.1	(4)	3.1	(3)	-	-	-	-	-	-	-	-	-	-	-	-
9	nerol	A,B,C	-	-	1.7	(3)	-	-	-	-	-	-	-	-	-	-	-	-
10	geraniol	A,B,C	53.6	(3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	3,7-dimethylocta-1,5-dien-3,7-diol (terpendiol I)	B,C	621.8	(3)	650.5	(3)	123.4	(5)	16.2	(7)	46.6	(9)	9.3	(3)	85.6	(3)	6.8	(9)
12	3,7-dimethylocta-1-en-3,7-diol	A,B,C	94.4	(4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	3,7-dimethylocta-1,7-dien-3,6-diol (terpendiol II)	B,C	23.5	(5)	13.8	(9)	-	-	-	-	6.4	(4)	-	-	-	-	-	-
14	3,7-dimethyloctane-1,7-diol	B,C	-	-	-	-	-	-	-	-	-	-	-	-	22.9	(6)	2.1	(8)
15	*(E)*-2,6-dimethylocta-2,7-dien-1,6-diol	B,C	-	-	27.2	(8)	-	-	-	-	-	-	-	-	-	-	-	-
16	*(Z)*-2,6-dimethylocta-2,7-dien-1,6-diol	A,B,C	95.2	(8)	-	-	48.8	(8)	-	-	139.3	(10)	-	-	-	-	-	-
17	geranic acid		35.5	(7)	-	-	17.0	(6)	-	-	-	-	-	-	-	-	-	-
	Sub-total (µg/L)		**1101.3**		**765.2**		**229.3**		**16.2**		**257.1**		**9.3**		**131.1**		**12.4**	
	C_{13} Norisoprenoids																	
18	vitispyrane	B,C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	*trans*-β-damascenone	A,B,C	94.6	(3)	1.6	(11)	6.7	(8)	-	-	-	-	-	-	-	-	1.3	(7)
20	dihydro-β-ionone	A,B,C	23.9	(8)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sub-total (µg/L)		**118.5**		**1.6**		**6.7**		**0.0**		**0.0**		**0.0**		**0.0**		**1.3**	
	Aromatic Alcohols																	
21	benzyl alcohol	A,B,C	78.0	(3)	25.6	(7)	153.5	(4)	14.3	(6)	52.3	(10)	6.0	(4)	21.3	(9)	1.7	(7)
22	2-phenylethanol	A,B,C	191.2	(3)	26.7	(4)	298.2	(5)	82.2	(13)	76.4	(10)	126.9	(3)	55.8	(2)	50.0	(7)
	Sub-total (µg/L)		**269.3**		**52.3**		**451.7**		**96.5**		**128.7**		**133.0**		**77.1**		**51.7**	
	TOTAL (µg/L)		**1489.1**		**819.1**		**687.7**		**112.7**		**385.8**		**142.2**		**208.3**		**65.4**	

[a] The reliability of the identification or structural proposal is indicated by the follows: A –mass spectrum and retention time consistent with those of an authentic standard; B – structural proposal given on the basis of mass spectral data (Wiley 275); C –mass spectrum consistent with spectra found in the literature. [b] Each value is the mean of six extraction replicates; number in parentheses is the corresponding coefficient of variation (%).

The two aromatic alcohols, benzyl alcohol and 2-phenylethanol, were present in the musts of all four varieties in both harvests. The highest amount of free aromatic alcohols fraction was found in Bic (0.45 and 0.096 mg/L in 1998 and 1999, respectively), followed by FP (0.27 and 0.052 mg/L, Cer (*ca*. 0.13 mg/L in both 1998 and 1999) and Ari (0.077 and 0.052 mg/L).

III.3.1.2. Potential varietal volatile fraction

As observed for the varietal volatile components in free form, the potential volatile volatile fraction exhibited differences among the four varieties studied, and also in the same varieties between the two harvests (Table III.3.2). In 1998, the potential varietal volatile fraction from FP accounted for 1.87 mg/L, a value higher than those obtained for Bic (0.56 mg/L), Cer (0.53 mg/L) and Ari (0.13 mg/L). In 1999, the highest amount of potential varietal volatile fraction was also found in FP (0.91 mg/L), followed by Cer (0.21 mg/L), Bic (0.14 mg/L) and Ari (0.049 mg/L) (Table III.3.2). For all four varieties, the amount of potential varietal volatile compounds was higher in 1998 than in 1999. In both harvests, the varietal volatile components of FP and Cer were predominantly in PVC form, accounting for *ca.* 55 and 59 %, respectively, of the total (F+PVC). In contrast, the PVC fraction of Ari represents only *ca.* 40% of the total (F+PVC), the varietal volatiles being mainly in free form. For the Bic variety, the PVC form represented 45 % of the total (F+PVC) in 1998 and 55% of the total (F+PVC) in 1999.

In the terpenoid fraction in potential form over the two harvests, 13 terpenoids were identified in common in FP musts, with terpendiol I being the major one, followed by 3,7-dimethylocta-1-en-3,7-diol, α-terpineol, hotrienol and linalool. As observed for the free fraction, the profile previously established for this variety in the 1998 harvest (section III.1) was maintained in 1999. Considering that the potential contribution of each component to the aroma properties should correspond to the amount in free form plus the amount in PVC form, hotrienol (0.21 and 0.11 mg/L in 1998 and 1999, respectively) and linalool (0.20 mg/L in 1998) were above their sensory perception limit (0.11 and 0.10 mg/L, respectively), suggesting their individual contributions to the FP wine aroma. Linalool has characteristic citrus-like, sweet and flowery notes, while hotrienol and α-terpineol exhibit flowery and sweet aromas (Marais, 1983). Musts from Bic had hotrienol as the only terpenoid present in potential volatile form in common between the two harvests. The amount of this component in F+PVC was below its sensory perception limit. All other terpenoids detected in this variety were below their sensory perception limits, confirming

the low terpenic character of Bic. Cer and Ari varieties had four and two terpenoids, respectively, in common between the musts obtained from the two harvests. With the exception of Bic from 1999, terpendiol I was the only compound common among all the varieties and harvests.

The C_{13} norisoprenoids vitispyrane, *trans*-β-damascenone and dihydro-β-ionone were detected in FP musts from both harvests. Vitispyrane and *trans*-β-damascenone were also present in musts of Bic in both harvests, and in musts of Cer in 1998. C_{13} norisoprenoids are considered grape-derived compounds that arise from carotenoids and are usually not present in free form (Strauss *et al.*, 1986; Williams *et al.*, 1980), which explains the higher amount of these components as PVC. In all these samples the amount of *trans*-β-damascenone in F+PVC was above its sensory perception limit (0.002 mg/L in water) (Belitz *et al.*, 2004), suggesting that this compound is important in explaining the aroma characteristics of these musts. The C_{13} norisoprenoids detected under the conditions of analysis used have no hydroxyl functional groups necessary for the glycosidic linkage. However, under these conditions, hydrolysis of the glycosidic precursor followed by other reactions is known to occur, resulting in the detected C_{13} norisoprenoids (Belitz *et al.*, 2004).

As observed for the free fraction, the two aromatic alcohols, benzyl alcohol and 2-phenylethanol, were present in all four varieties in both harvests. The highest amount of free aromatic alcohols was found in Bic (0.41 and 0.11 mg/L in 1998 and 1999, respectively), followed by FP (0.41 and 0.055 mg/L), Cer (0.20 and 0.13 mg/L) and Ari (0.13 and 0.050 mg/L). The presence of benzyl alcohol and 2-phenylethanol may lead to sweet and flowery notes (Belitz *et al.*, 2004), which could be considered a positive aroma characteristic in white wines, especially for the Bic variety, that exhibits the highest amount of these compounds.

Table III.3.2. Potential varietal volatile components identified in dichloromethane extracts of Fernão-Pires, Bical, Cerceal and Arinto musts from 1998 and 1999 harvests, grouped by chemical class

Peak nº	*Compound*	*Ident.*[a]	Concentration (µg/L)															
			Fernão-Pires				Bical				Cerceal				Arinto			
			1998		1999		1998		1999		1998		1999		1998		1999	
			$\bar{x}$ (n= 6)[b]		$\bar{x}$ (n= 6)		$\bar{x}$ (n= 6)		$\bar{x}$ (n= 6)		$\bar{x}$ (n= 6)		$\bar{x}$ (n= 6)		$\bar{x}$ (n= 6)		$\bar{x}$ (n= 6)	
	Terpenoids																	
1	*trans*-linalool oxide	B,C	25.8	(7)	30.3	(4)	-	-	-	-	8.2	(6)	-	-	7.5	(2)	-	-
2	*cis*-linalool oxide	B,C	36.3	(4)	47.1	(3)	-	-	-	-	9.4	(3)	-	-	-	-	-	-
3	linalool	A,B,C	132.6	(10)	29.9	(7)	17.4	(27)	-	-	52.9	(10)	-	-	-	-	-	-
4	hotrienol	B,C	152.9	(8)	96.6	(1)	20.9	(8)	8.0	(5)	-	-	-	-	18.9	(9)	-	-
5	*(E)*-2,6-dimethylocta-5,7-dien-2-ol	B,C	-	-	1.6	(4)	-	-	-	-	-	-	-	-	-	-	-	-
6	α-terpineol	A,B,C	148.7	(4)	103.7	(8)	5.6	(5)	-	-	31.9	(9)	3.9	(8)	12.0	(10)	0.7	(13)
7	linalool (*E*)-pyranic oxide	B,C	23.6	(6)	7.4	(7)	1.6	(8)	-	-	6.3	(3)	-	-	-	-	-	-
8	linalool (*Z*)-pyranic oxide	B,C	13.3	(8)	5.2	(4)	-	-	-	-	4.2	9.8	-	-	-	-	-	-
9	nerol	A,B,C	18.1	(2)	2.2	(7)	-	-	-	-	-	-	-	-	-	-	-	-
10	geraniol	A,B,C	66.8	(4)	50.8	(3)	-	-	-	-	24.9	(10)	6.2	(5)	-	-	-	-
11	3,7-dimethylocta-1,5-dien-3,7-diol (terpendiol I)	A,B,C	249.3	(6)	237.0	(6)	41.8	(4)	-	-	34.7	(1)	4.9	(9)	16.8	(5)	6.3	(2)
12	3,7-dimethylocta-1-en-3,7-diol	B,C	234.3	(5)	100.3	(6)	-	-	-	-	-	-	24.9	(10)	-	-	-	-
13	3,7-dimethylocta-1,7-dien-3,6-diol (terpendiol II)	A,B,C	53.9	(7)	21.2	(6)	-	-	-	-	27.7	(6)	-	-	-	-	-	-
14	3,7-dimethyloctane-1,7-diol	B,C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	*(E)*-2,6-dimethylocta-2,7-dien-1,6-diol	B,C	-	-	26.3	(4)	-	-	-	-	31.4	(10)	11.4	(14)	-	-	-	-
16	*(Z)*-2,6-dimethylocta-2,7-dien-1,6-diol	B,C	93.5	(6)	-	-	55.0	(8)	-	-	88.3	(6)	-	-	-	-	-	-
17	geranic acid	A,B,C	85.3	(10)	10.3	(6)	-	-	3.7	(3)	-	-	10.0	(9)	-	-	-	-
	Sub-total (µg/L)		**1334.3**		**769.8**		**142.3**		**11.7**		**319.8**		**61.2**		**55.1**		**7.0**	
	C_{13} Norisoprenoids																	
18	vitispyrane	B,C	12.1	(5)	9.6	(3)	5.4	(11)	12.9	(4)	5.4	(9)	-	-	-	-	-	-
19	*trans*-β-damascenone	A,B,C	17.2	(8)	8.8	(14)	5.9	(7)	4.6	(3)	9.6	(7)	-	-	7.7	(9)	-	-
20	dihydro-β-ionone	A,B,C	93.4	(4)	70.1	(10)	-	-	-	-	-	-	21.3	(5)	-	-	-	-
	Sub-total (µg/L)		**122.6**		**88.5**		**11.3**		**17.5**		**15.0**		**21.3**		**7.7**		**0.0**	
	Aromatic Alcohols																	
21	benzyl alcohol	A,B,C	142.2	(8)	29.7	(4)	149.7	(6)	18.2	(8)	84.3	(9)	5.7	(15)	20.9	(10)	2.0	(7)
22	2-phenylethanol	A,B,C	267.8	(5)	25.6	(2)	255.3	(8)	94.1	(10)	111.7	(8)	124.9	(5)	44.9	(6)	40.1	(11)
	Sub-total (µg/L)		**410.0**		**55.3**		**405.0**		**112.3**		**196.0**		**130.6**		**65.8**		**42.1**	
	TOTAL (µg/L)		**1867.0**		**913.6**		**558.6**		**141.5**		**530.8**		**213.1**		**128.6**		**49.1**	

[a] The reliability of the identification or structural proposal is indicated by the follows: A –mass spectrum and retention time consistent with those of an authentic standard; B – structural proposal given on the basis of mass spectral data (Wiley 275); C –mass spectrum consistent with spectra found in the literature. [b] Each value is the mean of six extraction replicates; number in parentheses is the corresponding coefficient of variation (%).

III.3.2. Multivariate data analysis

Since the aim of this study was to discriminate the four must varieties and hence characterize each of them, one possible approach was the application of factorial discriminant analysis (FDA) (Massart *et al.*, 1998); then, by examining the discriminant functions, one could characterize each of the observed clusters (must varieties). However, both the ratio between the number of samples and the number of must varieties (groups) and the strong effect of harvests means that a robust characterization of the samples in terms of discriminant functions would be difficult to achieve. Therefore, it was decided to use a more relaxed approach to uncover the profiles that characterize the must varieties. It was found that a pre-treatment procedure based on the contrasts between variables (in this case the compounds) could improve the ratio of the between- to the within-group variance (Wu *et al.*, 1999) in a very similar manner to the FDA objective function, though in the latter case, the objective function is inherently univariate, while in the former case the approach is multivariate. Thus, the contrast procedure was applied as a data pre-treatment seeking to suppress the harvest effect. The method starts by calculating all possible pairwise differences (contrasts) between all the variables (in this case volatile components). These new variables along with the original ones are then ranked in descendent order according to their Fisher ratios as a function of the sought discrimination (maximising the difference between must varieties). A set of the *m* largest variables (from the ranked Fisher ratios) is then selected for use in the application of PCA. The original data set comprised 22 varietal compounds identified by GC-MS (FP, Bic, Cer and Ari musts, from two harvests, both in free form and as PVC, in a total of 16 samples, each with six replicates).

A PCA was used to study the main sources of variability between the musts of the different varieties (in F and PVC forms from the two harvests) and to establish relationships between the varieties and the volatile components in both forms. Figure III.3.2a shows the scores scatter plot of the first two principal components (PC1 and PC2, accounting for 94% of the total variability) for the data of the PVC fraction of the four varieties. From Figure III.3.2a it is possible to distinguish FP from the other varieties along PC1. However, the separation among Bic, Ari and Cer is unclear. The loadings scatter plot (Figure III.3.2b) shows that all varietal PVC are in PC1 positive, as they have the highest concentration in FP. Also for the free varietal volatile components it was not possible to characterize each of the four varieties by a PCA (results not shown). Thus, the high amount of potential volatile components in FP variety compared with to the other

varieties did not allow the ascription of PVC (markers) to each of the varieties as initially proposed.

As PCA is a factorization procedure that recovers the main sources of variability, it sometimes gives results that are not directly related to the characteristics under study. Since the main aim of this work was to define the varietal volatile profile characteristic of each variety, the harvest effect should be minimised as much as possible. Therefore, the goal was to suppress or minimise this effect and to retrieve the main volatile compounds for each variety. Owing to the nature of this dataset, none of the data pre-treatment procedures for removing artefacts, such as multiplicative scatter correction (MSC), first derivative or second derivative, can be applied to remove the harvest effect. Therefore, a contrast data treatment was applied before the use of PCA, as it improves the ratio of between- to within- class variance, *i.e.* it seeks to maximise the difference between must varieties in the present work.

Figure III.3.3a shows the Contrast-PCA scores scatter plot of the first two principal components (accounting for 99.6% of the total variability) for the data of the PVC fraction of the four varieties. Compared with PCA (Figure III.3.2a), the utilization of Contrast-PCA on the varietal PVC improved the separation of varieties. Figure III.3.3b shows that this separation was characterized by 19 contrast variables and two original variables (2-0 and 11-0). The FP variety is located in PC1 positive, the Cer variety is located in the second quadrant (PC1 negative, PC2 positive), while the third quadrant (PC1 and PC2 negative) is characterized by Bic and Ari varieties, independently of the harvest.

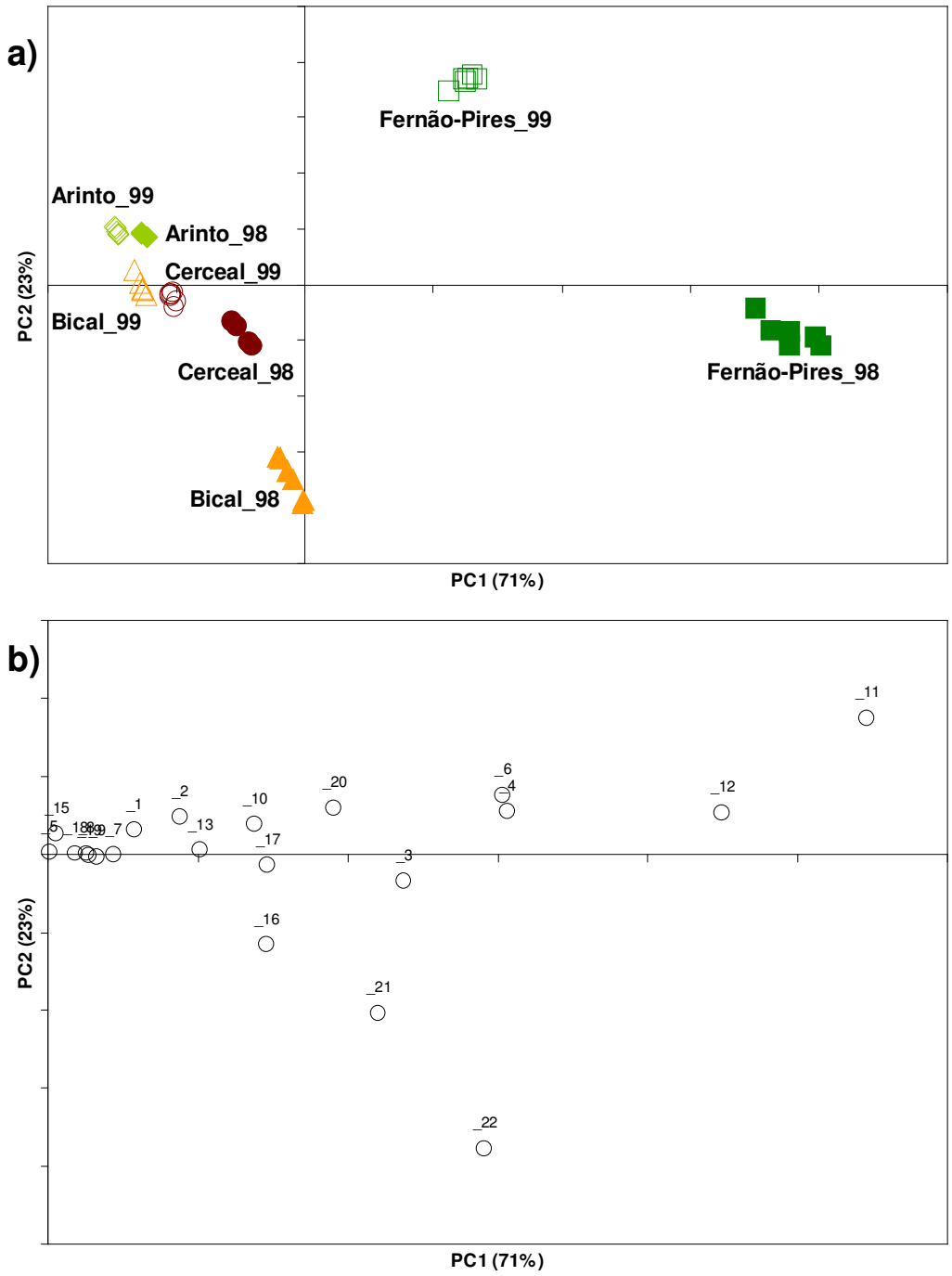


Figure III.3.2. PCA of varietal potential volatile compounds of the four *Vitis vinifera* L. varieties: a) scores; b) loadings (compounds are numbered according to Tables III.3.1 and III.3.2).

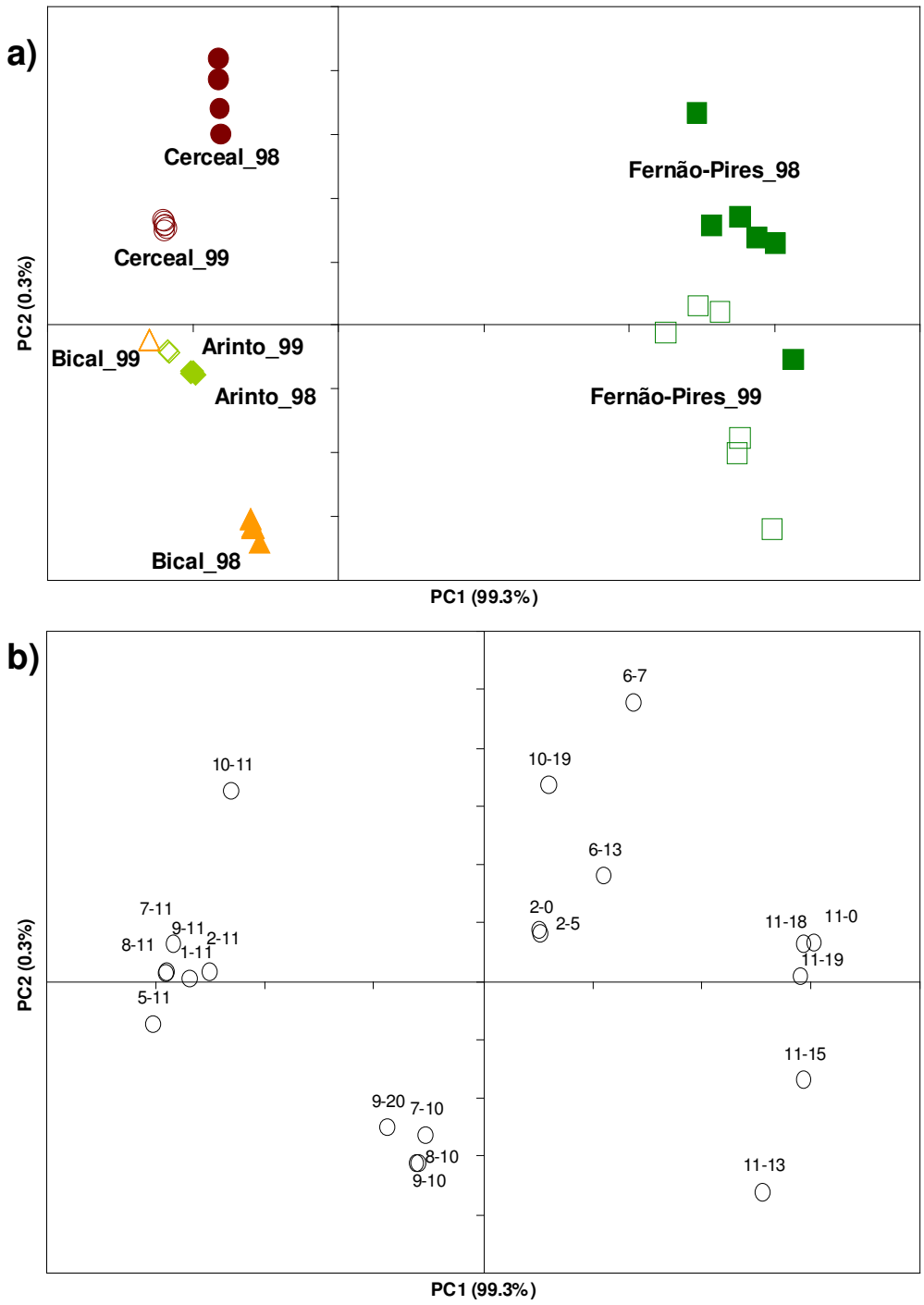


Figure III.3.3. Contrast-PCA of varietal potential volatile compounds of the four *Vitis vinifera* L. varieties: a) scores; b) loadings (compounds are numbered according to Tables III.3.1 and III.3.2).

In order to establish a characteristic varietal volatile profile for each of the must varieties, the loadings scatter plot was analysed. Concerning the varietal PVC data, contrasts were established for FP, Cer and Bic+Ari. Table III.3.3 summarises the findings from the loadings scatter plot (Figure III.3.3b) and related them to each variety. The numbers depicted in this plot correspond to the peak numbers shown in Tables III.3.2 and III.3.3. By comparing Figure III.3.3a (scores) and Figure III.3.3b (loadings), the most relevant variables (original and contrast) characterizing each variety were found. Table III.3.3 indicates that Bic and Ari varieties seem to be very similar owing to the absence of the majority of the compounds under study. Terpendiol I and contrasts of terpendiol I and two C_{13} norisoprenoid and terpendiol II characterized FP variety, which can be explained by the fact that terpendiol I is the major varietal potential component of this variety (section III.1). This is an important feature owing to the role of terpendiol I as the precursor of odourant monoterpenols such as hotrienol, linalool and α-terpineol (Williams *et al.*, 1980; Voirin *et al.*, 2000). For the Cer variety, contrasts of several monoterpenoids and terpendiol I were identified. These results indicate the lower contribution of terpendiol I to the volatile potential of this variety compared to the other monoterpenoids such as geraniol. The varieties Bic+Ari were characterized by the absence of geraniol as a PVC.

Table III.3.3. Original and contrast variables identified for potential volatile components of Fernão-Pires, Bical, Cerceal and Arinto musts

Contrasts identified for potential volatile components		
Fernão-Pires	**Cerceal**	**Bical+Arinto**
terpendiol I	*cis*-linalool oxide – terpendiol I	nerol – di-hydro-β-ionone
terpendiol I **-** vitispyrane	*trans*-linalool oxide **-** terpendiol I	*(E)*-2,6-dimethylocta-5,7-dien-2-ol – terpendiol I
terpendiol I **-** *trans*-β-damascenone	linalool-(*E)*-pyranic oxide – terpendiol I	linalool-(*E)*-pyranic oxide – geraniol
terpendiol I – (*E)*-2,6-dimethylocta-2,7-dien-1,6-diol	linalool-(*Z)*-pyranic oxide **-** terpendiol I	linalool-(*Z)*-pyranic oxide – geraniol
terpendiol I **-** terpendiol II	nerol **-** terpendiol I	nerol – geraniol
	geraniol **-** terpendiol I	

For the free varietal volatile components it was not possible to characterize each one of the four wine varieties by means of PCA or contrast-PCA (results not shown).

Hypothetical explanations for the fact that only contrast-PCA could be applied to the PVC are as follows.

1. The multivariate analysis was based on the data in Table III.3.1 and III.3.2, from which it can be seen that, for the F fraction, 69% of the values were zero, indicating that approximately two-thirds of the 22 compounds were absent from the must varieties. For the PVC fraction, only 48% of the values were zero, so there were more compounds in the PVC fraction than in the F fraction.

2. Since the FP variety was significantly different from the other varieties, it can be seen that, for the F fraction, six volatiles (32% of all varietal volatile components) were present in the FP variety that were not present in the other varieties. However, for the PVC fraction the majority (*ca.* 90%) of the components present in the FP variety were also present in all the other varieties.

3. It was observed that the results from the contrast-PCA loadings for the F fraction (in contrast to the PVC fraction) were highly influenced by terpendiol I, which was present in all the important pairwise differences. In fact, the terpendiol I present in the F fraction was almost three times higher than that found in the PVC fraction.

Therefore, these facts hindered the characterization of the separation between must varieties in the F fraction. Indeed, the results highlighted once more the difference between the FP variety and the other threevarieties, but the strong effect of harvest precluded any possible characterization of the varieties.

III.3.3. Conclusions

This work has shown that FP, Bic, Cer and Ari varieties have different volatile composition patterns in terms of the three varietal chemical groups studied (terpenoids, C_{13} norisoprenoids and aromatic alcohols). The varietal PVC fraction is more related to the varietal features than the free fraction. Besides the free fraction of volatile compounds, naturally non-odourous and non-volatile precursors also represent an important source of fragrant compounds in wine aroma. This aroma potential is naturally revealed during fruit maturation by endogenous enzymes identified as β-glucosidases (Gunata *et al.*, 1990). Since these endogenous enzymes have been reported to show low activities and high variability between harvests, they cannot release the whole aroma potential, which explains the higher variability in the free fraction between harvests than in the PVC fraction. Knowledge of potential aroma composition will allow the identification of the

adequate winemaking procedures for each variety/harvest for the improvement of appropriated winemaking procedures for each variety/harvest for the improvement of wine aroma quality.

The FP variety exhibits a profile significantly different from those of the other varieties, with a higher number and concentration of volatile compounds. The occurrence of odourless terpendiols allows the inference that this variety might be an important one if new strategies of winemaking technology are used, such as treatments to release the aroma components. Distinct aroma release methodologies seem to be effective for all four varieties owing to the fact that they have varietal volatile compounds predominantly in PVC form. The presence of aromatic alcohols, both in free form and as PVC, may be an interesting characteristic especially of the musts from Bic, owing to the possibility of the release of these flowery and sweet compounds. Furthermore, as a consequence of the fact that the four varieties exhibit different volatile composition patterns (different components and different distribution between free and PVC forms), winemaking technologies should be developed specifically for the characteristics of each variety, independently of their use in monovarietal wines or in blends.

III.4. Relationships between the varietal volatile composition of the musts and white wine aroma quality. A four year feasibility study

In wines produced from grapes of the same variety and from the same *terroir*, the factor that have a paramount influence on the maturation process and, consequently, on the composition of grapes and quality of wines are the climatic conditions, such as precipitation, temperature, wind, and sun exposure (Esteves and Orgaz, 2001, Jones and Davis, 2000). In particular, the sun exposure effect has been recognised to have a very important role in the variations of the grapes and wine volatile composition (Belacic *et al.*, 1997; Bergqvist *et al.*, 2001, Bureau *et al.*, 2000). Since these external factors, which may vary across the harvests, have a major influence on the wine quality, the possibility to assess the quality of the wine aroma from the must will allow the selection of the appropriated winemaking conditions for each harvest to achieve the best wine quality.

In this work, the harvest variability of the varietal composition of Fernão-Pires was evaluated using a liquid-liquid continuous extraction followed by analysis by gas chromatography coupled with mass spectrometry (GC-MS) (section II.2.2.1 and II.2.4) of the free (F) and potential varietal components (PVC), from the musts across 1998, 1999, 2000 and 2002 harvests (98_F, 99_F, 00_F, 02_F, 98_{PVC}, 99_{PVC}, 00_{PVC} and 02_{PVC}). Based on the data obtained, a PCA was applied to seek relationships between the varietal volatile composition of the musts and white wine aroma quality classification. This wine classification was conferred by the wine taster chamber of Comissão Vitivinícola da Região da Bairrada (CVRB), the official organism responsible for ruling, inspecting, crediting and promoting the Bairrada Appellation wines.

The varietal composition of the Fernão-Pires musts across four harvests and their different distribution in the F and PVC forms were shown in Table III.4.1. The PVC fraction contains the glycosidically-linked components released by the enzymes plus the compounds produced by the heat treatment (70 ºC, 15 min) at the must pH (3.2) (section III.1).

The free terpenoids, aromatic alcohols and C_{13} norisoprenoids extracted from the musts accounted for 1.5 mg/L in 1998, 0.8 mg/L in 1999, 0.9 mg/L in 2000 and 1.1 in 2002 harvest.

The PVC accounted for 1.9, 0.9, and 1.7 and 2.3 mg/L, which represented, respectively, 56%, 52%, 65 and 68% of the total varietal compounds in each one of the four years.

III.4.1. Free varietal compounds

A total of 14 terpenoid compounds were identified and quantified in the musts of Fernão-Pires. From these, only 9 were present in the four harvests: linalool, hotrienol, α-terpineol, linalool pyranic oxide isomers, 3,7-dimethylocta-1,5-dien-3,7-diol, 3,7-dimethylocta-1,7-dien-3,6-diol, (*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol, and geranic acid. The terpenoids accounted for 0.77-1.1 mg/L, which represent 74% of the total free varietal volatiles in 1998 (98_F), 94% in 1999 (99_F), 87% in 2000 (00_F), and 84% in 2002 (02_F) harvests.

For the four harvests, 3,7-dimethylocta-1,5-dien-3,7-diol (terpendiol I) was the major terpenoid, which was found in amounts ranging from 0.33 mg/L in 00_F to 0.65 mg/L 99_F. This terpendiol is an odourless compound but represents an important potential source of monoterpenoids aroma compounds, such as hotrienol and nerol oxide, by hydrolysis at the wine pH (Wilson *et al.*, 1984).

From all the terpenoids reported in Table III.4.1, based on the known aroma descriptors and SPL, a special attention should be given to linalool and hotrienol. Monoterpenoids are fragrant and are doubtless important to the general enhancement of the floral and fruity aromas (Marais, 1983). These compounds have been reported as having a determinant role in the wine aroma profile due to their aroma properties and low SPL (Simpson, 1979). Linalool has a characteristic citrus-like sweety and flowery notes, and hotrienol exhibit flowery and sweety notes (Marais, 1983). Linalool was only in 00_F (0.11 mg/L) above its SPL (0.10 mg/L) (Marais, 1983). Hotrienol was over its SPL (0.11 mg/L) in 00_F (0.16 mg/L) and in 02_F (0.24 mg/L) (Marais, 1983). These results suggest an individual contribution of linalool for the aroma of 2000 must and hotrienol to the 2000 and 2002 musts, conferring to their aroma characteristics not present in the musts of 1998 and 1999 harvests.

The aromatic alcohol fraction was composed by benzyl alcohol and 2-phenylethanol, which varied in these four harvests, respectively, from 0.026 to 0.078 mg/L and from 0.027 to 0.19 mg/L. The presence of benzyl alcohol and 2-phenylethanol contributes, respectively, with flowery-sweet and flowery/rose/honey notes (Belitz *et al.*, 2004). However, in these musts they were found below their sensory perception limits (200 and 10 mg/L, respectively) (Aznar *et al.*, 2003).

Table III.4.1. Free and potential varietal components identified in dichloromethane extracts of Fernão-Pires musts from 1998, 1999, 2000 and 2002 harvests, grouped by chemical class

peak	Compound	Ident.[a]	Concentration (µg/L) [b]														
			98_F $\bar{x}(n=6)$	(CV)	99_F $\bar{x}(n=6)$	(CV)	00_F $\bar{x}(n=6)$	(CV)	02_F $\bar{x}(n=6)$	(CV)	98_{PVC} $\bar{x}(n=6)$	(CV)	99_{PVC} $\bar{x}(n=6)$	(CV)	00_{PVC} $\bar{x}(n=6)$	(CV)	02_{PVC} $\bar{x}(n=6)$	(CV)	SPL [c] (mg/L)
	Terpenoids																		
6	*trans*-linalool oxide	A, B, C	tr. [d]		---		---		---		25.8	(7)	30.3	(4)	52.1	(7)	111.8	(9)	---
8	*cis*-linalool oxide	A, B, C	tr.		---		---		---		36.3	(4)	47.1	(3)	---		136.3	(8)	---
14	linalool	A, B, C	64.2	(4)	36.8	(3)	110.0	(6)	57.2	(3)	132.6	(10)	29.9	(7)	92.0	(9)	158.8	(9)	0.10
21	hotrienol	B, C	56.8	(13)	15.0	(13)	160.1	(5)	240.9	(1)	152.9	(8)	96.6	(1)	240.8	(4)	521.7	(7)	0.11
27	α-terpineol	A, B, C	22.4	(6)	7.9	(15)	32.8	(3)	14.6	(2)	148.7	(3)	103.7	(8)	216.6	(4)	258.4	(4)	0.4-0.5
32	linalool (*E*)-pyranic oxide	B, C	23.9	(5)	9.2	(13)	43.8	(4)	50.9	(2)	23.6	(6)	7.4	(7)	49.0	(3)	---		3-5
35	linalool (*Z*)-pyranic oxide	B, C	10.1	(4)	3.1	(3)	21.9	(5)	27.8	(4)	13.3	(8)	5.2	(4)	14.4	(4)	58.1	(9)	3-5
39	nerol	A, B, C	---		1.7	(4)	4.6	(6)	---		18.1	(2)	2.2	(7)	17.4	(3)	---		0.4-0.5
42	geraniol	A, B, C	53.6	(3)	---		8.0	(6)	---		66.8	(4)	50.8	(2)	84.0	(5)	44.8	(9)	0.13
53	3,7-dimethylocta-1,5-dien-3,7-diol	B, C	621.8	(3)	650.5	(3)	331.8	(9)	439.8	(1)	249.3	(6)	237.0	(6)	270.0	(6)	117.8	(8)	---
56	3,7-dimethylocta-1-en-3,7-diol	B, C	94.4	(4)	tr.		---		---		234.3	(5)	100.3	(6)	279.8	(3)	157.5	(8)	---
62	3,7-dimethylocta-1,7-dien-3,6-diol	B, C	23.5	(5)	13.8	(9)	51.7	(7)	29.4	(3)	53.9	(7)	21.2	(6)	69.5	(10)	---		---
72	(*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol	B, C	95.2	(8)	27.2	(8)	32.4	(9)	25.2	(3)	93.5	(6)	26.3	(4)	117.8	(7)	74.4	(8)	---
73	geranic acid	B, C	35.5	(7)	4.9	(11)	10.5	(10)	1.9	(4)	85.3	(9)	10.3	(6)	---		20.1	(10)	---
	Subtotal (µg/L)		**1101.3**		**770.1**		**807.6**		**887.7**		**1334.4**		**768.3**		**1503.4**		**1659.7**		
	Subtotal (%)		**74.0**		**93.5**		**86.8**		**83.5**		**71.5**		**84.0**		**88.3**		**73.0**		
	Aromatic alcohols																		
44	benzyl alcohol	A, B, C	78.0	(3)	25.6	(7)	56.7	(3)	72.1	(2)	142.2	(8)	29.7	(4)	92.5	(5)	211.5	(10)	200
48	2-phenylethanol	A, B, C	191.2	(3)	26.7	(4)	61.1	(5)	90.1	(3)	267.8	(5)	25.6	(2)	74.0	(4)	151.6	(11)	10
	Subtotal (µg/L)		**269.2**		**52.3**		**117.8**		**162.2**		**410.0**		**55.3**		**166.5**		**363.1**		
	Subtotal (%)		**18.1**		**6.3**		**12.7**		**15.3**		**22.0**		**6.1**		**9.8**		**16.0**		
	C_{13} norisoprenoids																		
11	vitispyrane	A, B, C	---		---		---		2.1	(16)	12.1	(5)	9.6	(3)	---		57.4	(9)	0.8
31	1,1,6-trimethyl-1,2-dihydronaphtalene	B, C	---		---		---		---		---		1.9	(5)	14.7	(9)	38.6	(11)	0.020
38	*trans*-β-damascenone	A, B, C	94.6	(3)	1.6	(11)	4.8	(6)	6.6	(11)	17.2	(8)	8.8	(14)	17.4	(4)	66.8	(8)	0.000002
75	3-hydroxy-β-damascone	B,C	---		---		---		4.5	(11)	---		---		---		---		---
84	dihydro-β-ionone	B, C	23.9	(8)	---		---		---		93.4	(4)	70.1	(10)	---		88.9	(12)	---
	Subtotal (µg/L)		**118.5**		**1.6**		**4.8**		**13.2**		**122.7**		**90.4**		**32.1**		**251.7**		
	Subtotal (%)		**8.0**		**0.2**		**0.5**		**1.2**		**6.5**		**9.9**		**1.9**		**11.0**		
	TOTAL (µg/L)		**1489.1**		**824.1**		**930.1**		**1063.1**		**1867.0**		**913.9**		**1701.5**		**2274.5**		

98_F - free varietal compounds from Fernão-Pires must of 1998 harvest, **99_F** - free varietal compounds from Fernão-Pires must of 1999 harvest, **00_{FV}** - free varietal compounds from Fernão-Pires must of 2000 harvest, **02_F** - free varietal compounds from Fernão-Pires must of 2002 harvest, **98_{PVC}** - potential varietal compounds of Fernão-Pires must of 1998 harvest, **99_{PVC}** - potential varietal compounds of Fernão-Pires must of 1999 harvest, **00_{PVC}** - potential varietal compounds of Fernão-Pires must of 2000 harvest and **02_{PVC}** - potential varietal compounds of Fernão-Pires must of 2002 harvest. [a] The reliability of the identification or structural proposal is indicated by the following: A- mass spectrum and retention time consistent with those of an authentic standard; B- structural proposals are given on the basis of mass spectral data (Wiley 275); C- mass spectrum consistent with spectra found in the literature. [b] Concentration- mean of six extraction replicates, CV correspond to the coefficient of variation (%). [c] Sensory perception limits found in the literature. [d] trace-concentration less than 1.6 µg/L.

From the four C_{13} norisoprenoids detected in the free form, *trans*-β-damascenone (0.002-0.095 mg/L) was the only one present in the musts of all four harvests. The C_{13} norisoprenoids are known to derive from carotenoids (Baumes *et al.*, 2002). The *trans*-β-damascenone contributes with flowery and exotic fruit notes and has a very small sensory perception limit (2 ng/L) (Winterhalter *et al.*, 1990). As it is above its own limit of perception in all harvests, it may have a contribution for the aroma of these musts.

III.4.2. Potential varietal compounds

A total of 14 terpenoid compounds were identified and quantified in PVC form. From these, only *cis*-linalool oxide, linalool *E*-pyranic oxide, nerol, 3,7-dimethylocta-1,7-dien-3,6-diol, and geranic acid were not present in the four harvests. The terpenoids accounted for 0.77-1.7 mg/L, which represent 72% of the total PVC varietal volatiles in 1998 (98_{PVC}), 84% in 1999 (99_{PVC}), 88% in 2000 (00_{PVC}) and 73% in 2002 (02_{PVC}) harvests.

As observed in the free varietal fraction, terpendiol I was the most abundant PVC terpenoid in the 98_{PVC} and 99_{PVC} harvests (0.25 and 0.24 mg/L, respectively). Beyond terpendiol I, in 00_{PVC}, 3,7-dimethylocta-1-en-3,7-diol (0.28 mg/L) and (*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol (0.12 mg/L), also occurred in higher relative amounts compared to 98_{PVC} and 99_{PVC}. Due to the relevance of terpendiols as aroma precursors, these results allow to predict a better aroma quality for the 2000 harvest wines when compared to the wines from the 1998 and 1999 harvests. In the 02_{PVC}, the major terpenoids were monoterpenols (α-terpineol, hotrienol, and linalool) being the 3,7-dimethylocta-1-en-3,7-diol (0.16 mg/L) and 3,7-dimethylocta-1,5-dien-3,7-diol (0.12 mg/L) the major terpendiols. The monoterpenols hotrienol, α-terpineol, and linalool were the most abundant terpenoid compounds in all four harvests (0.10-0.52 mg/L, 0.10-0.26 mg/L, and 0.030-0.16 mg/L, respectively). Previous studies of the volatile composition of the musts of Fernão-Pires variety showed that hotrienol, α-terpineol, and linalool, play a relevant contribution to the varietal aroma (section III.1). In order to assess their maximum contribution to the wine aroma, the quantification of these compounds in the F plus PVC forms were considered and related to their SPL. For hotrienol, the sum of the F plus PVC forms were 0.21, 0.11, and 0.40 and 0.76 mg/L, for the 1998, 1999, 2000 and 2002 harvests, respectively. According to its SPL (0.11 mg/L), for all harvests, it may be expected its potential individual contribution to the Fernão-Pires wine aroma. For α-terpineol, the sum of the F plus PVC forms were 0.17, 0.11, 0.25 and 0.27 mg/L, for the 1998, 1999, 2000 and 2002 harvests, respectively. According to its SPL

(0.4-0.5 mg/L) (Ribéreau-Gayon *et al.*, 1998), its individual contribution to the Fernão-Pires wine aroma cannot be expected in these harvests, but a contribution to the overall wine aroma cannot be excluded. For linalool, the sum of the F plus PVC forms were 0.20, 0.07, 0.20 and 0.22 mg/L, for the 1998, 1999, 2000 and 2002 harvests, respectively. Due to its SPL (0.10 mg/L), as observed for hotrienol, it was expected its individual contribution to the Fernão-Pires wine aroma for all harvests except 1998.

With the exception of 1999 harvest, the amount of terpenoids from PVC form for 1998, 2000 and 2002 harvests were, respectively, 17%, 46% and 47% higher than the amount in free form. The total amount of PVC terpenoids showed that the musts of 1998, 2000 and 2002 (1.3, 1.5 and 1.7 mg/L, respectively) have a greater aroma potential than the musts of 1999 (0.77 mg/L). As the musts of 2000 and 2002 had free terpenoid compounds above their SPL, it was expected that they origin wines with terpenic notes, indicating a minor dependence to the aroma release treatments during winemaking. The musts of 1998, and in minor extent, those of 1999, had the potential to provide wines with terpenic notes if adequate winemaking methodologies for aroma release were used.

The PVC aromatic alcohol fraction, as in the free form, was composed by benzyl alcohol and 2-phenylethanol, which varied, respectively, from 0.03-0.21 mg/L and from 0.03-0.27 mg/L. As observed for terpenoids, the levels of aromatic alcohols in 1999 harvest were similar in F and PVC forms. For the 1998, 2000 and 2002 harvests, the levels of the PVC forms were, respectively, 52, 41 and 53% higher than the levels of free form.

The amount and number of C_{13} compounds were higher in PVC form when compared to the F form. These compounds have been reported to be mainly in potential form and arise in juice and wines by hydrolytic degradation of precursor substances (Williams *et al.,* 1982c; Strauss *et al.*, 1987; Simpson *et al.*, 1983). In the musts from the three consecutive harvests, vitispyrane and 1,1,6-trimethyl-1,2-dihydronaphtalene (TDN) were present only in the PVC form. As observed for the F form, *trans*-β-damascenone (0.008-0.067 mg/L) was the only C_{13} norisoprenoid present in the musts of all four harvests. The amount of *trans*-β-damascenone was much higher in the PVC than in the free form, with the exception of the 1998 harvest, where the amount of *trans*-β-damascenone 98_{PVC} was much lower than in 98_{F}. Also, contrarily to the observed for terpenoids and aromatic alcohols, the total amount of PVC C_{13} norisoprenoids of 1999 harvest were higher (57 times) than the levels of F form, and were similar for 1998 harvest. As observed for

terpenoids and aromatic alcohols, the levels of the PVC forms for the 2000 and 2002 harvests were 7 and 19 times higher than the free forms.

III.4.3. Principal Component Analysis (PCA) model based on the musts varietal volatile composition to predict the quality of wine aroma

A PCA was applied to the normalised areas of the 21 varietal compounds identified and quantified by GC-MS (98_F, 99_F, 00_F, 98_{PVC}, 99_{PVC} and 00_{PVC}, each with six extraction replicates), in a total of 36 independent assays. This technique has been recently used in many wine applications (Marango *et al.,* 2001; Pozo-Bayón *et al.*, 2001; Escalona *et al.*, 2002; Morales *et al.*, 2002) (section III.1). This PCA was used to study the main sources of variability of varietal volatiles in F and PVC forms from the musts of 1998, 1999, and 2000 harvests and to build a model to relate the musts varietal volatile composition with the sensory classification of Fernão-Pires wine.

Figure III.4.1a shows the scores scatter plot of the first two principal components (PCs), which is related to the distinction between 98_F, 99_F, 00_F, and 02_F, explaining 93% of the variability between the 24 assays. The samples were dispersed along PC1 (73% of the variability) according to the year of harvest: 98_F and 99_F in PC1 positive and 00_F and 02_F in PC1 negative. According to the loadings plot (Figure III.4.1b), PC1 positive (98_F and 99_F) are related mainly to the occurrence of 3,7-dimethylocta-1,5-dien-3,7-diol (53), and in a lesser extent to 3,7-dimethylocta-1-en-3,7-diol (56), *trans*-β-damascenone (38), and 2-phenylethanol (48). The PC1 negative (00_F and 02_F) are described by the occurrence of hotrienol (21) and linalool (14). These results of the free varietal volatile suggest that the musts of 2000 and 2002 harvests can be distinguished from those of 1998 and 1999 due to the presence of hotrienol and linalool. According to Table III.4.1, hotrienol was above its SPL in 00_F and 02_F, and linalool was above its SPL in 00_F, which can allow to expect that better aroma quality wines could be produced from the 2000 and 2002 musts, specially for the 2000 harvest.

Concerning the PC2 (20% of the variability), it may be pointed out that the 98_F, located in the PC2 positive, was characterised by higher amount of the majority of the compounds than the 99_F (PC2 negative), with the exception of 3,7-dimethylocta-1,5-dien-3,7-diol (53) (Figure III.4.1c).

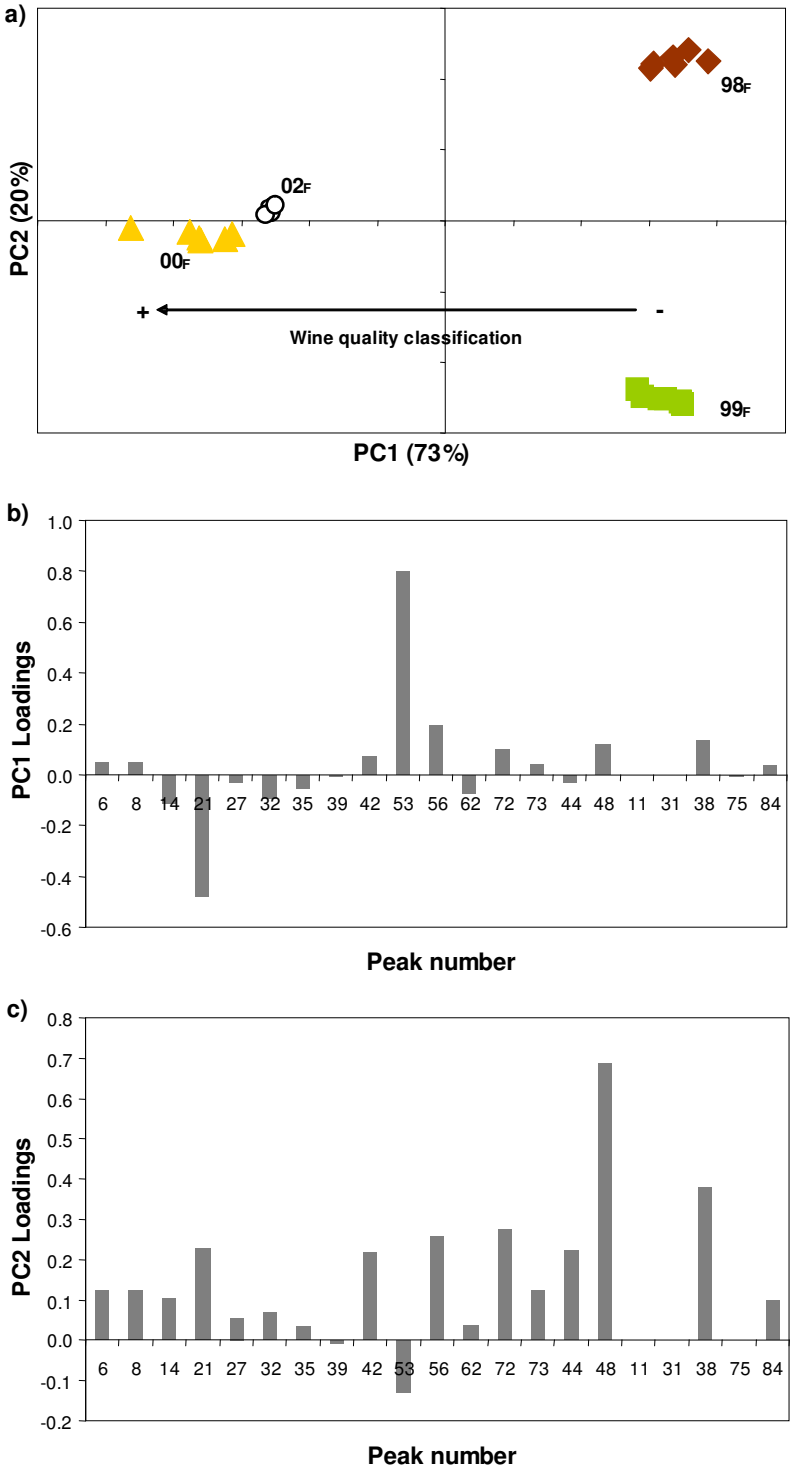


Figure III.4.1. PCA plots of the free forms of Fernão-Pires harvests. **a)** Scores: distinction between the samples of 98_F (◆), 99_F (■), 00_F (▲) and 02_F (O); **b)** PC1 loadings and c) PC2 loadings (attribution of peak numbers shown in Table III.4.1)

Table III.4.2 shows the quality classification of Bairrada white wines DOC-VQPRD (Portuguese designation for controlled quality wines produced in a specified geographical limited region) given by Comissão Vitivinícola da Região da Bairrada. This annual classification, in the range between 0 (very weak) and 5 (excellent), is based on the query annually made to the 96 CVRB associates. The quality classification of 1998, 1999, 2000 and 2002 harvests of Bairrada white wines, of which Fernão-Pires variety represents 80% of the white vineyard, showed that the white wines from the 2000 harvest were excellent wines (classification = 5), the white wines for the 2002 were classified as very good (classification = 4), and the white wines for the 1998 and 1999 harvests were classified as good (classification = 3). These results were closely related to the predicted aroma quality based on the analysis of the musts free varietal volatiles for these four years. According to this methodology developed from GC-MS in tandem with PCA, for the free volatile components, the wine quality classification can be explained by PC1 axis (Figure III.4.1a).

Table III.4.2. Quality classification of Bairrada white wines DOC-VQPRD (Portuguese designation for controlled quality wines produced in a specified geographical limited region) of 1998, 1999, 2000 and 2002 harvests by the wine tasters of CVRB (very weak = 0; weak = 1; average = 2; good = 3; very good = 4; excellent = 5).

Year of harvest	Wine quality classification*
1998	3
1999	3
2000	5
2002	4

* This annual classification is based on the query annually made to the 96 CVRB associates.

Figure III.4.2a shows the scores scatter plot of the first two PCs that represent the distinction between 98_{PVC}, 99_{PVC}, 00_{PVC} and 02_{PVC}, which explain 86% of the total variability. As observed for the free volatile fraction, the assays were grouped according to the year of harvest: 98_{PVC}, 99_{PVC} and 00_{PVC} in PC1 negative and in 02_{PVC} PC1 positive. According to the PC1 a clear distinction was observed between 02_{PVC} and the musts from other three harvests. Figure III.4.2b, shows the PC1 loadings plot: 02_{PVC} was described by the occurrence of hotrienol (21), α-terpineol (27), benzyl alcohol (44), linalool (14), and *trans*- and *cis*-linalool oxide (6 and 8), whereas 98_{PVC}, 99_{PVC} and 00_{PVC} were characterised by 3,7-dimethylocta-1,5-dien-3,7-diol (53) and 3,7-dimethylocta-1,7-dien-3,6-diol (62).

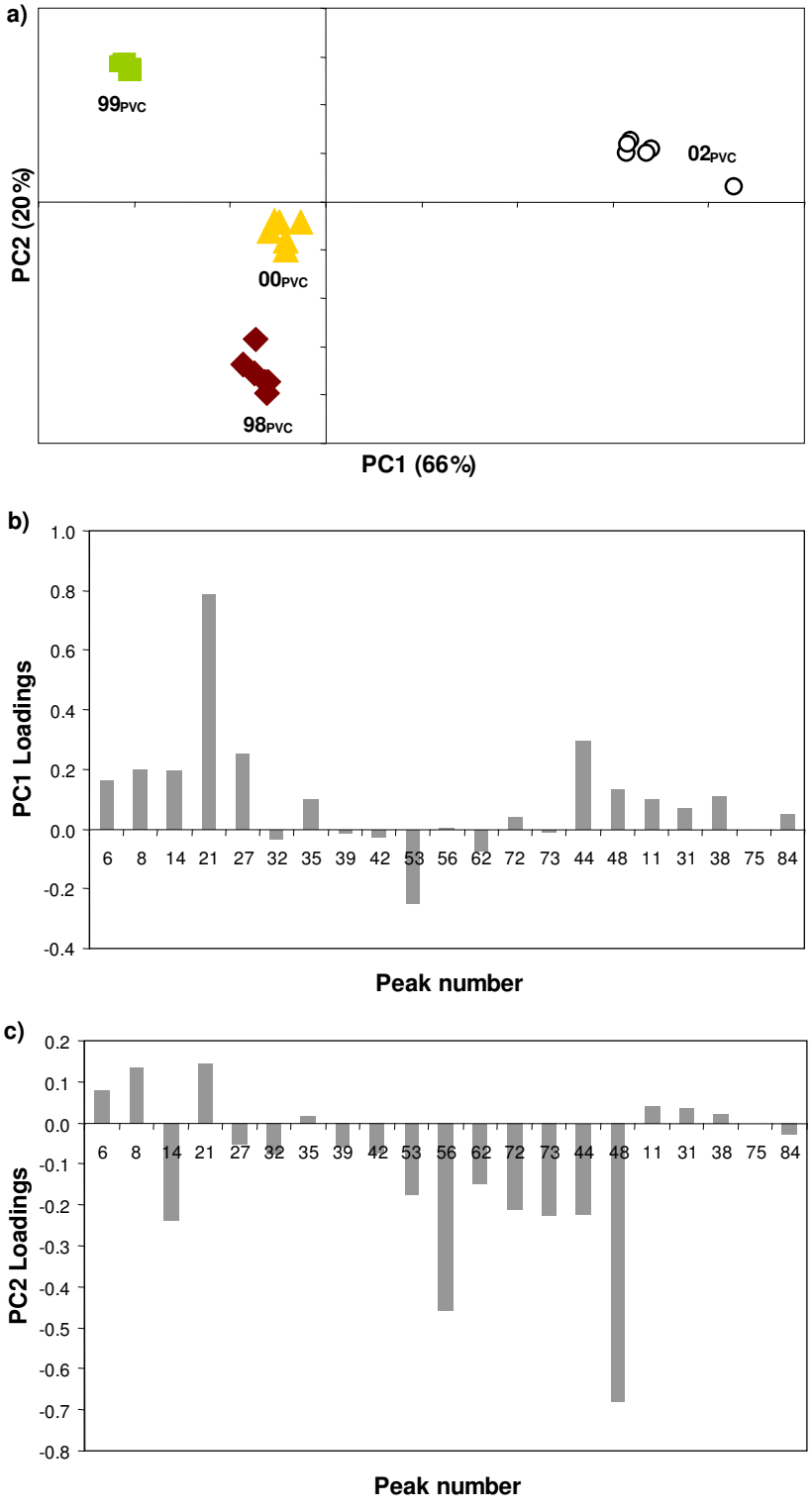


Figure III.4.2. PCA plots of the potential forms of Fernão-Pires harvests. **a)** Scores: distinction between the samples of 98_{PVC} (◆), 99_{PVC} (■), 00_{PVC} (▲), and 02_{PVC} (O); **b)** PC1 loadings (attribution of peak numbers shown in Table III.4.1)

As observed in Table III.4.1, in general, the 02_{PVC} exhibit the higher concentration of varietal volatiles, with exception of the 3,7-dimethylocta-1,5-dien-3,7-diol and 3,7-dimethylocta-1,7-dien-3,6-diol. As observed for the free fraction, according to the PC2, a distinction was observed specially between 98_{PVC} and 99_{PVC}, being the 98_{PVC}, located in the PC2 negative, characterised by higher amount of the majority of the compounds than the 99_{PVC} (PC2 positive), with the exception of hotrienol (21) and *cis*-linalool oxide (8) (Figure III.4.2c). These results about the PVC fraction, allows the distinction of the musts according to their potential aroma precursor's composition. This approach provides information, in real time, to winemakers concerning the winemaking methodologies that may be implemented to improve the wine aroma quality.

III.4.4. Conclusions

The study of the musts of Fernão-Pires variety across four harvests showed that it exhibited a varietal volatile composition variability along the years. The occurrence of compounds such as linalool and hotrienol in free form in amounts that can be higher than their SPL, as well as the occurrence of odourless terpendiols in potentially volatile form in amounts that can represent an important source of monoterpenoids, allows to infer that this variability can be determinant to explain the wine aroma quality observed along the harvests. Based on the data obtained, it was possible to use a GC-MS coupled with PCA to establish relationships between the varietal volatile composition of the musts and the official wine quality classification. This approach may provide two types of information: *i)* the analysis of the free volatile composition may offer direct information about the aroma quality of the wine to be produced without implementation of any specific methodology to improve the aroma quality, and *ii)* the analysis of the PVC provides information to the winemaker about the aroma potential of the musts, which may represent an additional helpful tool to support the winemaker decision. Thus, the producers can choose the adequate winemaking technology, in order to improve the wine aroma quality, if the musts exhibit aroma potential that may be managed. In the case under study, although the must from 2000 harvest did not need any improvement in the winemaking technology to produce wine with an excellent aroma quality, the musts of 1998 and 2002, and in minor extent, those of 1999, had the potential to provide wines with terpenic notes if adequate winemaking methodologies for aroma release were used. The effectiveness of winemaking technologies, namely the aroma release by enzymatic treatments is closely dependent on the varietal aroma potential, whose knowledge is a key determinant for full exploitations of wine's qualities (section III.2).

III.5. Free and glycosidically-linked varietal volatile composition of *Vitis vinifera* L. Fernão-Pires grape variety

The work previously developed made it possible to estimate the aroma potential of Fernão-Pires variety by the analysis of musts and wines. However, the varietal volatile composition of grape and its distribution throughout the different grape fractions (skin, solid fraction of pulp and liquid fraction of pulp) was remained unknown.

Hence, the aim of this work was: *i)* to study the varietal volatile composition of FP grape, and *ii)* to study its composition within the different grape fractions. To fulfil this approach the free (F) and glycosidically-linked (GL) varietal compounds were analysed using grapes of FP white variety from 2002 harvest (section II.2.1 and II.2.4).

The study of free and glycosidically-linked varietal compounds was focused on terpenoids, aromatic alcohols, C_{13} norisoprenoids and C_6 alcohols. The different grapes fractions were studied in order to evaluate the varietal volatile compounds within skins (SK), solid fraction of pulp (SP) and liquid fraction of pulp (LP). The glycosidically-linked fraction was found by releasing compounds by enzymatic hydrolysis from glycoside precursors. Only the compounds that appear at a concentration higher than 1 μg/kg (established as a quantification limit) have been displayed in Tables III.5.1 and III.5.2. The results were expressed in μg/kg of vegetal material (skins, liquid and solid pulp) and the total concentrations were calculated taking into account the different proportions of each fraction within FP berry (19% of skin, 10% of solid pulp and 68% of liquid pulp) (Figure III.5.1).

Globally, the F and GL compounds present in the SK accounted for a total of 24.2 mg/kg, 3.6 mg/kg in SP and 2.5 mg/kg in LP. These results are in accordance with the studies of other grape varieties, where skins are the major source of volatile compounds (Gunata *et al.*, 1985b; Wilson *et al.*, 1986; Park *et al.*, 1991; Gómez et al., 1994; Bayonove *et al.*, 1998a; Diéguez *et al.*, 2003). The C_6 alcohols were the main group of compounds found in the solid fractions (Figure III.5.2). In skins, the C_6 alcohols represent 79%, followed by aromatic alcohols (10%), terpenoids (9%) and C_{13} norisoprenoids (1%) and, in solid pulp, the C_6 alcohols represent 60% of the total FP varietal composition, followed by terpenoids (26%), aromatic alcohols (13%) and C_{13} norisoprenoids (2%). In

liquid pulp, terpenoids represent 69%, aromatic alcohols 18%, C_6 alcohols 10% and C_{13} norisoprenoids (2%). Concerning the different proportions of berry fractions, data shows that skin provided the majority of the total varietal compounds (4.6 mg/kg), followed by liquid pulp (1.7 mg/kg) (Figure III.5.3).

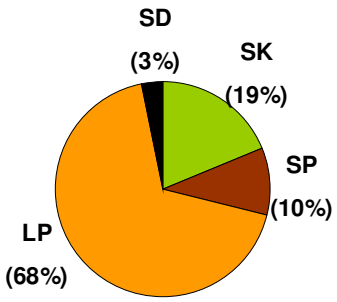


Figure III.5.1. Percentage of the different grape fractions: skin (SK), solid pulp (SP), liquid pulp (LP) and seeds (SD) of Fernão-Pires grape variety

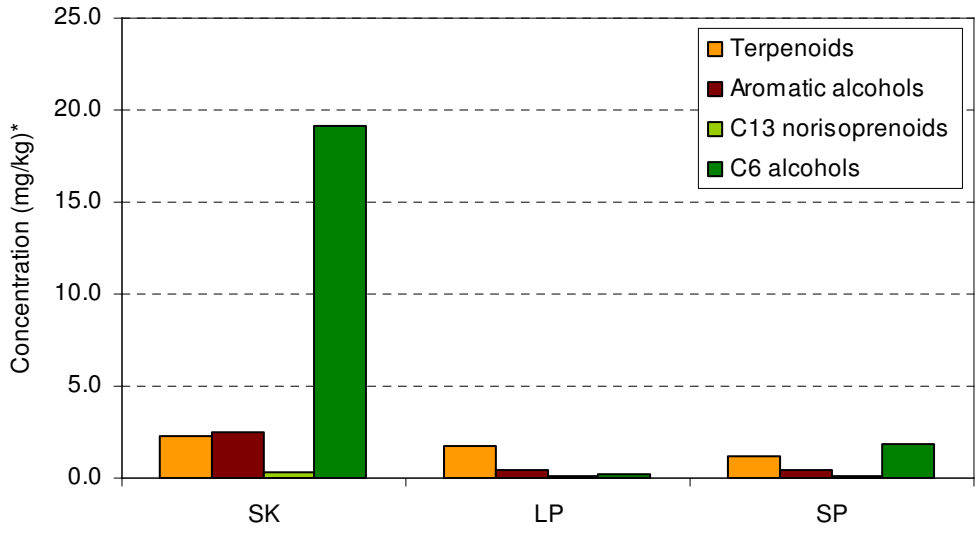


Figure III.5.2. Varietal volatile chemical groups (free+glycosidically-linked compounds) within skins (SK), solid (SP) and liquid pulp (LP), in grapes from Fernão-Pires variety; *(mg/kg of vegetal material)

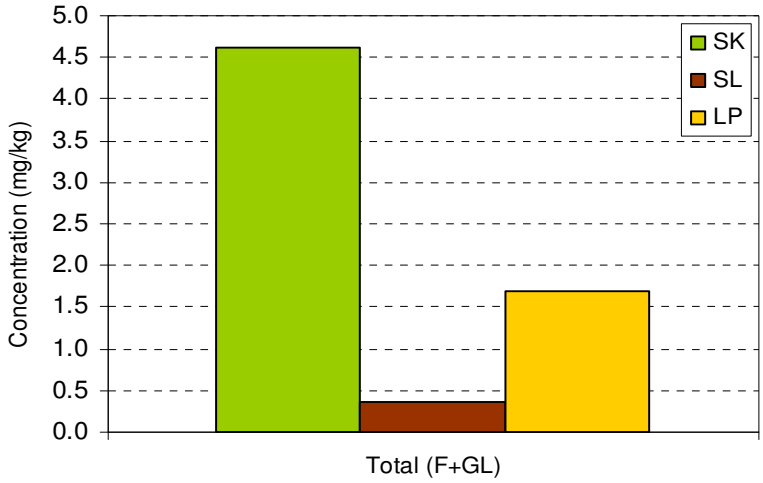


Figure III.5.3. Distribution of varietal compounds (free and glycosidically-linked) within skin (SK), solid (SP) and liquid pulp (LP), in Fernão-Pires berry (mg/kg of berries)

III.5.1. Free varietal compounds

Table III.5.1 shows the free varietal volatile compounds in each different grape fraction (skin, solid fraction of pulp and liquid fraction of pulp) for Fernão-Pires grape variety. For this variety the free varietal volatile compounds were more abundant in SK (20.8 mg/kg), followed by SP (2.1 mg/kg) and LP (0.4 mg/kg). This result is consistent with other studies performed for other white grape varieties, suggesting that varietal compounds are mainly associated to skins (Gunata *et al.*, 1985b; Wilson *et al.*, 1986; Park *et al.*, 1991; Gómez et al., 1994; Bayonove *et al.*, 1998a; Diéguez *et al.*, 2003). Furthermore, the volatile compounds are associated with solid parts of berries, where they are synthetized and/or stored. The results obtained, taking into account the contribution of each fraction on the global volatile composition of FP berry, indicated that the C_6 alcohols were the major chemical group identified in the free form (88.8%), followed by aromatic alcohols (7.8%). Terpenoids represented only 3.3%. Skin provides mainly C_6 alcohols and aromatic alcohols (91.8 and 82.3%) and liquid pulp provides mainly terpenoids (56.0%).

Table III.5.1. Free varietal volatile compounds identified in Fernão-Pires grapes, grouped by chemical class

compounds	Ident.[a]	Concentration (µg/kg of vegetal material)[b]						Berry Total[c]
		SK_F		LP_F		SP_F		
		$\bar{x}_{(n=6)}$	(CV)	$\bar{x}_{(n=6)}$	(CV)	$\bar{x}_{(n=6)}$	(CV)	(µg/kg)
Terpenoids								
trans-linalool oxide	A,B,C	---	---	---	---	---	**---**	**---**
cis-linalool oxide	A,B,C	---	---	1.9	(4)	---	**---**	1.3
Linalool	A,B,C	29.1	(9)	20.0	(3)	48.2	(5)	23.9
Citral	A,B,C	---	---	---	---	4.8	(10)	0.5
α-terpineol	A,B,C	---	---	1.9	(4)	---	**---**	1.3
linalool *(E)*-pyranic oxide	B,C	---	---	20.4	(2)	30.4	(5)	16.9
linalool *(Z)*-pyranic oxide	B,C	5.8	(7)	8.7	(1)	---	**---**	7.0
β-citronellol	A,B,C	---	---	---	---	---	**---**	---
Nerol	A,B,C	---	---	---	---	8.8	(15)	0.9
Geraniol	A,B,C	59.2	(6)	---	---	---	**---**	11.3
3,7-dimethylocta-1,5-dien-3,7-diol	B,C	---	---	51.1	(3)	61.3	(13)	40.9
3,7-dimethylocta-1-en-3,7-diol	B,C	---	---	---	---	---	**---**	---
3,7-dimethylocta-1,7-dien-3,6-diol	B,C	---	---	10.0	(6)	12.4	(5)	8.1
hydroxycitronellol	B,C	---	---	---	---	18.0	(14)	1.8
(*E*)-2,6-dimethylocta-2,7-dien-1,6-diol	B,C	---	---	---	---	---	**---**	---
(*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol	B.C	31.0	(10)	5.2	(9)	19.5	(11)	11.4
geranic acid	B,C	91.5	(10)	---	---	23.0	(8)	19.7
hydroxygeraniol	B,C	---	---	---	---	---	**---**	---
Sub-total (µg/kg)		**216.6**		**119.2**		**226.4**		**144.9**
Sub-total (%)		**1.1**		**28.9**		**10.8**		**3.3**
Aromatic Alcohols								
benzyl alcohol	A,B,C	811.1	(2)	66.4	(3)	52.5	(9)	204.5
2-phenylethanol	A,B,C	688.9	(9)	7.1	(3)	59.5	(5)	141.7
Sub-total (µg/kg)		**1500.0**		**73.5**		**112.0**		**346.2**
Sub-total (%)		**7.2**		**17.9**		**5.3**		**7.8**
C_{13} norisoprenoids								
β-ionone	A,B,C	---	---	---	---	7.6	(13)	0.8
3-hydroxy-β-damascone[d]	B,C	---	---	---	---	---	**---**	---
dihydro-β-ionone	B,C	25.7	(14)	---	---	---	**---**	4.9
3-oxo-α-ionol[e]	B,C	---	---	---	---	---	**---**	---
Sub-total (µg/kg)		**25.7**		**---**		**7.6**		**5.6**
Sub-total (%)		**0.1**		**0.0**		**0.4**		**0.1**
C_6 alcohols								
1-hexanol	A,B,C	16432.8	(8)	108.5	(3)	1530.5	(2)	3349.1
trans-3-hexen-1-ol	A,B,C	---	---	4.6	(3)	---	---	3.1
cis-3-hexen-1-ol	A,B,C	363.0	(4)	14.7	(1)	13.6	(9)	80.3
trans-2-hexen-1-ol	A,B,C	2241.4	(4)	91.5	(1)	209.5	(1)	509.0
Sub-total (µg/kg)		**19037.2**		**219.3**		**1753.6**		**3941.6**
Sub-total (%)		**91.6**		**53.2**		**83.5**		**88.8**
Total (µg/kg)		**20779.5**		**412.1**		**2099.6**		**4438.3**

SK_F, LP_F, SP_F- Free varietal volatile compounds from skin, liquid pulp, and solid pulp, respectively; Berry total= $0.19SK_F+0.68LP_F+0.10SP_F$.
[a] The reliability of the identification or structural proposal is indicated by the following: A mass spectrum and retention time consistent with those of an authentic standard; B structural proposals are given on the basis of mass spectral data (Wiley 275); C mass spectrum consistent with spectra found in the literature.
[b] Concentration- mean of six extraction replicates, numbers in parentheses correspond to the coefficient of variation (%).
[c] Total concentrations take into account the different proportions of skin, liquid pulp and solid pulp in the berry.
[d] 3-hydroxymegastigma-5,8-diene-7-one
[e] 9-hydroxymegastigma-4,7-diene-3-one

The composition of each group of compounds was not the same in each fraction. The terpenoid group was composed by 13 compounds in the free form. They are more abundant in SP (2.3 mg/kg) and SK (2.2 mg/kg). Although most studies report skins as the major source of terpenoids, one other showed that solid pulp can be, in some years, the major source of these compounds (Bayonove et al., 1998b). Globally, the major terpenoid

was geranic acid found in SK, followed by terpendiol found in SP. From the detected monoterpenols, the most abundants were geraniol and linalool in SK, and linalool and linalool (*E*)-pyran oxide in SP and LP. However, all these compounds were under their sensory perception limits, according to Marais (1983). Studies concerning other grape varieties suggest that geraniol is biosynthesized and/or stored in the hypodermic cells of skins and that it has a fundamental role on the monoterpenols metabolism (Gómez *et al.*, 1994). The origin of linalool, despite being spread throughout the grape, seems to be associated to the solid fractions (SK and SP). From the detected terpendiols, 3,7-dimethylocta-1,5-dien-3,7-diol was the major compound of SP and LP. This result agreed with earlier studies concerning FP must, where 3,7-dimethylocta-1,5-dien-3,7-diol was found to be the major terpenoid (section III.1). However, this compound was absent in skins. The 3,7-dimethylocta-1,5-dien-3,7-diol, although odourless, can represent a major potential source of grape flavour as precursor of flavorants such as hotrienol (Williams *et al.*, 1980; Marais, 1983; Wilson *et al.*, 1984).

Two aromatic alcohols were found: benzyl alcohol and 2-phenylethanol. They were clearly more abundant in SK. Benzyl alcohol was more abundant in SK and LP. In SP both compounds were present in similar amounts. They have been described as responsible for floral/sweet odours (Belitz *et al.*, 2004).

The presence of C_{13} norisoprenoids, in free form, is almost inexistent. Only one C_{13} compound was found in SK (dihydro-β-ionone) and one in SP (β-ionone). None was found in LP. This result is consistent with studies that reported small amounts of these compounds in grapes (Skouroumounis and Wintherhalter, 1994).

The C_6 alcohols were clearly more abundant in SK (16.4 mg/kg), followed by SP (1.8 mg/kg). This is due to the presence of the lipoxygenase enzyme and the fatty acids precursors of C_6 compounds in the solid parts of the berry (Gunata *et al.*, 1990; Gómez *et al.*, 1994). In the present work, the preliminary step in which the skins were manually separated from pulp before the analysis (berry preparation), must be considered. During that time, conditions for the formation of C_6 alcohols were created, mainly related to the lipoxygenase activity of the berry and air-contact, particularly in skin (Cayrel *et al.*, 1983; Gunata *et al.*, 1985; Cordonnier, 1989; Gunata *et al.*, 1990). Thus, it is possible that the amounts of these compounds, in particular 1-hexanol, were due to the varietal composition plus those who are formed by air-contact and endogenous enzymatic activity (pre-fermentative compounds). Globally, 1-hexanol was the main C_6 alcohol, followed by (*E*)-2-hexen-1-ol. In particular, 1-hexanol seems to be over the sensory perception limits

for this compound (4.8 mg/L in water) (Marais, 1983). It is responsible for herbaceous and greasy odours, which seem related to deleterious effect in the wine (Baumes *et al.,* 1986; Cordonnier, 1989). This value indicates that attention should be paid to avoid the passage of this component to musts. However, it must take into account that the berry preparation, before the analysis, promoted the presence of these compounds in higher amounts to those expected during winemaking. The results obtained for FP musts (section III.1) showed that (values below the SPL).

III.5.2. Glycosidically-linked varietal compounds

Table III.5.2 shows the glycosidically-linked varietal volatile compounds in each different grape fraction (skin, solid fraction of pulp and liquid fraction of pulp) for Fernão-Pires grape variety. For this variety the glicosidically-linked varietal volatile compounds were more abundant in skins (3.5 mg/kg), followed by liquid pulp (2.1 mg/kg) and solid pulp (1.5 mg/kg). The results obtained, taking into account the contribution of each fraction on the global volatile composition of FP berry, indicated that the terpenoids were the major chemical group identified in the glycosidically-linked form (71.0%), followed by aromatic alcohols (22.1%). The C_{13} norisoprenoids and C_6 alcohols represent only 4.8 and 2.0%. Terpenoids and aromatic alcohols were provided mainly by LP (69.1 and 53.4%, respectively) and SK (24.9 and 39.1% respectively). Skin provides mainly C_{13} norisoprenoids and C_6 alcohols (49.9 and 50.8%, respectively).

The terpenoid fraction of the glycosidically-linked form was composed by 15 compounds. They were more abundant in SK (2.0 mg/kg), followed by LP (1.6 mg/kg). The main compound identified in SK, SP and LP was (*Z)*-2,6-dimethylocta-2,7-dien-1,6-diol. It has been reported to appear naturally in musts of healthy grapes and, contrarily to other diols, is odourant (Gunata *et al*., 1990a). It can also be a precursor of other compounds. The second major terpendiol of all three fractions was 3,7-dimethylocta-1,5-dien-3,7-diol. This compound, despite being spread throughout the grape, was mainly found in LP.

The main monoterpenoids present in the linked-form of FP grape variety was geraniol in the LP (0.2 mg/kg) and in SK (0.07 mg/kg) and linalool in SK (0.06 mg/kg). In particular for geraniol, the sum of F and GL in LP (0.21 mg/kg) and SK (0.13 mg/kg) are over and equal to its sensory perception limits (0.13 mg/L), respectively (Marais, 1983). This compound is fragrant and is important to the general enhancement of the floral and fruity

aromas (Marais, 1983). Although in small amounts, linalool was the second major monoterpenol. Linalool is mainly associated to SK. The presence of a higher amount of terpenoids in the glycosidically-linked fraction compared to the free one shows the potential aroma properties of FP grape. The *trans*-linalool oxide, *β*-citronellol, (*E*)-2,6-dimethylocta-2,7-dien-1,6-diol, 3,7-dimethylocta-1-en-3,7-diol and hydroxygeraniol are only present in the glycosidically-linked form. It is expected that the glycosides of primary alcohols would be enzymatically formed faster than those of secondary and tertiary alcohols (Wilson *et al.*, 1984). This may explain the presence of some glycosides and the absence of others in the free form.

Concerning aromatic alcohols, as observed in the free form, benzyl alcohol is the most abundant for the three fractions. Aromatic alcohols are, once more, particularly abundant in SK. This result allows inferring that, for this variety, aromatic alcohols are synthesized in a greater scale in the skin.

The C_{13} norisoprenoids were distributed mainly by SK (0.28 mg/kg) and SP (0.15 mg/kg) and in small amounts in LP. The C_{13} norisoprenoids were identified mainly in glycosidically-linked form, which is consistent with studies that reported abundant amounts of their precursors (glycosides) in grapes (Skouroumounis and Wintherhalter, 1994). The C_{13} norisoprenoids appeared later in wines by hydrolysis of its precursor's components, playing in some cases a positive contribution on its aroma (Schneider *et al.*, 2001). Globally, 3-hydroxy-*β*-damascone was the main norisoprenoid compound, found in SK and SP but absent in LP. Thus, this compound is associated to the solid part of grapes. Because of the recognized very low sensory perception limit of 0.002 µg/L of C_{13} norisoprenoids in water (Belitz *et al.*, 2004), these compounds seem to be important for grape aroma characteristics. Two norisoprenoids identified in this work (*β*-ionone and dihydro-*β*-ionone) have no functional groups for the glycosylation reaction, their generation requires several steps (namely, hydrolysis of the glycosidic precursor and dehydration), and all these reactions are influenced by the composition of the crushed grapes and assay conditions (Coelho *et al.*, 2007).

Table III.5.2. Glycosidically-linked varietal volatile compounds identified in from Fernão-Pires grapes, grouped by chemical class

compounds	Ident.[a]	Concentration (µg/kg of vegetal material)[b]						Berry Total[c]
		SK_{GL}		LP_{GL}		SP_{GL}		
		$\bar{x}_{(n=6)}$	(CV)	$\bar{x}_{(n=6)}$	(CV)	$\bar{x}_{(n=6)}$	(CV)	(µg/kg)
Terpenoids								
trans-linalool oxide	A,B,C	---	---	1.2	(11)	1.4	(8)	0.9
cis-linalool oxide	A,B,C	tr.	---	---	---	---	**---**	---
linalool	A,B,C	55.6	(3)	8.5	(2)	11.7	(6)	17.5
citral	A,B,C	---	---	---	---	---	**---**	---
α-terpineol	A,B,C	---	---	---	---	---	**---**	---
linalool *(E)*-pyranic oxide	B,C	11.2	(8)	4.0	(7)	4.8	(9)	5.3
linalool *(Z)*-pyranic loxide	B,C	7.4	(7)	---	---	---	**---**	1.4
β-citronellol	A,B,C	8.1	(9)	1.9	(8)	1.5	(5)	3.0
nerol	A,B,C	23.2	(11)	4.2	(9)	2.9	(6)	7.5
geraniol	A,B,C	71.3	(11)	207.7	(4)	---	**---**	157.6
3,7-dimethylocta-1,5-dien-3,7-diol	B,C	85.2	(5)	194.1	(1)	97.4	(5)	157.9
3,7-dimethylocta-1-en-3,7-diol	B,C	tr.	---	2.3	(7)	4.3	(3)	2.0
3,7-dimethylocta-1,7-dien-3,6-diol	B,C	---	---	18.1	(2)	12.7	(7)	13.6
Hydroxycitronellol	B,C	61.5	(8)	6.6	(5)	---	**---**	16.2
(*E*)-2,6-dimethylocta-2,7-dien-1,6-diol	B,C	15.2	(10)	98.5	(1)	66.5	(3)	76.5
(*Z*)-2,6-dimethylocta-2,7-dien-1,6-diol	B.C	1646.5	(5)	1038.8	(2)	662.9	(2)	1085.5
geranic acid	B,C	75.9	(6)	10.6	(7)	27.0	(8)	24.3
hydroxygeraniol	B,C	**---**	---	---	---	15.5	(4)	1.6
Sub-total (µg/kg)		**2061.1**		**1596.7**		**908.6**		**1571.0**
Sub-total (%)		**59.4**		**77.4**		**63.7**		**71.0**
C_6 alcohols								
1-hexanol	A,B,C	80.8	(4)	21.5	(2)	27.8	(4)	32.8
trans-3-hexen-1-ol	A,B,C	---	---	---	---	---	---	---
cis-3-hexen-1-ol	A,B,C	9.4	(5)	5.0	(2)	5.6	(4)	5.7
trans-2-hexen-1-ol	A,B,C	31.1	(12)	---	---	9.1	(16)	6.8
Sub-total (µg/kg)		**121.2**		**26.5**		**42.5**		**45.3**
Sub-total (%)		**3.5**		**1.3**		**2.8**		**2.0**
Aromatic Alcohols								
benzyl alcohol	A,B,C	717.4	(5)	306.1	(1)	305.8	(3)	375.0
2-phenylethanol	A,B,C	287.7	(2)	77.8	(1)	63.9	(4)	114.0
Sub-total (µg/kg)		**1005.1**		**383.9**		**369.7**		**489.0**
Sub-total (%)		**29.0**		**18.6**		**24.7**		**22.1**
C_{13} norisoprenoids								
β-ionone	A,B,C	---	---	---	---	---	**---**	**---**
3-hydroxy-β-damascone[d]	B,C	204.0	(11)	---	---	59.0	(11)	44.6
dihydro-β-ionone	B,C	51.8	(11)	46.5	(13)	37.5	(8)	45.2
3-oxo-α-ionol[e]	B,C	24.0	(5)	10.1	(2)	52.1	(8)	16.6
Sub-total (µg/kg)		**279.8**		**56.6**		**148.5**		**106.5**
Sub-total (%)		**8.1**		**2.7**		**9.9**		**4.8**
C_6 alcohols								
1-hexanol	A,B,C	80.8	(4)	21.5	(2)	27.8	(4)	32.8
trans-3-hexen-1-ol	A,B,C	---	---	---	---	---	---	---
cis-3-hexen-1-ol	A,B,C	9.4	(5)	5.0	(2)	5.6	(4)	5.7
trans-2-hexen-1-ol	A,B,C	31.1	(12)	---	---	9.1	(16)	6.8
Sub-total (µg/kg)		**121.2**		**26.5**		**42.5**		**45.3**
Sub-total (%)		**3.5**		**1.3**		**2.8**		**2.0**
Total (µg/kg)		**3466.9**		**2063.7**		**1497.0**		**2211.7**

SK_{GL}, SP_{GL}, and LP_{GL}- Glycosidically-linked varietal volatile compounds from skin, solid pulp, and liquid pulp, respectively; Berry total= $0.19SK_{GL}+0.68LP_{GL}+0.10SP_{GL}$.

[a] The reliability of the identification or structural proposal is indicated by the following: A mass spectrum and retention time consistent with those of an authentic standard; B structural proposals are given on the basis of mass spectral data (Wiley 275); C mass spectrum consistent with spectra found in the literature.

[b] Concentration- mean of six extraction replicates, numbers in parentheses correspond to the coefficient of variation (%).

[c] Total concentrations take into account the different proportions of skin, liquid pulp and solid pulp in the berry.

[d] 3-hydroxymegastigma-5,8-diene-7-one

[e] 9-hydroxymegastigma-4,7-diene-3-one

The C_6 alcohols were distributed mainly by SK (0.12 mg/kg) and SP (0.04 mg/kg), and in small amounts in LP (0.03 mg/kg). These compounds were mainly associated with to the skins. It is important to refer that the amount of these compounds in the glycosidically-linked form was very low comparatively to the free fraction. Thus, these compounds are mainly present in the free fraction. Globally, as observed in the free fraction, 1-hexanol was the main C_6 alcohol followed by *trans*-2-hexen-1-ol, except in LP.

III.5.3. Conclusions

Eighteen terpenoids, two aromatic alcohols, four C_{13} norisoprenoids and four C_6 alcohols were identified as varietal volatile compounds of FP white grape variety. They were found in concentrations between 1.2 μg/kg and 16.4 mg/kg and were mainly related to the solid grape and the glycosidically-linked fractions. Compounds such as geraniol and 1-hexanol can be found over their sensory perception limits. The results obtained in this work showed that the liquid fraction of pulp provided a significant amount of terpenoids in both F and GL forms. However, skin that represents 19% of FP berry can be an important source of aromatic alcohols in both F and GL forms, and of C_{13} norisoprenoids and terpenoids in GL. This suggested that, in order to produce FP wines with higher aroma quality, adequate methodologies must be developed *(i)* to promote the transference of varietal compounds mainly from skins, to must and wine; *(ii)* avoiding, simultaneously, the transference of C_6 alcohols (mainly 1-hexanol) that may confer an unpleasant herbaceous odour. Both technological changes/improvements, related mainly with enzymatic preparations used during winemaking to release the linked compounds and skin contact time, may be taken into consideration as strategies to increase the aroma quality of FP wine variety. Optimal conditions could be attained.

III.6. Rapid tool for distinction of wines based on the global volatile signature

Recently, the technique using microextraction-mass spectrometry-multivariate analysis (SPME-MS-MVA) was developed for the rapid characterization of foods, as an alternative approach to current commercial *e*-nose instruments (electronic noses) (section I.3.4). The application of fast characterization techniques to wines and wine industry is almost inexistent. A kind of *e*-nose, using a headspace sampler coupled to a mass spectrometer, was proposed for the differentiation and classification of wines (Martí *et al.*, 2004) and other one was proposed to differentiate cork wine stoppers (Boudaoud and Eveleigh, 2003). However, as far as we known, SPME was never used for that purpose.

In this study a novel approach is proposed for the rapid distinction of wines based on the global volatile signature of the wine headspace obtained by headspace solid-phase microextraction-gas chromatography-mass spectrometry-principal component analysis (HS-SPME-GC-MS-PCA) (section II.3). This study comprises two steps: (*i*) optimisation of the SPME sampling conditions (coating fibre and temperature) and (*ii*) establishment of the global volatile signature of the wine headspace using the coating fibre and temperature of extraction previously selected. To obtain an adequate distinction between the wines in the shortest analysis time, the time of extraction was also optimised. This methodology was tested in two monovarietal wines: *Vitis vinifera* L. var. Fernão-Pires (FP) and Arinto (Ari). Principal component analysis (PCA) was applied to extract relevant chemical information by selecting the most significant mass fragments (*m/z*) that provide the better wine distinction.

III.6.1. Optimisation of SPME parameters

The wines contain volatile compounds in different amounts, which exhibit different contributions to the aroma properties. In order to increase the signals (markers) associated to the components and to decrease the signals associated to the components present in higher amounts and that can add considerable uninformative variability to the data, the SPME sampling conditions, namely the type of coating fibre and the temperature, were optimised. Several studies carried out on grapes characterization recognised a relationship between the wine varietal character and the grape and musts volatile and semi-volatile compounds, such as monoterpenoids, C_{13} norisoprenoids and aromatic

alcohols. The varietal grape composition is determined, in white grapes, mainly by terpenoids and aromatic alcohols (section III.1 and III.2). The aliphatic alcohols and, especially the ester, may contribute to the wine aroma properties with floral and fruity notes (section III.2). However, as they are common in the majority of the varieties, it is not really recognised if they have a potential contribution to the wines distinction.

In the first step of this work, the volatile and semi-volatile components present in the wine headspace were deeply analysed by HS-SPME followed by GC-MS, using GC conditions that allows an adequate chromatographic resolution. The aim of this step was the optimisation of the SPME conditions, in order to obtain a more balanced chromatogram, *i.e.* reduced weight signals associated to the major components, such as esters and aliphatic alcohols, and increased weight signals associated to the minor components, such as the varietal components.

III.6.1.1. Coating fibre and temperature of extraction

The volatile and semi-volatile components of FP wine were analysed using a polyacrylate (PA) and a carbowax-divinylbenzene (CW-DVB) fibres, both at 25 and 40 ºC. A total of 73 compounds were identified in the headspace of the FP wine, which were grouped in the following chemical classes: terpenoids+C_{13} norisoprenoids+aromatic alcohols, esters, acids and aliphatic alcohols (Figure III.6.1). For all the conditions tested the esters exhibited the higher chromatographic areas followed by the aliphatic alcohols. It was also observed that for both fibre coatings, the extraction temperature of 40 ºC provides the higher chromatographic areas for all the chemical groups, including the varietal compounds (terpenoids, C_{13} norisoprenoids and aromatic alcohols). The exception was the esters, where similar areas were obtained by the CW-DVB fibre at 25 and 40 ºC. The comparison of the two coating fibres showed that, for the same temperature, the CW-DVB fibre extracted fewer esters and alcohols than PA. The two coating fibres exhibited similar retention capacity for the varietal compounds (Figure III.6.1). Taking into account the signal levels associated to the varietal components and the lower signal intensities associated to the components present at significantly amounts, such as esters and aliphatic alcohols, the CW-DVB fibre and the temperature of 40 ºC were selected.

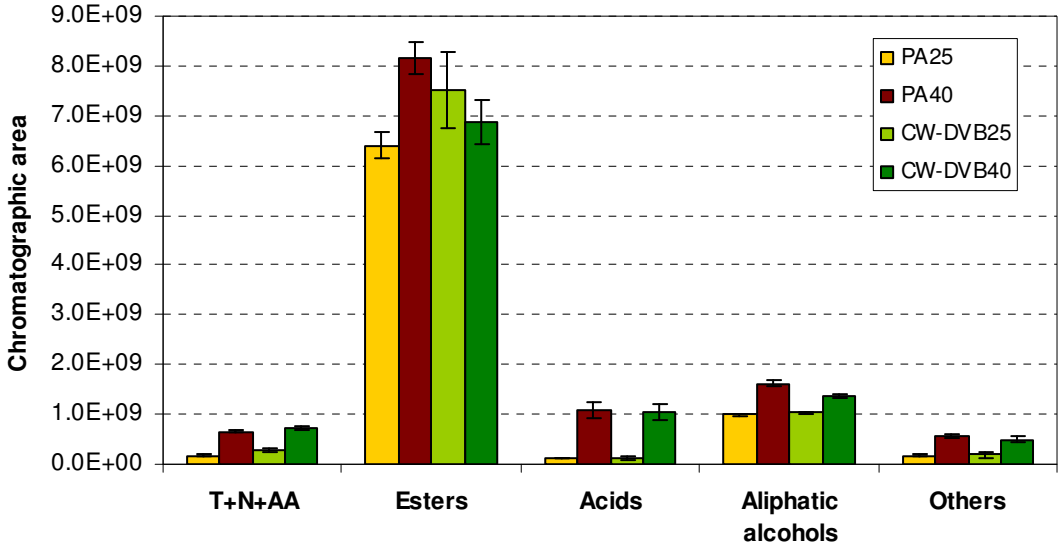


Figure III.6.1- Main chemical groups of Fernão-Pires wine obtained by PA and Carbowax-DVB coating fibre, using an extraction temperature of 25 and 40 ºC.

III.6.2. Study of the volatile components of FP and Ari wines

Following the establishment of the coating fibre and the temperature of extraction to be used, the volatile and semi-volatile components present in the headspace of FP and Ari wines were analysed. Although the wines from FP variety has been previously characterized (see III.1 and III.2), no study had yet been done for the wines of Ari variety. In this study, a total of 73 compounds were identified in FP and 56 in Ari wines (Table III.7.1). The total chromatographic area of FP wine (1.2×10^{10}) was 1.4 times higher than Ari (8.6×10^{9}). Esters and alcohols were the most abundant groups in the two varieties, respectively, 60% and 30% in FP and 66% and 28% in Ari. The esters ethyl octanoate and ethyl decanoate and the alcohols ethanol, 2-methyl-1-butanol and 2-phenylethanol, represented the major components in both varieties. The two esters may contribute with sweety and fruity notes; 2-phenylethanol may contribute with flowery, rose and honey notes (Belitz *et al.*, 2004), and 2-methyl-1-butanol should exhibit banana notes. 2-Phenylethanol was 17% more abundant in Ari than in FP wine, which may be a potential parameter for their distinction.

Table III.6.1. Volatile and semi-volatile components present in the wine headspace of Fernão-Pires (FP) and Arinto (Ari) varieties, obtained by HS-SPME (CW-DVB coating fibre, 40.0 ºC) followed by a GC–MS analysis, using GC conditions that allow an adequate chromatographic resolution.

Compounds	Identity[a]	*m/z*					FP						Ari					
							Area (n=3)	**CV**	**Extraction time (min)**				**Area** (n=3)	**CV**	**Extraction time (min)**			
									5	**10**	**15**	**20**			**5**	**10**	**15**	**20**
Esters																		
ethyl isobutyrate	B,C	43	71	116	88	101	-	-	-	-	-	-	74,781,076	7	x	x	x	x
2-methylpropylacetate	B,C	43	56	73	86		-	-	-	-	-	-	6,092,789	11	x	x	x	x
ethyl butanoate	B,C	71	43	88	60	101	22,337,640	8	x	x	x	x	16,254,844	13	x	x	x	x
ethyl 2-methylbutanoate	B,C	57	102	41	85	74	21,001,032	20	x	x	x	x	12,291,111	24	x	x	x	x
ethyl 3-methylbutanoate	B,C	88	85	57	60	70	20,137,103	24	x	x	x	x	6,865,349	23	x	x	x	x
3-ethyl-1-butanol acetate	B,C	43	70	55	61	87	70,810,342	12	x	x	x	x	11,705,921	11	x	x	x	x
ethyl hexanoate	A,B,C	88	99	43	60	70	351,774,015	3	x	x	x	x	209,621,895	7	x	x	x	x
ethyl acetate	A,B,C	56	61	69	84		26,827,932	2	x	x	x	x	-	-	-	-	-	-
cis-3-hexen-1-ol acetate	B,C	43	67	82			1,794,839	2	x	x	x	x	-	-	-	-	-	-
ethyl (*E*)-2-hexenoate	B,C	97	55	99	73	114	1,476,985	6	-	?	x	x	3,219,579	15	-	?	x	x
ethyl 2-hydroxypropanoate	A,B,C	45	75	103	118		13,089,358	2	-	x	x	x	233,752,446	9	-	x	x	x
methyl octanoate	B,C	74	87	127	115	101	-	-	-	-	-	-	38,040,804	14	-	x	x	x
ethyl octanoate	A,B,C	88	101	127	57	70	2,694,517,286	6	x	x	x	x	1,633,940,971	2	x	x	x	x
3-methylbutyl octanoate	B,C	70	99	43	71	55	5,111,554	11	x	x	x	x	5,964,086	12	x	x	x	x
ethyl 3-hydroxybutanoate	B,C	43	60	71	87	88	-	-	-	-	-	-	6,782,340	16	x	x	x	x
ethyl nonanoate	B,C	88	101	73	141	115	1,976,989	37	x	x	x	x	4,222,733	16	x	x	x	x
ethyl 2-hydroxyhexanoate	B,C	69	87	43	104		2,376,568	3	-	?	x	x	7,430,335	7	-	?	x	x
2-methylpropyl octanoate	B,C	56	57	127	145	73	1,619,820	6	?	x	x	x	3,295,285	17	?	x	x	x
methyl decanoate	B,C	74	87	143	155	101	1,742,795	14	x	x	x	x	4,635,182	14	x	x	x	x
ethyl decanoate	A,B,C	88	101	73	157	155	3,440,342,353	7	x	x	x	x	1,358,701,469	4	x	x	x	x
2-methylbutyl octanoate	B,C	70	55	57	127	145	29,341,578	8	x	x	x	x	27,928,585	28	x	x	x	x
diethyl succinate	A,B,C	101	129	55	73		75,334,680	4	x	x	x	x	411,049,381	13	x	x	x	x
ethyl 9-decenoate	B,C	41	55	88	69	84	36,063,125	10	x	x	x	x	-	-	-	-	-	-
isobutyl decanoate	B,C	56	57	155	173	71	2,955,982	28	?	x	x	x	2,172,557	40	?	x	x	x
2-ethylphenyl acetate	B,C	91	164	65	105	119	2,444,698	6	-	?	x	x	11,818,312	13	-	x	x	x
diethyl pentanedioate	B,C	143	45	115	87	55	tr.	-	x	x	x	x	-	-	-	-	-	-
ethyl 3-methylbutyl butanedioate	B,C	101	129				-	-	-	-	-	-	1,735,836	4	x	x	x	x
2-phenylethyl acetate	A,B,C	104	91				79,296,010	6	-	x	x	x	6,207,567	13	-	-	x	x
ethyl 4-hydroxybutanoate	B,C	87	88	43	102	69	2,284,022	17	x	x	x	x	-	-	-	-	-	-
ethyl dodecanoate	B,C	88	101	55	73	157	411,081,282	11	x	x	x	x	34,779,321	14	x	x	x	x
2-methylbutyl decanoate	B,C	70	43	71	155	55	13,045,191	14	x	x	x	x	10,048,678	6	x	x	x	x
2-phenylethyl-2-methylpropanoate	B,C	104	43	71	91	77	1,208,919	37	x	x	x	x	-	-	-	-	-	-
diethyl 2-hydroxybutane-1,4-dioate	A,B,C	117	71	89	145		2,286,400	29	-	x	x	x	-	-	-	-	-	-
ethyl tetradecanoate	B,C	88	101	55	73	157	1,558,984	54	-	x	x	x	-	-	-	-	-	-
ethyl 4-ethoxybenzoate	A,B,C	121	149	138	194	166	2,370,043	26	?	x	x	x	-	-	-	-	-	-
ethyl 2-hydroxy-3-phenylpropanoate	B,C	91	176	121	101	92	889,381	35	x	x	x	x	-	-	-	-	-	-
ethyl hexadecanoate	B,C	88	101	55	73	157	1,099,175	13	x	x	x	x	-	-	-	-	-	-
3,4-dimethoxy-benzaldehyde	B,C	166	165	95	51	77	1,000,456	87	?	?	x	x	-	-	-	-	-	-
Sub-total (area)							**7,339,196,537**						**5,706,564,372**					
Sub-total (%)							**59.9**						**66.1**					

Table III.6.1. *(Continued)* Volatile and semi-volatile components present in the wine headspace of Fernão-Pires (FP) and Arinto (Ari) varieties, obtained by HS-SPME (CW-DVB coating fibre, 40.0 ºC) followed by a GC–MS analysis, using GC conditions that allow an adequate chromatographic resolution.

Compounds	Identity[a]	*m/z*					FP						Ari					
							Area (n=3)	**CV**	**Extraction time (min)**				**Area** (n=3)	**CV**	**Extraction time (min)**			
									5	**10**	**15**	**20**			**5**	**10**	**15**	**20**
Alcohols																		
ethanol	A,B,C	45	46	43			1,721,796,667	4	x	x	x	x	1,477,259,931	3	x	x	x	x
1-propanol	B,C	59	42	60	41		22,079,140	12	?	x	x	x	18,246,248	7	?	x	x	x
2-methyl-1-propanol	B,C	43	74	59			56,319,916	13	-	x	x	x	106,528,660	19	-	x	x	x
1-butanol	B,C	56	41	43			13,330,415	35	x	x	x	x	4,134,207	13	x	x	x	x
2-methyl-1-butanol	B,C	57	41	56	70		1,206,660,038	3	x	x	x	x	1,601,266,724	7	x	x	x	x
4-methyl-1-propanol	B,C	56	69	41	84		-	-	-	-	-	-	1,998,080	15	x	x	x	x
3-methyl-1-pentanol	B,C	56	69	41	84		2,596,911	15	x	x	x	x	4,051,609	10	x	x	x	x
1-hexanol	A,B,C	56	43	55	69	84	35,175,731	3	?	x	x	x	51,208,669	7	x	x	x	x
trans-3-hexen-1-ol	A,B,C	41	67	55	69	82	931,489	4	x	x	x	x	1,373,631	5	?	x	x	x
cis-3-hexen-1-ol	B,C	67	41	55	82	100	6,232,469	3	x	x	x	x	-	-	-	-	-	-
1-octanol	A,B,C	56	41	70	84		2,899,269	3	x	x	x	x	4,485,247	23	x	x	x	x
(*R*,*R*) + (*S*,*S*)-2,3-butanediol	A,B,C	45	57	75	90		1,622,657	17	?	x	x	x	-	-	-	-	-	-
1-nonanol	B,C	56	55	70	69	83	-	-	-	-	-	-	5,885,926	10	x	x	x	x
1-decanol	A,B,C	55	70	83	97	112	3,169,152	12	-	?	x	x	4,872,007	17	-	-	x	x
2-phenylethanol	A,B,C	91	92	122	65		627,237,338	7	?	x	x	x	735,299,666	6	?	x	x	x
3,4-dimethoxybenzyl alcohol	B,C	168	139	151	109	65	6,956,290	8	x	x	x	x	-	-	-	-	-	-
Sub-total (area)							**3,708,053,995**						**2,415,343,881**					
Sub-total (%)							**30.2**						**28.0**					
Acids																		
acetic acid	A,B,C	43	45	60			47,134,835	1	x	x	x	x	77,203,467	8	x	x	x	x
propanoic acid	A,B,C	45	57	75	90		1,835,065	6	x	x	x	x	-	-	-	-	-	-
isobutyric acid	A,B,C	43	73	88			6,498,618	14	x	x	x	x	9,133,807	6	x	x	x	x
butanoic acid	A,B,C	60	73	88			4,971,189	6	x	x	x	x	-	-	-	-	-	-
3-methylbutanoic acid	B,C	60	87				15,953,892	4	?	x	x	x	13,652,047	5	?	x	x	x
hexanoic acid	A,B,C	60	73	87	55		16,576,311	3	x	x	x	x	62,081,999	15	x	x	x	x
octanoic acid	A,B,C	60	73	101	84	85	621,662,260	10	x	x	x	x	241,404,417	10	x	x	x	x
decanoic acid	A,B,C	60	73	129	55	87	360,017,945	28	x	x	x	x	-	-	-	-	-	-
Sub-total (area)							**1,074,650,115**						**403,475,737**					
Sub-total (%)							**8.8**						**4.7**					
Terpenoids																		
limonene	A,B,C	68	93	67	79	107	7,317,065	15	?	x	x	x	-	-	-	-	-	-
α-terpinene	B,C	93	121	136	91	79	1,313,775	12	x	x	x	x	-	-	-	-	-	-
linalyl acetate	B,C	93	136	121			3,859,908	21	x	x	x	x	-	-	-	-	-	-
α-terpinolene	B,C	93	136	121	79	105	1,313,775	12	x	x	x	x	1,963,652	38	x	x	x	x

Table III.6.1. *(Continued)* Volatile and semi-volatile components present in the wine headspace of Fernão-Pires (FP) and Arinto (Ari) varieties, obtained by HS-SPME (CW-DVB coating fibre, 40.0 ºC) followed by a GC–MS analysis, using GC conditions that allow an adequate chromatographic resolution.

Compounds	Identity[a]	*m/z*					FP						Ari					
							Area (n=3)	**CV**	**Extraction time (min)**				**Area** (n=3)	**CV**	**Extraction time (min)**			
									5	**10**	**15**	**20**			**5**	**10**	**15**	**20**
Terpenoids (cont.)																		
geranyl acetate	B,C	69	93	121	81	99	10,586,859	14	x	x	x	x	-	-	-	-	-	-
linalool	A,B,C	71	93	55	80	121	47,414,462	3	x	x	x	x	5,501,752	8	x	x	x	x
hotrienol	B,C	71	82	67	91		2,058,565	8	x	x	x	x	-	-	-	-	-	-
α-terpineol	A,B,C	59	93	121	136	81	37,368,237	8	x	x	x	x	16,478,330	6	x	x	x	x
citronellol	A,B,C	69	81	95	55	123	1,052,189	9	?	x	x	x	1,723,665	4	-	x	x	x
nerolidol	A,B,C	69	93	107	81	136	2,692,421	30	?	x	x	x	**-**	-	-	-	-	-
Sub-total (area)							**114,709,994**						**25,667,399**					
Sub-total (%)							**0.9**						**0.3**					
C_{13} noriprenoids																		
theaspirane	A,B,C	93	69	121	80	112	912,122	38	?	x	x	x	-	-	-	-	-	-
vitispirane I	B,C	93	192	121	136	177	3,214,117	16	x	x	x	x	17,397,627	4	x	x	x	x
vitispirane II	B,C	93	192	121	177	136	2,004,915	26	x	x	x	x	17,929,974	5	x	x	x	x
1,2-dihydro-1,1,6-trimethyl-naphthalene	B,C	157	142	172	115	128	10,122,707	12	x	x	x	x	16,971,622	5	x	x	x	x
trans-β-damascenone	B,C	69	121	105	190	91	**-**	-	-	-	-	-	7,992,575	12	x	x	x	x
Sub-total (area)							**16,253,861**						**60,291,798**					
Sub-total (%)							**0.1**						**0.7**					
Others																		
3–hydroxy-2-butanone	A,B,C	45	43	88			-	-	-	-	-	-	3,894,390	8	x	x	x	x
benzaldehyde	A,B,C	106	105	77	51		-	-	-	-	-	-	8,052,310	12	x	x	x	x
γ-butyrolactone	A,B,C	42	86	56			3,624,245	2	-	-	x	x	5,859,167	11	-	x	x	x
methionol	A,B,C	106	61	57	47	73	2,786,405	8	x	x	x	x	4,568,600	18	x	x	x	x
phenol	A,B,C	94	66	65	55		572,089	12	-	-	x	x	-	-	-	-	-	-
benzophenone	B,C	105	182	77	51		879,582	45	-	-	x	x	-	-	-	-	-	-
Sub-total (area)							**7,862,321**						**22,374,467**					
Sub-total (%)							**0.1**						**0.3**					
Total (area)							**12,260,726,823**						**8,637,538,958**					

CV, coefficient of variation (%); tr, trace; x, presence and identification of the *m/z* fragment in the global volatile signature of the wine headspace for the time of extraction under study; ?, uncertainty of the presence of the *m/z* fragment in the global volatile signature of the wine headspace for the time of extraction under study; –, absence of area, CV and/or *m/z* fragment. For each compound the chromatographic area and the respective *m/z* fragment obtained in full scan, ordered by decreasing order of intensity are indicated. The compounds are grouped in chemical classes and ordered by increasing retention time. For each extraction time (5, 10, 15 and 20 min), it was tentatively identified, from the global volatile signature of the wine headspace, the *m/z* corresponding to the compounds identified in the wines (first column).

[a] The reliability of the identification or structural proposal is indicated by the following: A, mass spectrum and retention time consistent with those of an authentic standard; B, structural proposals are given on the basis of mass spectral data (Wiley 275); C, mass spectrum consistent with spectra found in the literature.

Acids and terpenoids were more abundant in FP than in Ari wine. However, C_{13} norisoprenoids were more abundant in Ari than in FP. The chromatographic area of acids and terpenoids were, respectively, 62% and 77% higher in FP than in Ari wine, which is in accordance with the terpenic character of FP variety shown in previous studies (see III.1 and III.2). The C_{13} norisoprenoids were 73% higher in Ari than in FP. This different varietal profile of FP and Ari may also be a powerful parameter that can contribute to the distinction of the varieties, in particular, terpenoids and C_{13} norisoprenoids, which may have an important contribution on the varietal aroma of the wines. The most abundant terpenoids reported in Table III.7.1 were linalool and α-terpineol in FP and α-terpineol in Ari. Linalool was already found in earlier studies to be the major monoterpenoid in FP wine (see III.2). Monoterpenoids are fragrant and are no doubt important to the general enhancement of floral and fruity aromas (Marais, 1983). These compounds have been reported as having a determinant role in the wine aroma profile due to their aroma properties and low sensory perception limit (Simpson, 1979). From the five C_{13} norisoprenoids found, the two varieties have in common the two vitispiranes isomers and 1,2-dihydro-1,1,6-trimethyl-naphthalene (TDN). Theaspirane was only detected in FP and *trans*-β-damascenone was only detected in Ari variety. The C_{13} norisoprenoids are known to derive from carotenoids (Baumes *et al.*, 2002), and may contribute with camphor, honey-like, black currant or cassis notes (Belitz *et al.*, 2004; Winterhalter *et al.*, 1990; Rapp, 1998).

III.6.3 Establishment of the global volatile signature of the wine headspace

III.6.3.1. Extraction time

In order to obtain a global volatile signature of the wine headspace, *i.e.* a chromatographic profile and a *m*/*z* pattern of fragmentation in each scan, adequate for distinction between the varieties in the shortest possible time of analysis, four extraction times were tested (5, 10, 15 and 20 min). Figure III.6.2 shows the global volatile signature of the wine headspace of FP and Ari wines obtained by HS-SPME-GC–MS for 5 min of extraction. Similar profiles were obtained for 10, 15 and 20 min (data not shown). The signal (total ion current) obtained exhibited unresolved and asymmetrical wide peaks through 0.8 min (0.7–1.5 min). PCA was applied to extract useful chemical information by selecting the most significant mass fragments (*m*/*z*) that allow a better wine separation in

the shortest time of extraction, according to the *m*/*z* fragments previously established for the compounds identified in both varieties (Table III.6.1).

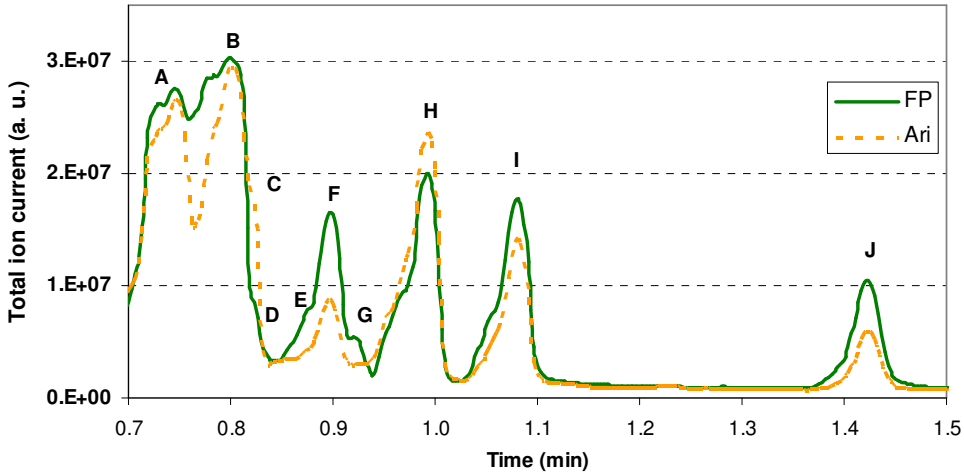


Figure III.6.2. Global volatile signature of the wine headspace of Fernão-Pires and Arinto wines obtained by HS-SPME-GC–MS, for 5 min of extraction (peak assignments in Table III.6.2). a.u.: arbitrary units

Figure III.6.3 shows the scores scatter plot of the global volatile signature of FP and Ari wines using different times of extraction. The two first principal components accounted for 78% of the total variability present in the dataset. A clear distinction was observed between the two wine varieties along PC1 (53% of the total variability) for all extraction times used, being Ari located in the PC1 positive and FP in the PC1 negative. Along PC2, which contains 25% of the total variability, the samples, independently of the wine variety, were distributed as a function of the extraction time. The lower extraction times were located in the PC2 positive, and the higher ones were located in the PC2 negative. These results showed clearly that this methodology has the power to distinguish FP from Ari, and 5 min of extraction are enough for that distinction.

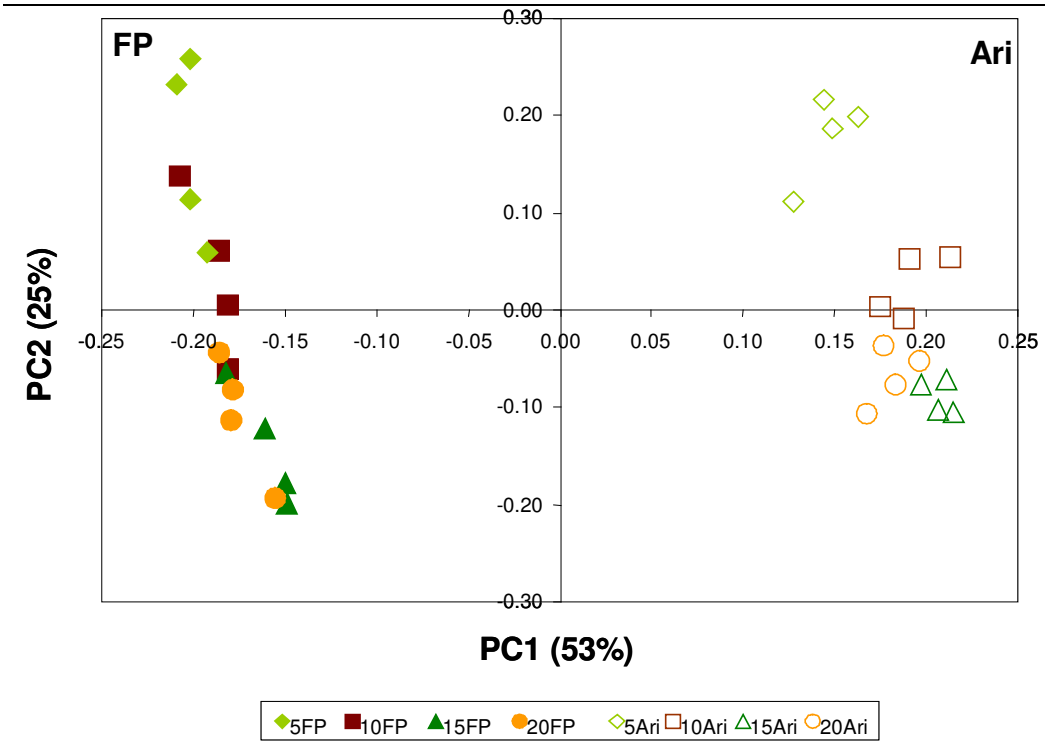


Figure III.6.3. PC1×PC2 scores scatter plot of the global volatile signature of Fernão-Pires and Arinto wines for 5, 10, 15 and 20 min of extraction

III.6.3.2. Chemical information provided by the global volatile signature of the headspace of FP and Ari wines

An exhaustive study was carried out on the global volatile signatures, in order to tentatively identify the *m*/*z* fragments related to the wine components. The exercise provides the *m*/*z* pattern of fragmentation in each scan for the period of 0.8 min (0.7–1.5 min), which was carried out for the two wine varieties and for the extraction times of 5, 10, 15 and 20 min. This approach allows: (*i*) to tentatively identify the *m*/*z* fragments corresponding to the compounds detected in the FP and Ari wines (Table III.6.1) and (*ii*) to define 10 chromatographic zones (A–J, Figure III.6.2). According to their *m*/*z* fragments, they could be related only with one compound or with a group of compounds (Table III.6.2). The zones D and H seem to be particularly interesting as they exhibit contributions of the terpenoids and 2-phenylethanol, respectively.

It was observed that the *m*/*z* fragmentation pattern changed along the chromatographic profile, providing a fingerprint for each wine based on its volatile composition (Table III.6.1). As the compounds involved in this work are volatile and semi-volatile, thus with low molecular weight, they exhibit small *m*/*z* ions. From these, a large

number are common to several compounds. To detect the *m*/*z* fragments associated to all compounds perceived in both varieties, in particularly the varietal ones, such as 2-phenylethanol, limonene and citronelol (Table III.6.1), the extraction time of 10 min was considered as the minimum required. However, this information seems to be unnecessary to obtain a distinction between the varieties, when a PCA was applied to data. Considering that this approach, involving the exhaustive study of the GC–MS data, is time-consuming, a statistical data treatment was applied.

Table III.6.2. Relation between the chromatographic zones defined in Figure III.6.2, the *m*/*z* characteristic fragments and their tentatively attributed compounds or group of compounds.

Chromatographic zone (From Figure III.6.2)	**Main *m/z* fragments**	**Compounds or group of compounds**
A	88, 101, 127	Esters (ethyl octanoate)
B	88, 101, 157	Esters (ethyl decanoate)
C	101, 129	Diethyl succinate
D	59,93,121,136	Terpenoids
E	60,73,88,101	Acids and esters
F	88,101	Esters
G	104,91	2-phenylethyl acetate
H	91,92,122	2-phenylethanol
I	60,73,101	Acids (octanoic acid)
J	60,73,129	Acids (decanoic acid)

To extract relevant information by selecting the most significant mass fragments (*m*/*z*) that provide the better wine distinction, a PCA was applied. Figure III.6.4 represents the 2D PC1 loadings map which characterizes the distinction of the samples observed in the scores scatter plot (Figure III.6.3) by combining the chromatographic data of the global volatile signatures and the *m*/*z* fragmentation data of each scan. The analysis of the 2D loadings map allowed the identification of the *m*/*z* fragments that mainly contribute to the separation of the FP and Ari varieties. As was seen in Figure III.6.3, the Ari variety was characterized by PC1 positive values which, according to Figure III.6.4, corresponded to the spots identified by the numbers 1, 6, 7 and 10. On the other hand, the FP variety,

characterized by PC1 negative values, corresponded to the spots identified by the numbers 2, 3, 4, 5, 8 and 9. The intensity of spot numbers 2 and 8 are close to zero, which allowed to infer that these two spots had a very small contribution to the varieties distinction.

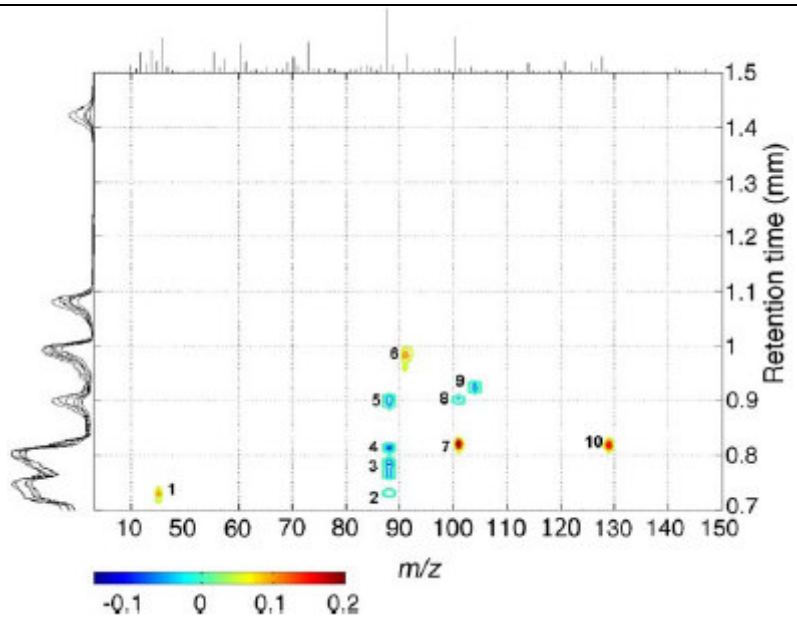


Figure III.6.4. 2D PC1 loadings map combining the chromatographic data of Figure III.6.3 and *m*/*z* fragmentation data of each scan

From the 2D loading map of spot numbers 3, 4, 5 and 9 (Figure III.6.5, right), representing the PC1 negative values that characterize the FP variety, the *m*/*z* fragments identified were the 88 (present in spots 3, 4 and 5) and the *m*/*z* fragment 101 (spot 5), related to esters, ethyl octanoate, ethyl decanoate and ethyl dodecanoate; the *m*/*z* fragments 60 and 73, related to acids, octanoic and decanoic acids (spot 5) and the *m*/*z* 91 and 104 (spot 9) linked to 2-phenylethylacetate. According to Table III.6.1, all these compounds were present in higher amount in FP variety than in Ari. On the other hand, the *m*/*z* fragments that characterize the Ari variety are, in spot 1, fragments with *m*/*z* 43 and 45, which presented the lower retention time (0.73 min) that are probably associated to highly volatile compound. In spot 6, *m*/*z* fragments 91, 92 and 122, ascribed to 2-phenylethanol, as previously suggested. This compound has a higher chromatographic area in Ari than in FP variety (Table III.6.1). The *m*/*z* fragments 101, 129 in spots 7 and 10, related to diethyl succinate and/or ethyl 3-methylbutyl butanedioate, can also be related to

Ari variety. Both of these two compounds, according to Table III.6.1, were present in higher amounts in Ari than in FP variety. However, the detection of the *m*/*z* 55 and 73 in the loadings corresponding to these two spots suggests that they were related with diethyl succinate.

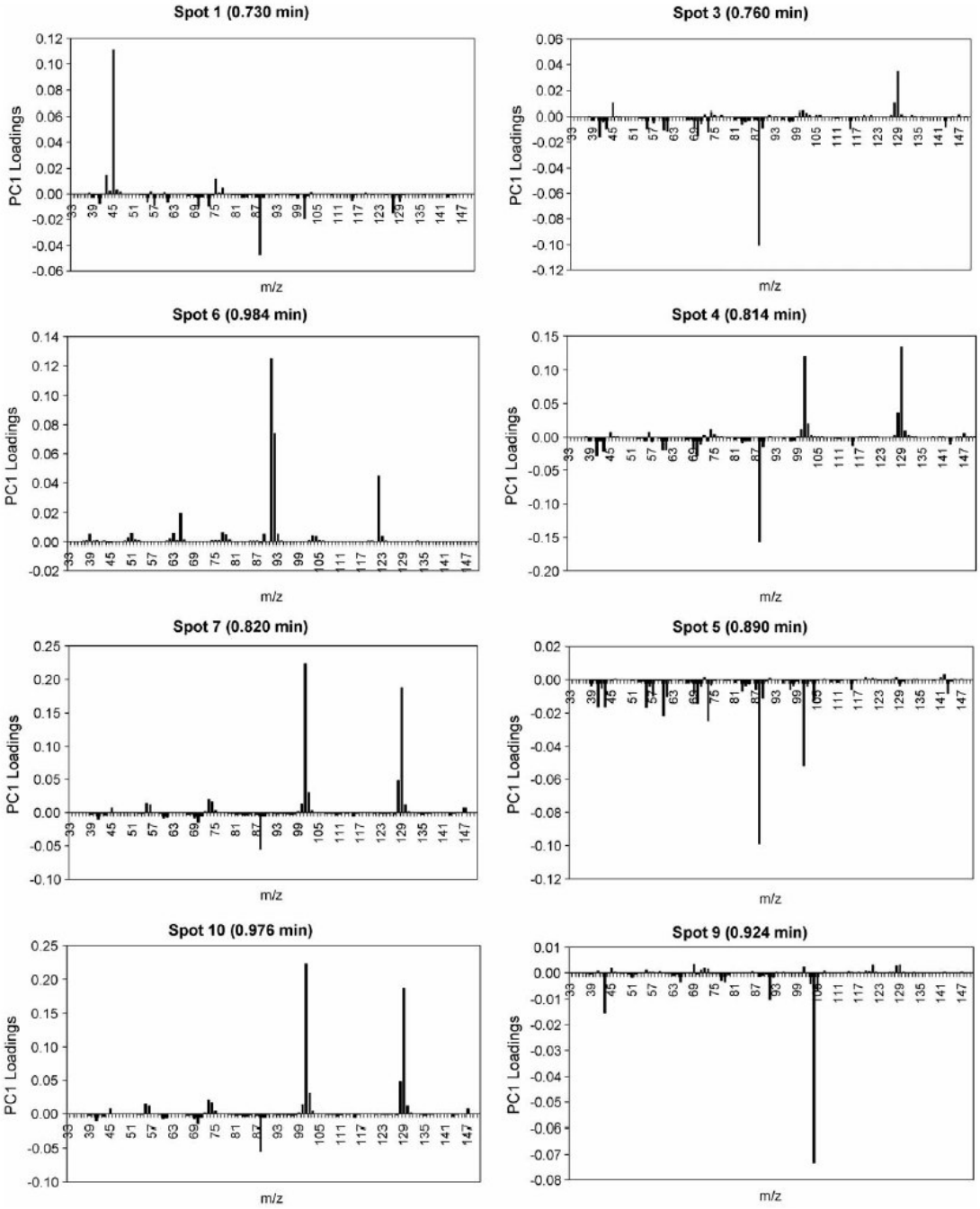


Figure III.6.5. Loadings profiles through the spots observed in the 2D PC1 loadings map: the spots 3, 4, 5 and 9 are located at PC1 negative and characterize the FP variety whereas spots 1, 6, 7 and 10 are located at PC1 positive and characterize the Ari variety

III.6.4. Conclusions

PCA based on HS-SPME-GC–MS data provided rapid distinction of the two wines, independently of the extraction time used, which allowed to establish *m*/*z* fragments associated to the wine components of FP and Ari varieties. This methodology represents a simple and effective tool for the wine distinction by rapid analysis of their headspace volatile fraction. The total time of analysis of 6.5 min (5 min for SPME plus 1.5 min of GC–MS analysis) seems to be sufficient for the distinction of wine varieties. Associated to the fast character of the proposed methodology and robustness taking into account the extraction time, it is also important to focus the higher sensibility and the lower effect of the sample moisture of the MS sensor response when compared to the conventional *e*-noses.

Moreover, the mass fragmentation data also provides a fingerprint of each wine that contains the chemical information about its volatile composition. The *m*/*z* fragments associated to esters and acids (ethyl octanoate, ethyl decanoate, ethyl dodecanoate; 2-phenylethylacetate, diethyl succinate and/or ethyl 3-methylbutyl butanedioate, octanoic and decanoic acids) and to 2-phenylethanol seems to be responsible for the wine distinction. Despite the optimisation of the experimental methodology, in order to increase the signal weight associated to varietal compounds, the improvement obtained was not enough to extract varietal chemical information. However, the results indicate that the wine distinction was achieved without this information. The applicability of the proposed methodology can certainly be wider than that presented in this study. In future studies it will be essential to apply it to a higher number of wine samples and focus the analysis in the *m*/*z* ion fragments associated to the varietal components or others that may contribute with odour descriptors.

The results suggest that it is possible to focus the analysis only in some zones of the chromatographic profiles that revealed to be more interesting in the varietal composition point of view. This approach allows to extract specific information about chemical composition. Hence, it is possible to define varietal markers that will allow the distinction and the possible classification of wines. In this present work, the region defined by the spot D, with *m*/*z* fragments characteristics of terpenoids and some C_{13} norisoprenoids and by the spot H, with *m*/*z* fragments characteristic of 2-phenylethanol, may be a potential chromatographic region to take into consideration in future applications of this methodology.

III.7. Headspace-solid phase microextraction-gas chromatography as a tool to define an index that establishes the retention capacity of the wine polymeric fraction towards ethyl esters

The macromolecules present in wines have the ability to retain volatile compounds (section I.2.6). This occurrence may effect the perception of wines aroma and, consequently, its quality.

In this study, a novel approach was applied to study the interactions between wine polymeric fraction (obtained from *Vitis vinifera* L. var. Fernão-Pires monovarietal wines from the Portuguese Bairrada Appellation) and three ethyl esters (ethyl hexanoate, ethyl octanoate, and ethyl decanoate) based on headspace-solid phase-microextraction followed by gas chromatographic analysis (HS-SPME-GC) (section II.4), allowing definition of an index that establishes the retention capacity of the wine polymeric fraction towards each one of the three esters.

III.7.1. Characterization of the wine polymeric material

The polymeric material used in this study was composed by *ca*. 58% of sugars, *ca*. 38% of proteins and *ca*. 4% of phenolic compounds (Table III.7.1). Mannose accounted for *ca*. 49% of the sugar residues, followed by uronic acid and galactose (20% and 17%, respectively), glucose (6%), arabinose (5%) and rhamnose (3%). Thus, the fraction used in the study was mainly composed by mannoproteins, a group of macromolecules produced by the yeasts and that has been reported as playing an important role on volatile-macromolecule interactions (Lubbers *et al.*, 1994b).

III.7.2. Headspace-SPME-GC study of the behaviour of the esters towards the polyacrylate fibre

The esters, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, if present above their sensory perception limits (14, 5 and 200 μg/L, respectively) (Ferreira *et al.*, 2000), may contribute individually with fruity notes, such as apple, banana, ripe fruits, pear and sweet (Table III.7.2) (Gurbuz *et al.*, 2006; Selli *et al.*, 2006). The relevant physico-chemical characteristics of these esters, that are structurally distinguished only by the number of

carbons (C_8, C_{10}, and C_{12}), are listed in Table III.7.2. Ethyl decanoate is the more hydrophobic, shown by the higher Log *P* (octanol-water) and is the less volatile compound, shown by the lower boiling point. In the opposite position appears the ethyl hexanoate, and ethyl octanoate is in an intermediate position. The balance between these two characteristics, volatility and solubility, is determinant to the transference of the compounds from the liquid phase to the headspace.

Table III.7.1. Material accounted for phenolics, protein and polysaccharides present in the wine polymeric material (WP).

	Wine polymeric material	
	mg/g [a]	% [c]
Phenolics	30.9±0.1 [b]	4.3
Protein	272.6±6.1	37.7
Polysaccharides	420.5±7.6	58.1
Rhamnose	12.7±0.8	3.0
Fucose	1.4±0.3	0.3
Arabinose	21.0±1.9	5.0
Xylose	3.2±1.1	0.8
Mannose	205.0±14.9	48.8
Galactose	69.3±7.7	16.5
Glucose	23.7±3.0	5.6
Uronic Acid	84.2±19.8	20.0

[a] Values are expressed as mg of component/g dry wine polymeric material (WP).

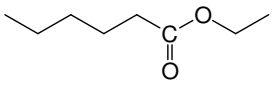
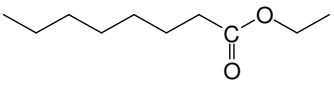
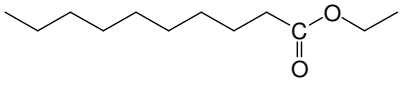
[b] Mean of three replicates ± standard deviation

[c] Values are expressed as percentage of each component in relation to the total dry wine polymeric material (WP).

The objective of this section is to evaluate the behaviour between the polyacrylate coating fibre and the three esters under study. In general, absorption-type coatings, like polyacrylate, are less sensitive to matrix effects and offer wider linear ranges (Pawliszyn, 2000). In this case, a linear dependence between the amount of an analyte extracted by the fibre and its concentration in a sample should be observed. The confirmation that this

linear dependence occurs is crucial when performing quantitative analysis. In the present study, as a first approach, the linear behaviour of the ethy hexanoate, ethy octanoate and ethyl decanoate towards a polyacrylate fibre was studied under experimental conditions previously defined (Rocha *et al.*, 2001). According to Table III.7.3, the linear isotherms that characterize the absorption-type coatings were achieved for ethyl hexanoate (R^2= 0.992), ethyl octanoate (R^2= 0.992) and ethyl decanoate (R^2= 0.970) under the concentration tested: 0.11-6.0 mg/L for ethyl hexanoate, 0.59-7.0 mg/L for ethyl octanoate and 0.053-8.4 mg/L for ethyl decanoate. The lower linearity observed for ethyl decanoate (lower R^2) should be explained due to the fact that the concentration range used for this compound included the lowest concentrations when compared to the other standards used.

Table III.7.2. Physico-chemical characteristics of the chemical standards used: structure, molecular weight (MW), boiling point (BP), solubility in water and ethanol, Log *P*, Henry's law constant and aroma descriptor (AD).

Compounds	Chemical structure	Characteristics
ethyl hexanoate		MW= 144.21 BP= 168 ºC Insoluble in water Miscible with ethanol Log *P* (octanol-water)= 2.83 Henry's Law constant 0.000723 atm m^3/ mol AD [a,b]: fruit, apple, banana
ethyl octanoate		MW= 172.27 BP= 208 ºC Insoluble in water Miscible with ethanol Log *P* (octanol-water)= 3.81 Henry's Law constant 0.00127 atm m^3/ mol AD [b]: ripe fruits, pear, sweety
ethyl decanoate		MW= 200.31 BP= 245 ºC Insoluble in water Miscible with ethanol Log *P* (octanol-water)= 4.79 Henry's Law constant 0.00225 atm m^3/mol AD [b]: sweety, fruit, dry fruits

[a]- Gurbuz *et al.*, 2006 ; [b]- Selli *et al.*, 2006

Table III.7.3. Statistical data for calibration curves for the three ethyl esters under study.

Compounds	Concentration range (mg/L)	Regression equation	Correlation coefficient (R^2) (n=18)*
ethyl hexanoate	0.11 - 6.0	y = 22557x	0.992
ethyl octanoate	0.59 - 7.0	y = 367011x	0.992
ethyl decanoate	0.053 – 8.4	y = 1777915x + 828391	0.970

* resultant of 18 independent assays (6 concentration levels x 3 replicates each).

The repeatability of the method, expressed as relative standard deviation (RSD), was estimated for three consecutive analyses of the RWM containing only one ester. The results obtained gave RSD values that ranged from 0.7 % for ethyl decanoate (2.1 and 8.4 mg/L) to 15.1 % for ethyl hexanoate (0.11 mg/L), which is considered acceptable for this type of analysis.

III.7.3. Headspace-SPME-GC study of the interactions of the wine polymeric material with esters

A reference wine model (RWM), without wine polymeric material, and wine models with different amounts of polymeric fraction (PWM_1, PWM_{10} and PWM_{30}) were analysed at the same time, throughout 11 extractions in order to study the outcome of the presence of the wine polymeric material on the retention of the ethyl esters. The PWMs and the corresponding RWM contained only one ester.

The headspace-SPME was used to quantify the amount of each ester present in the headspace, *i.e.*, the amount not retained by the WP. After the addition of each ester to the WMs, two situations may occur: *i*) $C_{RWM} \cong C_{PWM}$ or *ii*) $C_{RWM} > C_{PWM}$, where C_{PWM} is the concentration of the compound in PWM and C_{RWM} is the concentration of the compound in RWM. This second situation indicate that a retention occurs when the WM contain WP fraction.

Ethyl hexanoate. According to Figure III.7.1a, a decrease occurred in the amount of the ethyl hexanoate along the sampling moments due to its extraction in each analysis. Because no significant differences were observed between the RWM (control) and the PWM_{30}, the experiment was stopped after eight extractions. These results indicate that no retention effect was observed for ethyl hexanoate, even when a saturated wine polymeric

solution was used. Due to this fact, no studies have been made for the wine polymeric concentrations of 1.0 and 10.0 g/L.

Ethyl octanoate. As observed for ethyl hexanoate, the amount of ethyl octanoate decreased along each sampling moment in all experiments (RWM, PWM_1, PWM_{10} and PWM_{30}) throughout the 11 extractions (Figure III.7.1b). However, with this ester, differences between the RWM and the PWMs were observed. The difference between the concentration in RWM and the concentration in PWM, corresponding to the concentration retained by the polymeric material, are shown in Table III.7.4. At the first extraction, a *ca*. 4% decrease in the concentration of the ester was observed in PWM_1 compared to the RWM, and for PWM_{10} and PWM_{30} a reduction of *ca*. 24 and 30% occurred (Table III.7.4). Throughout all the sampling moments, a slight difference was observed between the headspace concentration of the ester in the RWM and PWM_1 (Figure III.7.1b and Table III.7.4), indicating a small retention of ethyl octanoate by the wine polymeric concentration of 1 mg/L. For the higher concentrations a reduction was observed from the first until the 7^{th} extraction in the headspace concentration of ethyl octanoate when compared to the concentration in the RWM (Figure III.7.1b and Table III.7.4). These results indicate that a retention effect occurs for this volatile compound by the WP at 10 mg/L and by the WP in a saturated solution (PWM_{30}). The PWM_{30} promoted a *ca*. 5-11% higher retention of ethyl octanoate than PWM_{10}. From the 8^{th} to the 11^{th} extraction, it was observed that the headspace concentration of ethyl octanoate was higher in PWM_{10} and PWM_{30} than in RWM (Figure III.7.1b and Table III.7.4), corresponding to the negative values reported in Table III.7.4. This suggests that after a certain number of extractions, when the amount of volatile compound is reduced, the retained ethyl octanoate has an important contribution in its dosage to the headspace. The crossing-points observed in Figure III.7.1b (from the 7^{th} to the 8^{th} extraction) between the RWM and the PWM_{10} and between RWM and PWM_{30} are unambiguous indications that retention of the ethyl octanoate occurs, which was followed by its dosage to the headspace.

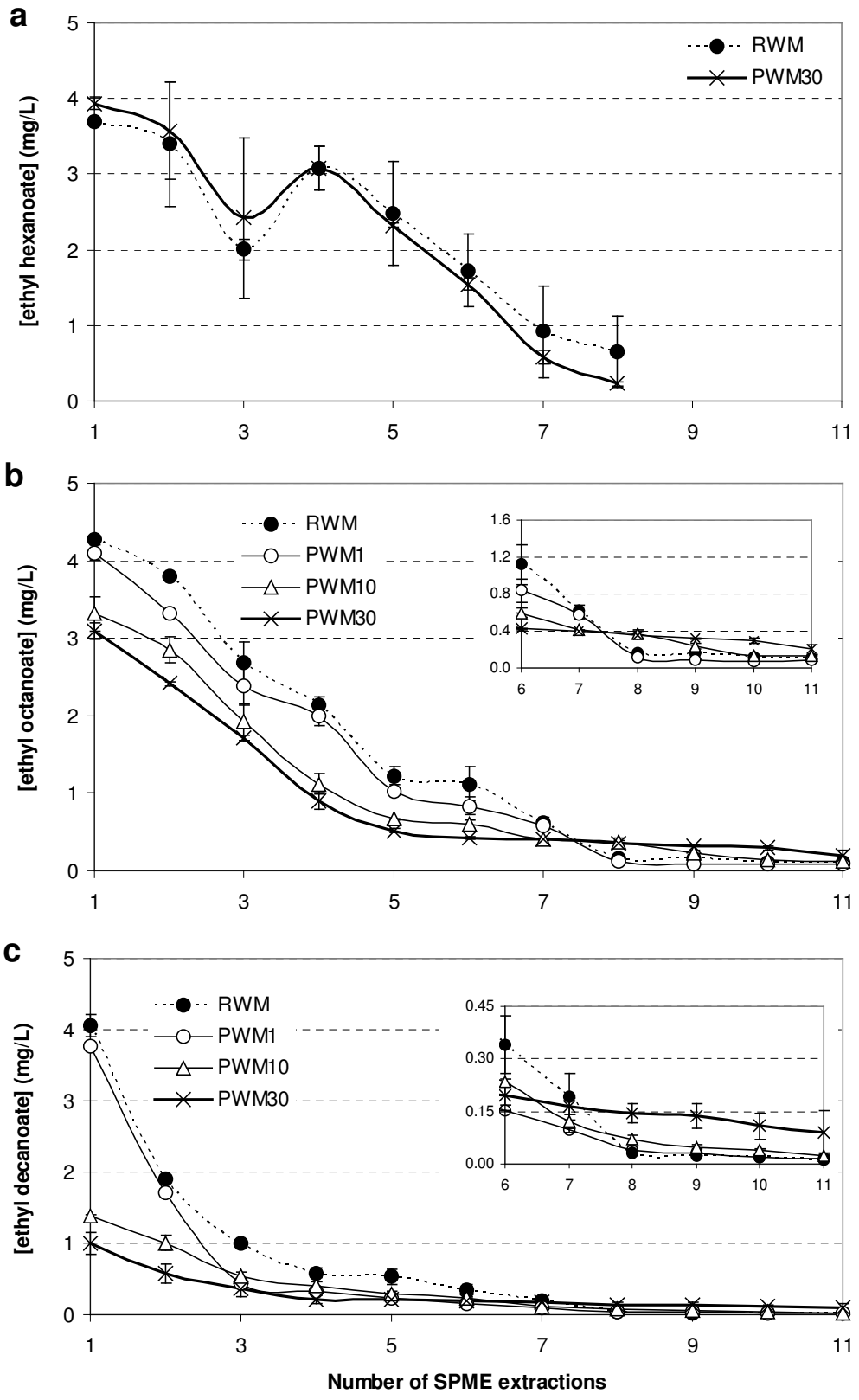


Figure III.7.1. Headspace concentration (mg/L) across SPME extractions for: a) ethyl hexanoate, b) ethyl octanoate, c) ethyl decanoate.

Ethyl decanoate. As observed for ethyl hexanoate and ethyl octanoate, a decrease occurred in the amount of the ethyl decanoate along each sampling moment for all experiments (RWM, PWM_1, PWM_{10} and PWM_{30}) throughout the 11 extractions (Figure III.7.1c). For this ester, differences in its amount in the headspace between the RWM and the PWMs were also observed. At the first extraction, a *ca*. 7% of decrease in the concentration of ethyl decanoate was observed for PWM_1 when compared to the RWM. For PWM_{10} and PWM_{30} the reductions were 67 and 76% (Table III.7.4). From the 1st to the 3rd extraction a decrease occurred in the ethyl decanoate concentration for RWM and for PWM_1 in relation to the initial concentration of 4 mg/L (Figure III.7.1c and Table III.7.4). For the higher concentrations, PWM_{10} and PWM_{30}, from the 1st to the 3rd headspace extraction the concentration accounted from 1.4 to 0.4 mg/L. The PWM_{30} promote a *ca*. 1-11% higher retention than PWM_{10}. These results may suggest that in the RWM and for PWM_1 solutions the ethyl decanoate, a hydrophobic compound, was predominantly in the headspace and was sorbed by the fibre. However, in the presence of the polymeric material in higher concentration, the ethyl decanoate was predominantly in the liquid matrix, which is suggestive of the retention capacity of the polymeric material. From the 8th to the 11th extractions, it was observed that the headspace concentration of ethyl decanoate was higher in PWM_1, PWM_{10} and PWM_{30} than in RWM (Figure III.7.1c and Table III.7.4). As was observed for ethyl octanoate, these results suggest that the amount of retained ethyl decanoate was regularly dosed into the headspace during a larger number of extractions. Furthermore, it was also observed the crossing-points between the RWM and the PWMs (Figure III.7.1c, from the 7th to the 8th extraction), which represent a clear indication that retention of the ethyl decanoate occurs, which was followed by its dosage to the headspace.

Table III.7.4. Concentration of volatile compounds retained by the wine polymeric material (values obtained by the difference between the concentration in RWM and the concentration in PWM)

	Number of SPME extractions										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Concentration of volatile compounds retained (mg/L)											
ethyl octanoate											
WP_1	0.17	0.48	0.29	0.15	0.20	0.28	0.04	0.04	0.07	0.03	0.02
WP_{10}	0.96	0.95	0.76	1.03	0.56	0.52	0.20	-0.21	-0.07	-0.03	-0.03
WP_{30}	1.18	1.39	0.97	1.25	0.71	0.70	0.22	-0.19	-0.16	-0.18	-0.10
ethyl decanoate											
WP_1	0.29	0.19	0.55	0.25	0.31	0.19	0.09	-0.01	-0.01	0.00	0.00
WP_{10}	2.68	0.90	0.46	0.18	0.24	0.11	0.07	-0.04	-0.02	-0.02	-0.01
WP_{30}	3.05	1.34	0.63	0.37	0.32	0.15	0.03	-0.11	-0.11	-0.09	-0.08

III.7.4. Estimation of the retention index

The amount of retained compounds was determined against RWM, which allows to calculate a retention index RI= 1-[(C_{RWM}-C_{PWM})/C_{RWM}], where C_{PWM} is the concentration of the compound in the wine model with polymeric fraction (PWM) and C_{RWM} is the concentration of the compound in the reference wine model without polymeric material (RWM). The value obtained from the difference C_{RWM}-C_{PWM} corresponds to the concentration retained by the polymeric material. This index was used to establish the retention capacity of the wine polymeric material towards the three esters. The index ranged from 0, when no retention occurs, to 1, when occurs a total retention of the compound, as schematically can be observed in Figure III.7.2. The values used in the computation of the RI correspond to the concentrations obtained in the first extraction. This approach allows calculating the amount of each standard retained by the WP fraction after the first period of contact with both.

Table III.7.5 shows the RI values obtained for the three ethyl esters under study. As no retention was observed for ethyl hexanoate under the conditions studied, a RI equal to zero was obtained. The results show that the higher retention was observed for ethyl decanoate, the more hydrophobic compound. The RI value increased with WP concentration. The PWM_{30} exhibited the higher values: RI= 0.75 for ethyl decanoate and

RI= 0.28 for ethyl octanoate. Furthermore, this study also suggests that the retained compounds should be dosed to the headspace, which may promote the perception of its aroma for a longer period of time.

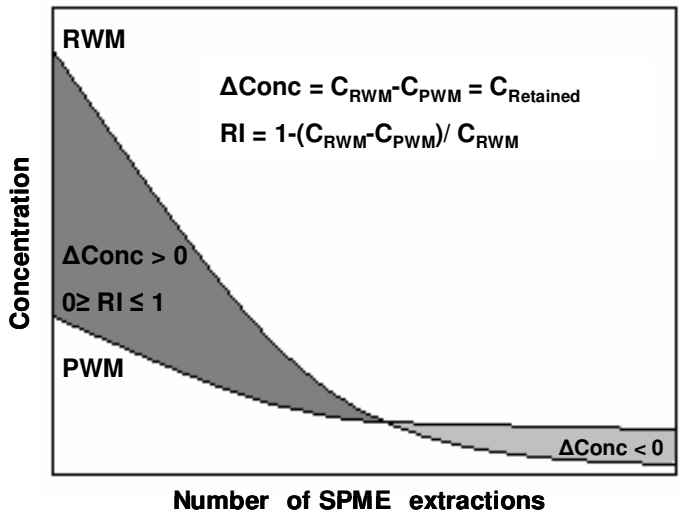


Figure III.7.2. Proposed theoretical representation of the initial retention of volatile compounds by the wine polymeric fraction (0≥RI≤1) followed by their dosage to the headspace.

Table III.7.5. The retention index (RI) of each wine polymeric fraction towards the three ethyl esters under study

		RI
Ethyl hexanoate	WP_1	---*
	WP_{10}	---*
	WP_{30}	0.00
Ethyl octanoate	WP_1	0.04
	WP_{10}	0.22
	WP_{30}	0.28
Ethyl decanoate	WP_1	0.07
	WP_{10}	0.66
	WP_{30}	0.75

*- not calculated.

III.7.5. Conclusions

The novel approach proposed in this study, based on headspace-solid phase-microextraction followed by gas chromatographic analysis, seems to be adequate to evaluate the interactions in a wine model solution between wine polymeric fraction and three ethyl esters. The level of retention of each compound by the WP fraction affects the amount of volatile compound in the headspace. These changes in the amount of the ethyl esters under study may be computed by the proposed methodology. The definition of the RI can be a suitable approach to establish the retention capacity of the wine polymeric fraction due to its easier and objective interpretation. It should be stressed that the use of the RI approach can be extended to other studies of retention capacities, including different matrices and/or aroma compounds.

The results show that the higher retention was observed for ethyl decanoate, the more hydrophobic compound. The RI value increased with WP concentration. Furthermore, this study also suggests that the retained compounds should be dosed to the headspace, which may promote the perception of its aroma for a longer period of time. This suggests that, in a real wine, some hydrophobic compounds can be retained by the polymeric material and be slowly released into the headspace when the bottle is opened, contributing to the perception of its odour for a longer period of time.

IV

Conclusions and Future Work

This work allows us to conclude that *Vitis vinifera* L. Fernão-Pires variety, that is the most cultivated white variety from Bairrada Appellation, exhibits a profile significantly different from those of the other most representative white varieties (Bical Arinto and Cerceal), with a higher number and concentration of volatile compounds.

The results obtained by the analysis of grapes, must and wine of FP contributed to its characterization. In grapes, the varietal volatile content is mainly associated to the solid parts of berries, skin (69.3%) and liquid pulp (25.3%), and is distributed by free (66.7%) and glycosidically-linked (33.3%) forms. This grapes variety exhibited terpenoids, aromatic alcohols, C_{13} norisoprenoids and C_6 alcohols. It is mainly characterized by the presence of monoterpenoids, one of them over sensory perception limit (geraniol), as well as the presence of an odour terpendiol in the glycosidically-linked form (*Z*-2,6-dimethylocta-2,7-dien-1,6-diol). Similarly, the musts are characterized by the presence of monoterpenoids, some of them over their sensory perception limits (hotrienol and linalool), as well as the presence of odour/odourless terpendiols in the potential form (*Z*-2,6-dimethylocta-2,7-dien-1,6-diol and 3,7-dimethylocta-1,5-dien-3,7-diol), which may also represent an important source of monoterpenoids. The terpenoid potential of grapes, which is mainly associated to skin and mainly present in the glycosidically-linked form, as well as the presence of C_6 alcohols in skin and in the free fraction, suggests that both technological changes/improvements, related mainly with enzymatic preparations used during

winemaking to release the linked compounds and skin contact time must be taken into consideration as strategies to increase the aroma quality of wine from Fernão-Pires variety. In fact, the effect of an aroma release enzyme in FP wine was tested in this work, demonstrating the real improvement of its aroma. There was an increase of 9% in the total amount of volatile compounds of Fernão-Pires, due mainly to the increase of geraniol (67%), terpendiols (96%), phenols and aromatic alcohols (26%) and esters (32%). Some of them were within their sensory perception limits and may have a contribution to the floral and fruity notes.

These studies also allowed to conclude that, as a consequence of the fact that the four main white varieties of Bairrada Appelation (Fernão-Pires, Bical, Arinto and Cerceal) exhibit different volatile composition patterns (different components and different distribution between free and PVC forms) winemaking technologies should be specifically developed for each variety for the improvement of wine aroma quality. In particular for Bical variety, the studies concerning the effect of an aroma release enzyme in this wine variety did not show an improvement of its aroma. The presence of aromatic alcohols in Bic variety seems to be an interesting characteristic of it. Studies also suggested that one could establish markers (volatiles) for the characterization of must varieties independently from the harvest effect. As these varieties are all grown in the same region (Bairrada Appellation), knowledge of their varietal composition will allow winemakers to plan their use in monovarietal wines or in blends, providing the potential of the high volatile organic acid composition of Ari and Cer (data not shown) together with the terpenic characteristics of FP and the aromatic alcohols of Bic.

The harvest variability of the varietal composition of FP grape variety was also evaluated from the musts across four harvests. Based on the data obtained, using GC-MS tandem with PCA, relationships were established between the varietal volatile composition of the musts and the white wine aroma quality classification conferred by the wine taster chamber of Comissão Vitivinícola da Região da Bairrada (CVRB). The results of the volatile analysis were consistent with the wine quality classification given by CVRB, indicating that the wine aroma quality is clearly linked with the musts free varietal volatile composition. On the other hand, the PVC fraction allowed the distinction of the musts according to their potential aroma precursor's composition. The proposed approach provides information to winemakers concerning the winemaking methodologies that can be implemented to improve the wine aroma quality.

The emergence of techniques for the rapid characterization of food products, with the use of mass spectrometry, leaded us to the development of a methodology for the rapid distinction of wines by volatile fraction analysis. This technique based on headspace solid-phase microextraction- gas chromatography- mass spectrometry- principal component analysis (HS-SPME-GC-MS-PCA), allows one to evaluate the global volatile signature of the wine headspace (chromatographic profile and *m/z* pattern of fragmentation in each scan) without complete chromatographic separation of its components. In order to retrieve from the data as much chemical information as possible and to extract *m/z* fragments (markers) for the characterisation and distinction of the wines varieties, a PCA was applied to the data resultant from the unresolved volatile fraction. Two different monovarietal white wines (*Vitis vinifera* L. var Fernão-Pires and Arinto) were tested. Associated to the fast character of the proposed methodology and robustness taking into account the extraction time, it is also important to highlight the higher sensibility and lower effect of the sample moisture of the MS sensor response when compared to the conventional *e*-noses.

Finally, the interactions that volatile compounds can establish with the wine macromolecules and the importance of this ocurrence for the sensory quality of wines leaded us to the development of a headsapce-solid phase microextraction followed by gas chromatography analysis (HS-SPME-GC) for the study, in model wines, of the interactions between three ethyl esteres (ethyl hexanoate, ethyl octanoate and ethyl decanoate) and diferent amounts of polymeric fraction extracted from the Fernão-Pires wine. The methodology allows to calculate a retention index (RI) for each compound, which is the retention capacity of each wine polymeric fraction towards the three esters established. The higher retention indexes were observed for ethyl decanoate, the more hydrophobic compound, and for the the wine polymeric material with higher concentration. Ethyl decanoate was founds to be retained for the wine polymeric fraction concentration. Furthermore, this study also suggested that the retained compounds are dosed to the headspace, which may promote the perception of their aroma for a longer period of time.

Future work

The results gathered in this thesis open up an array of future research.

- Studies with skin and/or pomace contact time must be done in order to promote the enhancement of the Fernão-Pires musts in free volatile compounds and, particularly, in glycosidically-linked compounds.

- Studies with grapes and wines of the Bical, Arinto and Cerceal must be done in order to fulfil these varieties characterization.
- Wines submitted to adequate winemaking technologies (skin contact time, and enzymatic preparations) should be analysed, chemically and sensory, in order to attest the improvement of the sensory quality of these wines.
- The number of years of study for the Fernão-Pires volatile composition must be increased in order to improve the built model to predict the quality of wines.

Concerning the developed methodologies:

- Enlarge the study of the interactions between volatile compounds-macromolecules for other relevant volatile compounds of wines. Study the interactions between volatile compounds with each type of macromolecule, in order to evaluate their importance in this process.
- To apply the developed method for rapid distinction of wines by HS-SPME-GC–MS-PCA to a higher number of wine samples. Focus the analysis in the *m*/*z* ion fragments associated to the varietal components or others that may contribute with odour descriptors.

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