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Characterisation of the aroma components of clonal grapes and wines from Aragonez and Trincadeira *Vitis vinifera* L. cultivars

UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO
VILA REAL, 2008

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This work was elaborated expressly as an original dissertation with the aim of obtaining a *PhD* degree in Food Science, in accordance with Law 216/92 of October 13.

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Há ainda muito a dizer sobre os efeitos do vinho no trabalho intelectual, ou seja, nos trabalhos de imaginação, porque são sem dúvida os únicos em relação aos quais nos podemos interrogar sobre a utilidade ou a inutilidade da embriaguez. O vinho foi definido como o cavalo do poeta. E efectivamente não se pode negar que na sela desse cavalo, o poeta, se não vai devagar, pelo menos vai longe. As primeiras vezes em que se escreve num estado leve de embriaguez, sente-se um grande entusiasmo. Sob as ondas de sangue ardente que irrigam o cérebro, já não se produz a chamada dança das células, mas um verdadeiro turbilhão, já não é um sopro, mas antes um furacão de inspiração.

In: Il vino – Un discorso sui suoi effetti psicologici
Edmondo de Amicis, 1880

Aos meus Pais por tudo...
Ao Marco por quase tudo...

Um brinde ao Amor!

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Who am I?

Where am I going?

Sincerely I don't know how to answer these questions. But I do know who I have to thank for the help and support that led me through this sinuous but wonderful walk. Indeed, no research work is the making of a single person. So, I would like to thank all the persons who have contributed, directly or indirectly, to my work and to this thesis.

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RESUMO

A grande variabilidade e diversidade dos vinhos tintos produzidos em Portugal provenientes de castas nacionais *Vitis vinifera* L. justificam a sua caracterização aromática e físico-química. Essa caracterização, tem por objectivos, preservar a qualidade e a tipicidade dos vinhos de diferentes regiões, bem como, contribuir para aprofundar o conhecimento de castas que proliferam no mundo vitivinícola. Além disso, a caracterização do aroma de vinhos clonais apresenta um interesse inegável para a indústria vitivinícola, devido à importância que assume nos diversos aspectos qualitativo, produtivo e financeiro.

De entre algumas centenas de compostos voláteis pertencentes a diversas famílias químicas e existentes em diferentes gamas de concentração, apenas uma parte contribui efectivamente para o aroma, aumentando a sua intensidade e complexidade no vinho. O conhecimento sobre a identidade e a concentração de tais compostos é fundamental para uma melhor compreensão do seu papel no aroma do vinho. Esta abordagem deverá ser complementada com a análise sensorial descritiva, para que se possa obter uma visão mais abrangente do aroma dos vinhos e da apreciação da sua qualidade.

O programa Português de Selecção Clonal, criado em 1978, cujos objectivos são os de conhecer e seleccionar as melhores castas, visando aumentar a qualidade dos vinhos produzidos em Portugal, possui actualmente diversos clones certificados que são cultivados por viticultores. As castas Aragonez e Trincadeira estão entre as oito variedades tintas mais plantadas em Portugal, encontrando-se já certificados 7 e 6 clones, respectivamente.

O conhecimento sobre as relações entre o papel individual de cada composto odorante e o papel global dos compostos responsáveis pelo aroma, são metas específicas a alcançar, de modo a ser possível realizar a escolha dos melhores clones para produzir vinhos de elevada qualidade.

Este estudo teve como objectivo, contribuir para a caracterização dos componentes do aroma de vinhos tintos clonais e dos respectivos mostos e uvas, das castas Aragonez e Trincadeira *Vitis vinifera* L.

O desenvolvimento e a aplicação de um método de cromatografia em fase gasosa – olfactometria (GC-O), designado método de intensidade posterior, permitiu, pela primeira vez, estabelecer os perfis odorantes de cada casta e, simultaneamente, diferenciar os vinhos clonais de ambas as castas e os mostos dos clones de Aragonez. Assim, em todos os vinhos clonais foram detectados diversos compostos odorantes, tendo apresentado intensidades médias mais elevadas: o ácido 3-metilbutanóico, o 2-feniletanol, o Furaneol™ e o 4-vinilguaiacol. A quantificação de alguns compostos odorantes utilizando a cromatografia em fase gasosa acoplada ao detector de ionização de chama (GC-FID) encontrados nos vinhos clonais e

respectivos mostos e uvas, demonstrou a existência de diversas diferenças estatísticas entre os clones.

Os compostos Furaneol™ e homofuraneol, caracterizados com os descritores de odor a *açúcar queimado* (tipo *caramelo*) e *algodão doce*, foram identificados nos vinhos clonais de Aragonez e Trincadeira bem como nas fracções livres e ligadas dos mostos de Aragonez, indicando a sua origem varietal.

Demonstrou-se por GC-O, GC-FID e análise sensorial descritiva que o ano de vindima apresentou uma influência relevante nos vinhos clonais da casta Trincadeira. A análise discriminante linear aplicada aos dados obtidos, revelou a existência de algumas variáveis discriminantes que poderão ser utilizadas para se obter uma correcta classificação dos vinhos clonais provenientes das duas vindimas em estudo.

Os atributos de aroma, *adocicado*, *herbáceo*, *animal*, *frutos secos*, *frutos vermelhos*, *especiarias* e *madeira*, utilizados pelo painel sensorial, foram úteis para a obtenção do perfil de aroma dos vinhos clonais de Aragonez e de Trincadeira.

A não quantificação de compostos monoterpénicos e a escassez de norisoprenóides em C_{13} nas uvas e nos mostos são indicadores de que as castas Aragonez e Trincadeira podem ser classificadas como castas neutras.

A informação obtida por GC-O e pela análise sensorial descritiva sugere uma elevada utilidade destas "ferramentas sensoriais" para o controlo da qualidade dos vinhos.

Palavras-chave: vinhos tintos clonais, mostos, uvas, Aragonez, Trincadeira, compostos odorantes, aroma, qualidade.

ABSTRACT

The large variability and diversity of red wines produced in Portugal with Portuguese *Vitis vinifera* L. cultivars fully justify their chemical and aroma characterisation. The objectives of this characterisation are to preserve the quality and tipicity of the wines from different regions and to contribute to the deeper knowledge of grape varieties in the world. Furthermore, the characterisation of the aroma of clonal wines is of undeniable interest to the winemaking industry, due to its productive, financial and qualitative aspects.

Among hundreds of volatile compounds of distinct classes and a wide range of concentrations present in red wines, only a part contribute effectively to the aroma, enhancing the intensity and complexity of the wine flavour. Knowing the identity and the concentration of such compounds in wines is crucial for a better understanding of their role in defining wine aroma. This approach should be complemented with descriptive sensory analysis in order to give an overview of the aroma of wines and appreciation of the general quality of wines.

The Portuguese Clonal Selection Program was created in 1978 with the objectives of getting to know and select our best varieties of grapes and to increase the quality of wines produced in Portugal. Nowadays it has several certified clones commonly used by grape-growers. Aragonez and Trincadeira are among the eight more planted red grape varieties in Portugal and respectively seven and six clones of both cultivars have already been certified.

The knowledge of the relationships between the individual role of each odourant compound and the global role of the overall aroma compounds are specific targets that we need to know in order to be able to choose the best clones to produce the best wines.

The aim of this study was to contribute for the characterisation of the aroma of distinct clonal red wines, musts and grapes, from *Vitis vinifera* L. cultivars Aragonez and Trincadeira.

The development and application of a gas chromatography-olfactometry (GC-O) posterior intensity method allowed, for the first time, the establishment of the odourant profiles of each cultivar and, simultaneously, the differentiation of the clonal wines among Aragonez and Trincadeira varieties and clonal musts from Aragonez.

Several odourant compounds were detected, having the highest average intensities in all clonal wines: 3-methylbutanoic acid, 2-phenylethanol, Furaneol™, and 4-vinylguaiacol. The quantification by gas chromatography-flame ionization detector (GC-FID) of some of the odourant compounds found in the clonal wines as well as those found in musts and grapes showed several statistical differences among clones.

Furaneol™ and homofuraneol, described with a *burnt sugar* (*caramel-like*) and *candy-cotton* odour descriptors, were identified in Aragonez and Trincadeira clonal wines as well as in both free and bound fractions of Aragonez musts, indicating their grape-derived origin.

Vintage had a significant influence on Trincadeira clonal wines, as was demonstrated by GC-O, GC-FID and by descriptive sensory analysis. Stepwise linear discriminant analysis applied to data obtained from the previous analyses revealed some discriminating variables that can be used to obtain a correct classification of the clonal wines from the two distinct vintages.

The aroma attributes *sweet*, *herbaceous*, *animal*, *dried fruits*, *red fruits*, *spicy* and *woody*, used by the sensory panel, were useful in obtaining the aroma profile of the Aragonez and Trincadeira clonal wines.

The inexistence of quantified monoterpenic compounds and the poorness in C_{13}-norisoprenoids found in musts and grapes indicated that Aragonez and Trincadeira can be classified as neutral cultivars. The information obtained by GC-O and descriptive sensory analysis suggested the usefulness of these "sensory tools" for controlling wine quality.

Key-words: clonal red wines, musts, grapes, Aragonez, Trincadeira, odourant compounds, aroma, quality.

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ABBREVIATIONS AND SYMBOLS LIST

*****: significant ($p < 0.05$)
******: highly significant ($p < 0.01$)
*******: very highly significant ($p < 0.001$)
α: significance level
% v/v: percent volume per volume
% vol.: percentage of alcohol in the total wine volume
A 3MH: 3-mercapto-hexanol acetate
2-D: two dimentional
3-D: three dimentional
3 MH: 3-mercapto-hexan-1-ol
3 MMB: 3-mercapto-3-methylbutan-1-ol
4 MMP: 4-mercapto-4-methylpentan-2-one
4 MMPOH: 4-mercapto-4-methylpentan-2-ol
ACIII: adenylyl cyclase
DCO: Denomination of Controlled Origin is the translation of "DOC: Denominação de Origem Controlada"
AEDA: aroma extract dilution analysis
AMP: adenosine monophosphate
ANOVA: analysis of variance
ASTM: American Society for Testing and Materials
ATP: adenosine triphosphate
CaBP: calmodulin-binding protein
cAMP: cyclic adenosine monophosphate
CHARM: combined hedonic aroma response measurement
cm: centimeters
CNEVV: Comissão Nacional para o Exame das Variedades de Videira
CNG: cyclic nucleotide-gated channel
CoA: acetyl coenzyme A
cv.: cultivar
DFs: discriminant functions
DGADR: Direcção Geral de Agricultura e Desenvolvimento Rural
DMAPP: dimethylallyl pyrophosphate
DOC: Denominação de Origem Controlada
EAN: Estação Agronómica Nacional
e.g.: from Latin "exempli gratia" means "for example"
EVN: Estação Vitivinícola Nacional
FD: flavour dilution factor
FID: flame ionization detector
FTIR: fourier transform infrared spectroscopy
GC: gas chromatography
GC-FID: gas chromatography – flame ionization detector
GC-MS: gas chromatography-mass spectrometry
GC-O: gas chromatography-olfactometry
G_{olf}: G protein olfactory
GPP: geranylpyrophosphate
h: hour
HCA: hierarchical cluster analysis
HSSE: headspace sorptive extraction
i.d.: internal diameter
INRB, I.P.: Instituto Nacional dos Recursos Biológicos
IPP: isopentenyl pyrophosphate
i.e.: from Latin "id est" means "that is"
IS: internal standard

ISO: International Organization for Standardization
IUPAC: International Union of Pure and Applied Chemistry
JND: just noticeable difference
L-INIA: Instituto Nacional de Investigação Agrária
LRI: linear retention indices
LSD: least significant difference
mV: millivolt
min: minute
m/z: mass (m)-to-charge (z) ratio in mass spectrometry
MIBP: 2-methoxy-3-isobutylpyrazine
MLF: malolactic fermentation
n: number of samples
NIF: nasal impact frequency
nq: not quantified compound
ns: not significant
OAV: odour active value
OBP: olfactory binding protein
ODP: olfactory detection port
ORK: olfactory receptor kinase
OSNs: olfactory sensory neurons
p.: page
PCA: principal component analysis
PCs: principal components
PDE: phosphodiesterase
PDMS: polydimethylsiloxane
PKA: protein kinase A
PT: Portugal
***p*-value**: statistical significance of difference between the samples
QDA: Quantitative descriptive analysis
r: correlation coefficient
R: functional group in a molecule
RGS: regulator of G proteins
rpm: revolutions per minute
SBSE: stir bar sorptive extraction
SD: standard deviation
SFE: supercritical fluid extraction
Sig.: significance level
SIM: selected ion monitoring
SLDA: stepwise linear discriminant analysis
SNIF: surface of nasal impact factor
SO_2: sulfur dioxide
SPME: solid-phase microextraction
SPSS: statistical package for the social sciences
syn: synonym
TA: titratable acidity (expressed as $g.L^{-1}$ tartaric acid)
TCA: 2,4,6-trichloroanisole
TDN: 1,1,6-trimethyl-1,2-dihydronaphthalene
™: Trademark
USA: United States of America
vs: from Latin “versus” means “against”
x: average

Objectives and outline of thesis

OBJECTIVES AND OUTLINE OF THESIS

The identification of the compounds that can contribute to the aroma and flavour of wines still remains as one of the most significant challenges in the future of wine research and industry.

Up now few reports on the characterisation of the Portuguese grape varieties were found. Regarding the clones of Aragonez or Trincadeira *Vitis vinifera* L., and in spite of their clonal selection, scarce information was found on the volatile fraction of both cultivars. The aim of the present work is to study and, if possible, identify the characteristic aroma compounds of those cultivars. For that purpose, red wines from three Denominations of Controlled Origin (DCO), namely five Aragonez clonal wines from the Alentejo (DCO), three Aragonez clonal wines from the Estremadura (DCO) and five Trincadeira clonal wines from two different vintages (2001 and 2003) from the Ribatejo (DCO), were studied.

The analysis were also carried out in musts and grapes from all clones mentioned above, except for the five Trincadeira clonal musts and grapes from the 2001 vintage because they were already not available at the time.

The specific goals of this thesis are:

- To characterise the odourant compounds responsible for the aroma of the clonal wines and musts in order to establish their odourant profiles.
- To study the main sources of variability present in the data obtained from GC-O, GC-FID or descriptive sensory analysis, and if possible to establish relationships between samples (objects) and compounds (variables) for better samples differentiation. Both objectives were achieved by the application of multivariate statistical analyses, such as principal component analysis (PCA), hierarchical cluster analysis (HCA) and stepwise linear discriminant analysis (SLDA).

A sequence of four techniques was used: gas chromatography-olfactometry (GC-O), gas chromatography-mass spectrometry (GC-MS), gas chromatography-flame ionisation detector (GC-FID) and descriptive sensory analysis. The extracts of wines and respective musts and grapes were obtained by solvent extraction with ultrasound and they were used successively in those gas chromatography analyses. The extracts were the same for the different analyses, which minimised variability.

GC-MS is a powerful tool for the separation and identification of volatile compounds whether they are odour-active or not. In the analysis of aroma, GC-MS can selectively focus on the odour-active compounds once their spectral and chromatographic properties are known. However, the task of determining which compounds in a sample are odour-active requires a bioassay. In other words, we first determined which constituents were contributing to the characteristic sensory properties of the clonal wines and musts, by the GC-O analysis. In fact, GC-O is a bioassay that reveals odourant descriptors and odour intensities thus eliminating odourless compounds.

An overview of the literature published in the last decades is presented (chapter 1) and it refers mainly to eight aspects: wine aroma, the sense of smell, aroma evaluation, sensory analysis coupled with instrumental analysis, GC-O application in wines and musts, reconstitution, addition and omission sensory tests, extraction methods for quantitative analysis, and finally, clonal wines characterisation around the world.

Prior to the characterisation of clonal wines from both cultivars by GC-Olfactometry, a comparative study of two GC-O methods, detection frequency and posterior intensity methods, was carried out in order to select one (chapter 2). After the selection of the suitable method, the GC-O posterior intensity method, the clonal wines from Aragonez and Trincadeira were studied in order to find and characterise the odourant compounds (chapter 3) which defines the odourant profiles of each wine. The same GC-O method was also applied with free and glycosidically-bound volatile compounds (chapter 4) with the aim of characterising Aragonez clonal musts.

Descriptive sensory analysis was used to complement the GC-O and GC-MS analyses of Aragonez and Trincadeira clonal wines (chapter 5). In fact, GC-O only gives restrict information about the possible contribution of each individual odourant compound to the aroma, while descriptive sensory analysis gives global information about the aroma of wines and allows the characterisation of the aroma from a quantitative and qualitative point of view.

After the information regarding odourant compounds and aroma profiles, a quantitative determination by GC-FID analysis (chapter 6) of individual volatile compounds found in grapes, musts and wines is presented. Finally, the main conclusions and the future perspectives of this work are presented (chapter 7).

Divulging the knowledge acquired with the present work

DIVULGING THE KNOWLEDGE ACQUIRED WITH THE PRESENT WORK

Papers in international scientific periodicals with referees

(1) Botelho G., Mendes-Faia A., Clímaco M. C., 2008. Odourant profile differentiation of five Trincadeira clonal wines by GC-O posterior intensity method. *J. Agric. Food Chem.* (Submitted)

(2) Botelho G., Caldeira I., Mendes-Faia A., Clímaco M. C., 2007. Evaluation of two quantitative gas chromatography-olfactometry methods for clonal red wines differentiation. *Flavour Fragrance J.* **22**, 414-420.

Papers in national scientific periodicals with referees

(1) Botelho G., Paulino C., Mendes-Faia A., Clímaco M. C., 2007. A method to analyse bound aroma compounds in non-aromatic red grape juices. *Ciência Téc. Vitiv.* **22** (1), 21-26.

Papers in international/national conference proceedings

(1) Botelho G., Mendes-Faia A., Clímaco M. C., 2008. GC-O posterior intensity method in discrimination of clonal wines from two vintages. *In:* Wine Active Compounds 2008, Beaune, France. (Accepted)

(2) Botelho G., Mendes-Faia A., Clímaco M. C., 2007. GC-O Posterior intensity method in clonal red wine evaluation. *In:* OENO 2007 – 8th International Enology Symposium, Bordeaux, France. (In press)

(3) Clímaco M. C., Caldeira I., Botelho G., Avelar M. L., 2005. OGA – Metodologia de caracterização de odorantes-chave. *In:* 7º Encontro de Química dos Alimentos. Alimentos: Tradição e Inovação, Saúde e Segurança, P1-24, 8 p. CD-ROM, Viseu, Portugal.

(4) Caldeira I., Botelho G., Belchior A. P., Bruno de Sousa R., Mendes-Faia A., Clímaco M. C., 2004. Aplicação da Cromatografia em Fase Gasosa - Olfactometria no Conhecimento dos Odorantes - Chave em Vinhos e Aguardentes. *In:* 6º Simpósio de Vitivinicultura do Alentejo, **2** (114-120), Évora, Portugal.

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(2) Botelho G., Mendes-Faia A., Clímaco M. C., 2007. GC-O Posterior intensity method in clonal red wine evaluation. *In:* OENO 2007 – 8th International Enology Symposium, p. 262, Bordeaux, France.

(3) Botelho G., Mendes-Faia A, Clímaco M.C., 2005. Comparison of two olfactometric methods for the identification of impact odourants in red wines. *In: In Vino Analytica Sciencia* 2005, p. 54, Montpellier, France.

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(2) Clímaco M. C., Botelho G., Avelar M. L., 2005. A caracterização do aroma das uvas por métodos olfactométricos. Colóquio ALABE 2005, Anadia, Portugal.

(3) Caldeira I., Botelho G., Belchior A. P., Bruno de Sousa R., Mendes-Faia A., Clímaco M. C., 2004. Aplicação da Cromatografia em Fase Gasosa - Olfactometria no Conhecimento dos Odorantes - Chave em Vinhos e Aguardentes. 6º Simpósio de Vitivinicultura do Alentejo, Évora, Portugal.

Posters in international/national conferences

(1) Botelho G., Mendes-Faia A., Clímaco M. C., 2008. GC-O posterior intensity method in discrimination of clonal wines from two vintages. Wine Active Compounds 2008, Beaune, France. (Accepted)

(2) Botelho G., Mendes-Faia A., Clímaco M. C., 2007. GC-O Posterior intensity method in clonal red wine evaluation. Poster: V.07. OENO 2007 – 8th International Enology Symposium, Bordeaux, France.

(3) Caldeira I., Botelho G., Avelar M. L., Clímaco M. C., 2005. OGA – Metodologia de caracterização de odorantes-chave. Poster: P1.24. 7º Encontro de Química dos Alimentos. Alimentos: Tradição e Inovação, Saúde e Segurança, Viseu, Portugal.

CHAPTER 1

General introduction

1. GENERAL INTRODUCTION

Wine aroma characterisation has been carried out by several researchers around the world. Several volatile compounds resulting from grapes, pre-fermentative, fermentative and ageing of wines are recognised today as odour-active compounds with an effective contribution towards the aroma complexity of wines.

The human sense of smell is a useful tool both in gas chromatography-olfactometry (GC-O) and in descriptive sensory analysis. Thus being, the current status regarding the understanding of human olfaction will be explored. This part is particularly interesting and relevant in order to improve the knowledge regarding the crucial role of (i) the sniffers (the individuals that sniff the capillary column effluent), who are the detectors in the GC-O experiments; (ii) the panelists, who are the individuals that carry out the sensory evaluation of wines or other beverages or foods.

Different GC-O methods have been developed in order to increase the knowledge about volatile compounds in beverages and foods, allowing the distinction between odour-active and no odour-active compounds. They can be classified in four main categories: dilution methods, time-intensity methods, detection frequency methods and posterior intensity methods.

It is of great interest to identify odour-active compounds as well as to quantify them. Thus, different extraction and quantification methods of volatile compounds are described in the literature review.

All of these aspects will be focused on in the following sections; the aim, however, is not to be too exhaustive.

1.1. WINE AROMA

Wine aroma is made up of several hundreds of volatile compounds, in concentrations ranging from several $mg.L^{-1}$ to a few $ng.L^{-1}$, or even less (Schreier, 1979; Nykänen, 1986; Etiévant, 1991; Bertrand *et al*., 1994; Ebeler, 2001). The complexity of wine aroma (Figure I.1) makes it particularly difficult to study, probably due to the diversity of the factors involved in their appearance: i) grape metabolism, depending on grape variety, climate, soil and vineyard management techniques; ii) biochemical phenomena (oxidation and hydrolysis) occurring prior to fermentation, during extraction of the juice and maceration; iii) metabolic activity of the microorganisms responsible for alcoholic and malolactic fermentations, and iv) chemical or enzymatic reactions that occur after fermentation, particularly during ageing in vat, barrel or bottle (Drawert, 1975; Cordonnier and Bayonove, 1979). Early, Drawert and Rapp (1966), Cordonnier and Bayonove (1979) and Crouzet (1986), considered wine aroma the result of four stages of the biotechnological processes of winemaking: varietal aroma, pre-fermentative aroma, fermentative aroma and post-fermentative aroma.

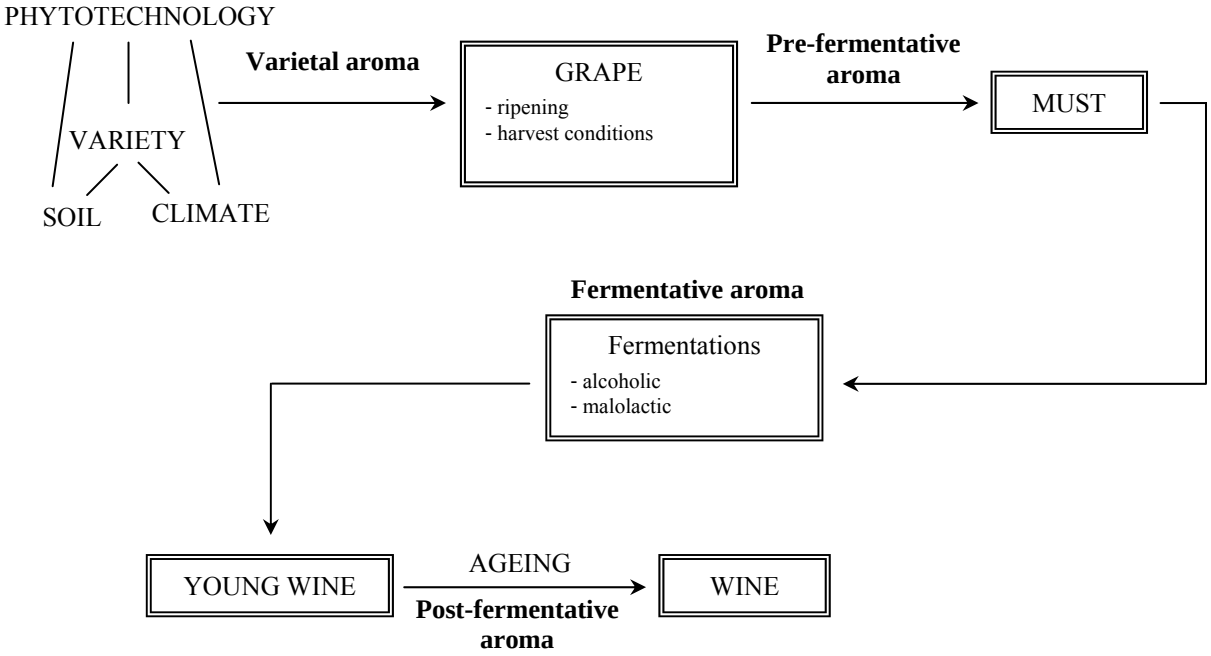


Fig. I.1 – Biotechnological sequence and components of the wine aroma (adapted from Drawert, 1975).

1.1.1. Varietal aroma

Numerous studies have shown that the terpenoid compounds were the essential compounds of the sensory expression of varietal wine aroma. As an example, the floral aroma characteristic of the wines obtained from Muscat grape varieties, results mainly from their monoterpoids (Bayonove and Cordonnier, 1970a,b; Ribéreau-Gayon *et al*., 1975; Williams *et al*., 1980, 1981), such as linalool, geraniol, nerol, citronellol, α-terpineol and hotrienol. Several authors have

devoted their research work to the identification and study of varietal aroma compounds in grapes and musts as well as its evolution in wines to characterise the aromatic potential of the grape varieties (Cordonnier and Bayonove, 1974; Clímaco, 1978; Noble *et al*., 1980; Rapp *et al*., 1980; Noble, 1981; Augustyn and Rapp, 1982; Clímaco, 1982; Marais, 1983; Rapp, 1984; Rapp and Mandey, 1986; Rapp, 1988; Darriet *et al*., 1991; Canal-Llaubers, 1993; Le Chevanton *et al*., 1993; Oliveira, 2000; Câmara, 2004; Câmara *et al*., 2004; Ferreira and Guedes de Pinho, 2004; Oliveira *et al*., 2004). A number of surveys have been made regarding monoterpene concentration in different grape varieties (Di Stefano, 1981; Dimitradis and Williams, 1984; Gunata *et al*., 1985). However, since the reported quantitative data were obtained by different techniques and from samples of grapes from different origins, direct comparison between different analytical results has not been feasible. Nevertheless, according to Mateo and Jiménez (2000), in their review about monoterpenes in grape juice and wines, it is possible to classify them based on the general classification of those varieties that have been screened: (1) intensely flavoured muscats, in which total free monoterpene concentrations can be as high as 6 $mg.L^{-1}$; (2) non-muscat but aromatic varieties with total monoterpene concentration of 1-4 $mg.L^{-1}$; and (3) more neutral varieties not dependent on monoterpenes for their flavour (Table I.1).

Table I.1 – Classification of some grape varieties based on monoterpene content (adapted from Mateo and Jiménez, 2000).

(1) Muscat varieties	(2) Non-muscat aromatic varieties	(3) Neutral varieties
Canada Muscat	Traminer	Aryan
Gewurztraminer	Huxel	Bacchus
Muscat of Alexandria	Kerner	Bobal
Muscat of Frontignan	Morio-Muskat	Cabernet Sauvignon
Muscato Bianco del Piemonte	Müller-Thurgau	Carignan
Muscat Hamburg	Riesling	Cencibel
Muscat Ottonel	Achurebe	Chardonnay
Moscato Italiano	Schonburger	Chasselas
	Siegerebe	Chenin Blanc
	Sylvaner	Cinsault
	Wurzer	Clairette
		Dattier de Bevrouth
		Doradillo
		Forta
		Merlot
		Nobling
		Rkaziteli
		Ruländer
		Sauvignon blanc
		Semillon
		Shiraz
		Sultana
		Terret
		Trebbiano
		Verdelho
		Viognier

In the cultivars listed under (3), monoterpenes are at such low concentration, generally below the perception threshold, that these compounds could only play a minimal role in the varietal aroma of wines (Mateo and Jiménez, 2000). Regardless of this situation, a large volume of the world's wine is produced from several cultivars of group (3).

With respect to the Portuguese cultivars, Oliveira (2000) has considered that the Loureiro variety may be classified among the aromatic varieties, as the concentration of linalool in these grapes is always above its perception threshold. More recently, Câmara (2004) and Câmara *et al*. (2004) showed that Boal, Malvasia, Sercial and Verdelho white grape varieties, grown in the Island of Madeira, have different profiles of terpenoids. Malvasia had a total amount of these compounds higher than the others. However, the authors do not present a final classification of the four varieties according to their terpenoid content. In the same year, López *et al.* (2004), in a study about Tempranillo (syn. Aragonez) and Grenache confirmed that these two red grape varieties should be considered as neutral cultivars. Accordingly, the small number of terpenes as well as their amounts found in these grape varieties confirmed their non-floral character.

1.1.2. Pre-fermentation aroma

The destruction of the grape cells during pre-fermentation treatments results in airing despite the precautions taken. Two enzyme categories, oxido-reductases and oxygenases, are responsible for many grape constituent transformations. Alcohols and aldehydes of C_6 chain length are quite common components of several fruits and vegetables, and are known as being enzymatically originated from linoleic and linolenic acids by aerobic oxidation (Stone *et al*., 1975; Cayrel *et al*., 1983). Four enzymatic activities are sequentially involved (Figure I.2). First, an acylhydrolase releases the fatty acids from membrane lipids. Next, the lipoxigenase catalyzes the fixation of oxygen on these C_{18} unsaturated fatty acids, and the peroxides obtained are then cleaved into C_6 aldehydes. Some of them are reduced to their corresponding alcohols by specific alcohol dehydrogenases from the grapes (Crouzet, 1986). Their occurrence in wines has been extensively studied as they have been related to the so-called *leaf grassy*, *herbaceous* odours initially attributed to leaves mixed with grapes which are collected by mechanical harvesting (Joslin and Ough, 1978; Ramey *et al*., 1986). Several C_6 compounds have been identified in grape and must, namely hexanal, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol, and 1-hexanol (Rapp *et al*., 1976; Schreier *et al*., 1976).

In Muscat-type varieties, a considerable proportion of their aromatic potential is in the form of terpenic heterosides – non-odourous in ripe grapes. During pre-fermentation treatments, enzymatic hydrolysis of these compounds increases must aroma intensity. This phenomenon is enhanced by maceration of grape solids because higher concentrations of bound terpenic compounds are found in skins (Ribéreau-Gayon *et al*., 1998).

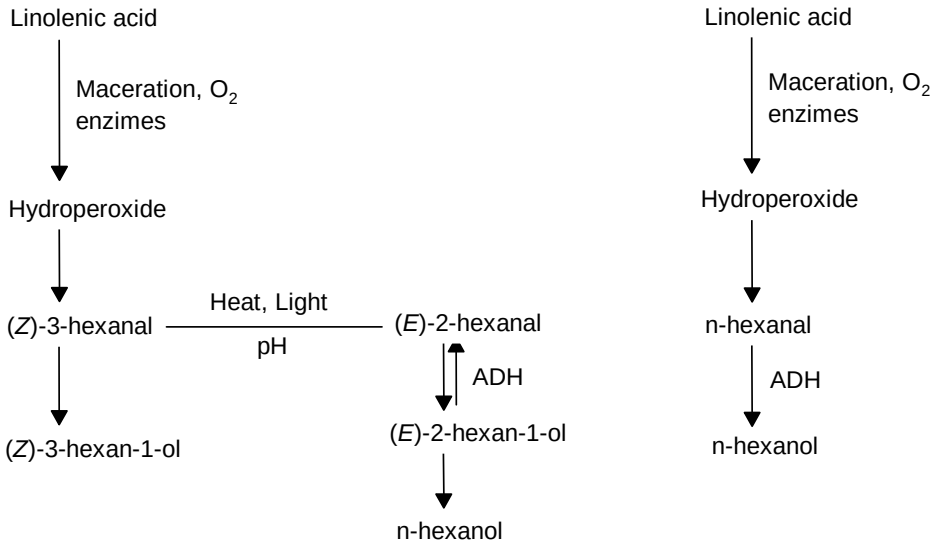


Fig. I.2 – Pathway of C_6 alcohols (adapted from Joslyn *et al*., 1978).

1.1.3. Fermentation aroma

Alcoholic fermentation represents the main process in the development of flavour-active compounds since when compared to wine, the aroma and flavour of grape juice/must is relatively low (Rapp, 1988; Jackson, 2000; Lambrechts and Pretorius, 2000). The aroma of young wines, red or white, is highly influenced by the secondary products of alcoholic fermentation, such as, esters, alcohols, volatile acids, or volatile phenols. Organic acids, higher alcohols, low-volatile organic sulphur compounds and esters are significant sensorial components of wine and constitute the main group of compounds that form the "fermentation bouquet". Additionally, in red wines, malolactic fermentation also plays an important role in the aroma complexity (Cordonnier and Bayonove, 1979; Bayonove *et al*., 1998; Ribéreau-Gayon *et al*., 1998).

The complexity of flavour development during alcoholic fermentation is still relatively poor understood. Three main routes of flavour development can be identified during fermentation, namely, the fact that while some grape-derived compounds remain essentially chemically intact, others are metabolised to form flavour-active metabolites, and others undergo hydrolytic or biotransformation reactions either intra- or extra-cellularly, which modify their flavour-active contributions. Figure I.3 provides a summary of how these compounds are formed during alcoholic fermentation by *Saccharomyces cerevisiae.*

The fatty acid ethyl esters (ethyl butanoate, ethyl hexanoate and ethyl octanoate) have very pleasant odours, described as *fruity* and *sweet* character. Also the esters of acetate have pleasant odours: isoamyl acetate and isobutyl acetate have an aroma like *fresh banana*, hexyl acetate smells like *fruit*, and *sweet*. Both groups of esters contribute to the fuitiness of wines and their

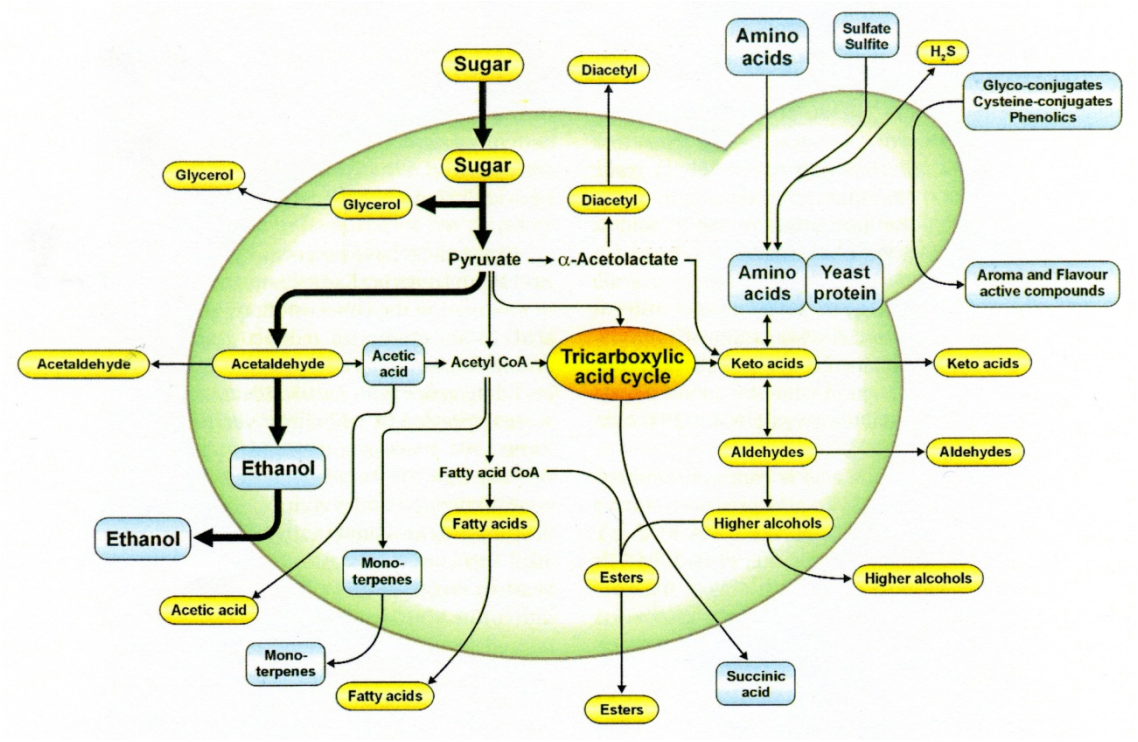


Fig. I.3 – A schematic representation of derivation and synthesis of flavour-active compounds from sugar, amino acids and sulfur metabolism by wine yeast (Swiegers *et al.*, 2005).

concentrations slowly decline due to non-enzymatic hydrolysis during storage and ageing (Meilgard, 1975; Etiévant, 1991).

With the exception of 2-phenylethanol, which has a *floral* and *rose-like* aroma descriptors (Simpson, 1979; Nykänen, 1986) higher alcohols do not have pleasant odours; for example, isoamyl alcohol has an aroma described as *alcohol, burnt*, 2-methylpropan-1-ol is described as *solvent* and *alcohol* (Meilgard, 1975; Simpson, 1979). In this line, higher alcohols when present in excess concentrations may also be regarded as undesirable.

In order to control the fermentation process, the development of active dried yeast for the alcoholic fermentation and commercial malolactic starters for malolactic fermentation has been the focus of several research studies. Nevertheless, recent studies (Renouf *et al.*, 2005) suggest that the use of commercial starters has no significant effect on the development of indigenous microflora. This indigenous microflora plays an important role in winemaking and its beneficial effects on wine properties have now been established (Jolly *et al.*, 2003). Furthermore, winemakers are increasingly focusing on preserving the *terroir* characteristic of each wine (Pretorius *et al.*, 1999). Actually, the microbiological life of wines starts before reception and fermentation of the grapes at the winery, since yeast and bacteria cover the grape berry with a complex microbial system. This microbial community is very large and diverse. The populations change according to the stage of grape development. Veraison, the key stage during grape ripening, is also an important step in the evolution of the microbial community on the berry surface (Renouf *et al.*, 2005).

Lactic acid bacteria play an important role in red winemaking. Only four genera of the lactic acid bacteria, *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*, are able to survive under the unfavourable conditions (low pH, high ethanol concentration and low nutrients) present in wine to any extent. *Oenoccocus oeni* is the most well adapted wine-associated species and is used almost exclusively for the induction of malolactic fermentation (MLF) in red, white and sparkling base wines (Wibowo *et al*., 1985; Henick-Kling, 1993; Henschke, 1993).

Research in progress is showing that these bacteria can modify some of the components and sensory properties of wine (Figure I.4), providing a new opportunity to alter the chemistry and possibly the aroma and flavour perception of wine (Henick-Kling, 1993; Bartowsky *et al*., 2002; Mattews *et al*., 2004; Swiegers *et al*., 2005).

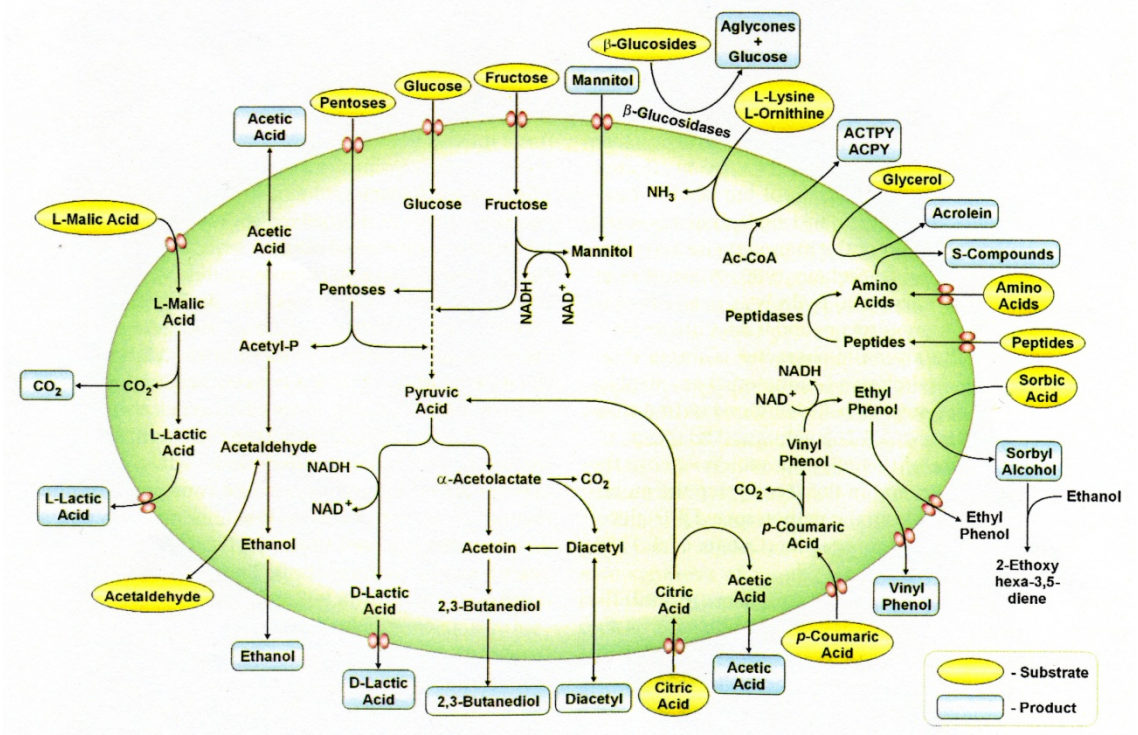


Fig. I.4 – A schematic representation of the biosynthesis and modulation of flavour-active compounds by malolactic bacteria (Swiegers *et al.*, 2005).

1.1.4. Post-fermentation aroma

The post-fermentation aroma, called ageing aroma or bouquet (Bayonove, 1993) includes the volatile compounds derived from and/or transformed by enzymatic, chemical or physical ways, which occur during wine ageing (Bayonove, 1993; Ribéreau-Gayon *et al*., 1998; Bayonove *et al*., 1998). The intensity of these reactions is strongly dependent on the storage conditions, and in particular, on the type of the container (stainless steel, wood, glass bottle) (Clímaco *et al*., 1988; Chatonnet *et al*., 1990; Clímaco and Borralho, 1996; Clímaco *et al*., 1997; Pérez-Prieto *et al*., 2003). Several volatile compounds can be provided by the wood to the wine. These compounds can be quantitatively influenced by the wine's own composition, by the wood and

by its botanical and geographical origin, by the technological treatments accomplished in cooperage, and by the technologies of wood utilisation applied in ageing (Chatonnet *et al*., 1990; Francis *et al*., 1992; Bertrand *et al*., 1997; Masson *et al*., 1997a; Botelho, 2000; Clímaco *et al*., 2001, 2004; Garde-Cérdan *et al*., 2004; Clímaco and Rodrigues, 2005; Clímaco *et al*., 2005; Câmara *et al*., 2006; Eiriz, 2006).

1.2. THE SENSE OF SMELL

1.2.1. Human olfactory system

The anatomy of the nose is such that only a small fraction of inspired air reaches the olfactory epithelium via the nasal turbinates, or via the back of the mouth on swallowing (Maruniak, 1988).

The sensitivity and range of the olfactory system is remarkable, enabling organisms to detect and discriminate between thousands of low molecular mass, mostly organic compounds, which we commonly call odours (Firestein, 2001).

The sense of smell is a primal sense for humans as well as animals. From an evolutionary standpoint it is one of the most ancient of senses. Smell (or Olfaction) allows vertebrates and other organisms with olfactory receptors to identify food, mates, predators, and provides both sensual pleasure (the odour of flowers and perfume) as well as warnings of danger (*e.g.*, spoiled food, chemical dangers). For both humans and animals, it is one of the important means by which our environment communicates with us (Leffingwell, 2002).

The human olfactory system is the detector in the GC-O analysis and is a precious tool for the detection and recognition of the odourant compounds eluting from a GC column.

However, the sensitivity of each individual's olfactory system is widely varied. Some individuals possess a heightened sensitivity to odours (hyperosmic) while others are physically unable to detect odourant compounds (anosmics). This wide variance in an individual's ability to detect odours, as well as the variability and complex nature of odours themselves, makes the determination of odours very difficult to standardise and measure quantitatively.

With regard to terminology, the terms "subject", "panelist", "judge", and "assessor" are used interchangeably as suggested by Meilgaard *et al*. (1991) and Stone and Sidel (1993).

1.2.1.1. Olfaction organ

The sense of smell enables us to analyse chemical molecules coming from the outside ambient air. The organs of olfaction (Figure I.5) include peripheral neuroceptors or "sensors" located in the olfactory mucous membrane of the nasal fossae, a peripheral nervous organ, the olfactory bulb, receiving the fibres from the first neurone (first cranial pair), and finally from the central connections (Portmann, 1999).

The olfactory region of each of the two nasal passages in humans is a small area of about 2.5 cm^2 containing in total approximately 50 million primary sensory receptor cells (Leffingwell, 2002). It is much larger in animals; the dog for example, has a sensory surface area which varies according to the breed from 30 to 100 cm^2 (Portmann, 1999).

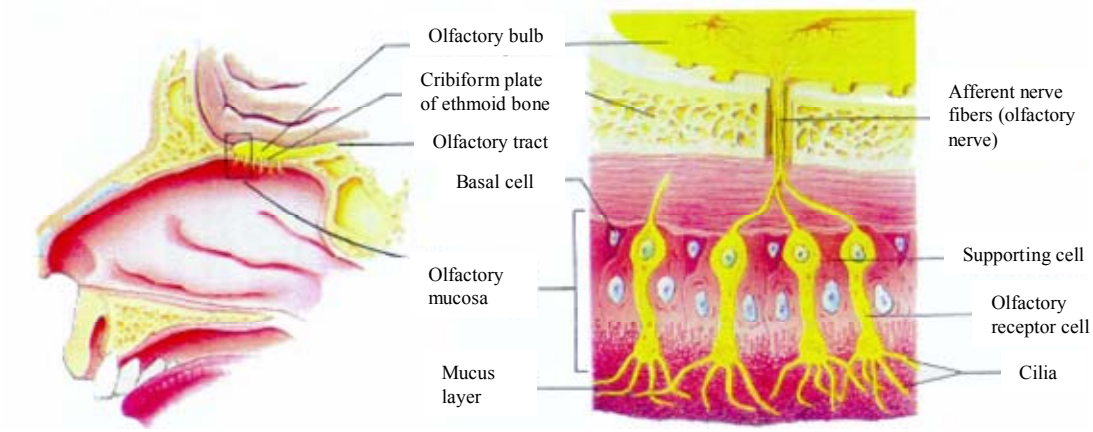


Fig. I.5 – Location and structure of the olfactory receptors (adapted from Kehoe *et al*., 1996).

1.2.1.2. Olfaction mechanism

According to Dautry (1988) the olfaction involves four steps: reception and transduction, transmission, encoding and perception.

Reception and transduction

The cells involved in odour detection are neurones, that is to say, cells which are capable of transmitting information in electric form. In humans, there are about 50 million of them. In the olfactory mucous membrane, the neurons are specialised and have many cilia (hair-like lashes) at their extremity. These cilia bathe in the mucus. Inserted in the membrane of the cilia, the olfactory receptors are the tools which transform the "message" of the odourant molecule into a physiological message. Each neurone has only a single molecular type of olfactory receptor (Morrot and Brochet, 1999).

In 1991, Linda Buck and Richard Axel discovered both the family of transmembrane proteins that were believed to be the odour receptors and some of the genes that encode them. They cloned and characterised 18 different members of an extremely large multigene family that encodes the seven transmembrane proteins whose expression was restricted to the olfactory epithelium. This was a seminal breakthrough in our potential understanding of the olfactory system (Buck and Axel, 1991). The proteins found all contained the 7 helical transmembrane structures and contained a sequence similar to other members of the "G-protein" linked receptor family (Figure I.6).

It is now known that there are about 350 odourant receptor genes and about 560 odourant receptor pseudogenes in humans (Glusman *et al*., 2000, 2001; Zozulya *et al*., 2001). This number of genes and pseudogenes, specific to the olfactory system, comprises nearly 2% of the 50,000 or so genes of the human genome. This number is second only to the receptors of the immune system (Leffingwell, 2002).

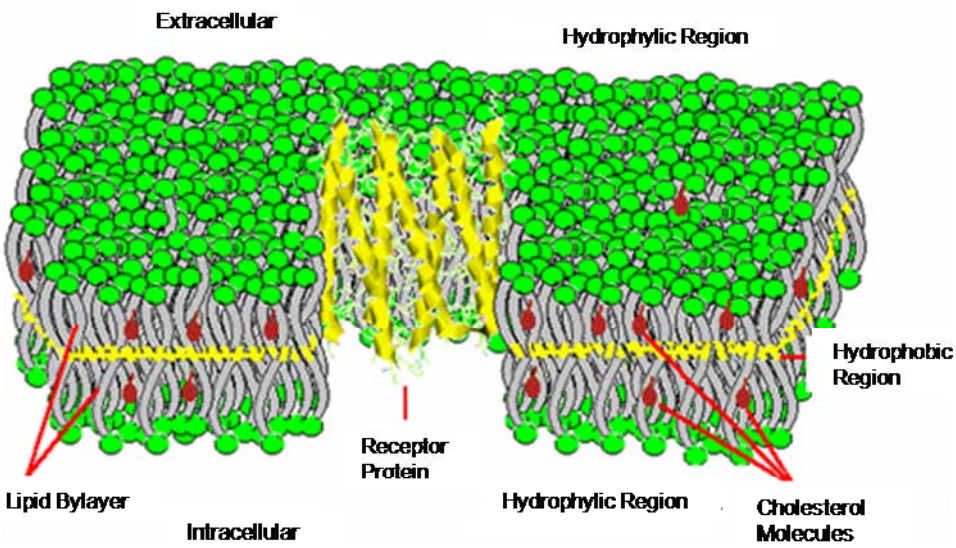


Fig. I.6 – Cut away view of 7-Helical Olfactory G-protein coupled "receptor protein" transversing cellular membrane (adapted from Leffingwell, 2002).

In 1998, Firestein and coworkers at Columbia University clearly demonstrated that transgenic expression of these proteins in mice allowed them to acquire new olfactory capacities. The structure of the olfactory neurons with their cilia enables a substantial increase in the contact surface between the neurone membranes, and therefore of the receptors, and of the odourant molecules present in the mucus. The 2.5 cm^2 of apparent surface area is in fact composed of a surface of 500 cm^2.

Our nose is therefore forever young, totally renewed every 100 days (half-life 50 days). Of course, this renewal is constantly in progress and we are never deprived of our sense of smell (Morrot and Brochet, 1999).

There is not a specific receptor for a given odour. For example, there is not a receptor entirely devoted to the *banana* odour of isoamyl acetate. Each molecule can bond with one or several olfactory receptors, depending on its concentration and its affinity with that receptor (Figure I.7).

Transmission

Any synthetic or natural molecule can be captured and can induce a physiological signal. This bond causes a modification in the conformation of the receptor and sets off a cascade of amplification of the signal coming from the odourant molecule in the neuroreceptor cell.

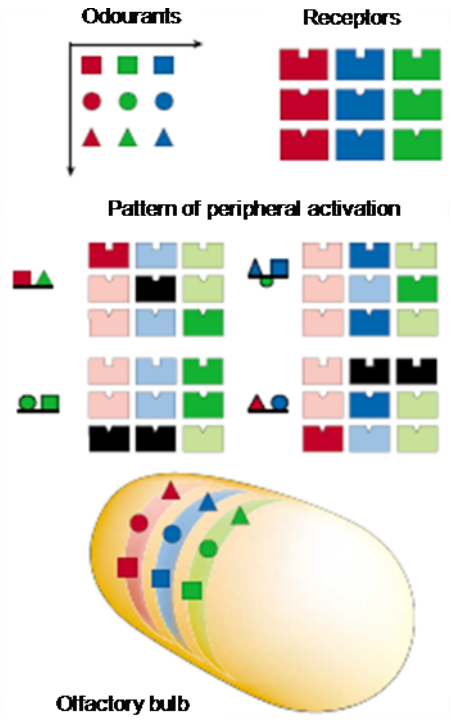


Fig. I.7 – A code in the nose (adapted from Firestein, 2001).

Once the receptor has bound an odourant molecule (Figure I.8), a cascade of events is initiated that transforms the chemical energy of binding into a change in the membrane potential of the OSN (olfactory sensory neurons).

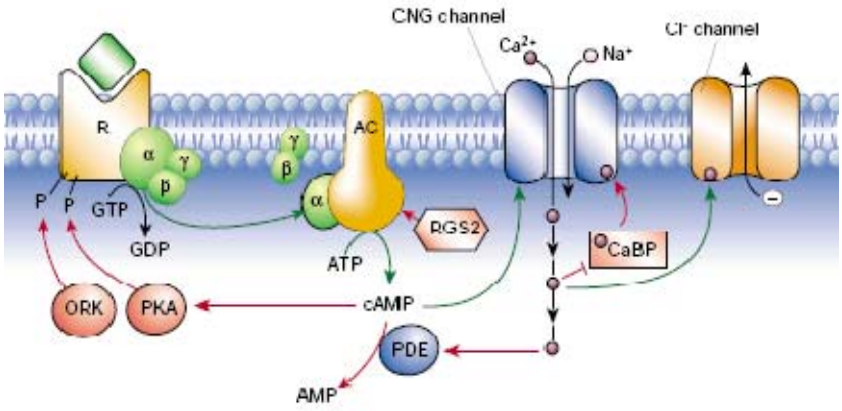


Fig. I.8 – Sensory transduction (Firestein, 2001). AC, adenylyl cyclase; CNG channel, cyclic nucleotide-gated channel; PDE, phosphodiesterase; PKA, protein kinase A; ORK, olfactory receptor kinase; RGS, regulator of G proteins (but here acts on the AC); CaBP, calmodulin-binding protein. Green arrows indicate stimulatory pathways; red indicates inhibitory (feedback).

The ligand-bound receptor activates a G protein (an olfactory-specific subtype, G_{olf}), which in turn activates an adenylyl cyclase (ACIII). The cyclase converts the abundant intracellular molecule ATP into cyclic AMP, a molecule that has numerous signalling roles in cells. In the case of OSNs the cAMP binds to the intracellular face of an ion channel (a cyclic nucleotide-gated, CNG) channel closely related to that found in photoreceptors, enabling it to conduct cations such as Na^+ and Ca^{2+} (Firestein *et al*., 1991). Inactive OSNs normally maintain a resting

voltage across their plasma membrane of about -65 mV (inside with respect to outside). When the CNG channels open, the influx of Na^+ and Ca^{2+} ions causes the inside of the cell to become less negative. If enough channels are open for long enough, causing the membrane potential to become about 20 mV less negative, the cell reaches threshold and generates an action potential. The action potential is then propagated along the axon, which crosses through a thin bone known as the cribiform plate, and into the forebrain where it synapses with second-order neurons in the olfactory bulb (Firestein, 2001).

Encoding

The odourant molecules bond with weak affinity with a great number of olfactory receptors. The nature of the odours is thus encoded by a combination of sensors. The signal resulting from an odour (or from a set of odours) is the activation of a large number of olfactory neurones. The intensity of this activation in each neurone (encoded as a frequency of action potentials) depends on the concentration of the odourant molecule and on the affinity between this molecule and the receptor. If we were to imagine that we had about 1,000 sensors working in a binary manner (sensor which can take on two states: active or inactive), then we would be capable of encoding $2^{1,000}$ different odours. But each sensor can itself have a great number of different states, bringing the number of actual possible combinations to a level which we cannot even imagine (Morrot and Brochet, 1999).

Perception

According to Kehoe *et al*. (1996), the human perception of odours consists of more than just "smell". It represents a complex series of psychological and physiological responses to the quality of the odourant detected.

The properties of protein sensors can also provide explanations of the particularities of the sense of smell. Certain molecules thus change "tone" at very high concentrations. For example, the compound 4-mercapto-4-methyl-pentan-2-one has an aroma of *passion fruit* at low concentration, which develops into strong notes of *cat urine* at higher concentration (Darriet *et al*., 1999). When the quantity of a molecule increases, not only are the receptors with strong affinity for this molecule all occupied and highly activated, but new receptors are also "recruited" and modify the nature of odour (Morrot and Brochet, 1999).

The olfactory system is sometimes capable of making the difference between isomers and between stereoisomers. In fact, it is well accepted that in humans certain specific chemical enantiomers such as carvone, menthol, limonene, linalool, citronellol, 7-hydroxy citronellol, 1-octen-3-ol, δ-decalactone, γ-decalactone, *p*-menthene-8-thiol, α-damascone, α-ionone, 3-mercapto-2-methylpentanol, (*E*)- and (*Z*)-nerolidols, α-terpineol, the theaspiranes, 2-ethylhexanoic acid, *cis*-rose oxide, nerol oxide, ethyl 2-methylbutyrate, methyl 2-

methylbutyrate, ethyl 2-oxo-3-methylpentanoate and 2-methylbutyric acid can be distinguished as they possess varying degrees of olfactory differences (Leffingwell, 2001). This author, Leffingwell, has published an extensive data base on the internet that provides over 100 enantiomeric pairs of odourants that have different odour properties. This site provides both 2-D and 3-D molecular structures along with odour descriptors, odour thresholds and original references.

Good sensory measurements require that we look at the subjects as measuring instruments, somewhat variable over time and among themselves, and very prone to bias. In order to minimise variability and bias, the subject must understand the basic physiological and psychological factors which may influence sensory perception. The main physiological and psychological factors are presented in Table I.2 (Meilgaard *et al*., 1991; Stone and Sidel, 1993).

Table I.2 - Physiological and psychological factors which may influence sensory perception (adapted from Meilgaard *et al*., 1991; Stone and Sidel, 1993).

Physiological factors	**Description**
Adaptation	Adaptation is a decrease in or change in sensitivity to a given stimulus as a result of continued exposure to that stimulus or a similar one. In sensory testing this effect is an important unwanted source of variability of thresholds and intensity ratings.
Enhancement	The effect of the presence of one substance increases the perceived intensity of a second substance.
Synergy	The effect of the presence of one substance increases the perceived combined intensity of two or more substances, such that the perceived intensity of the mixture is greater than the sum of the intensities of the components.
Suppression	The effect of the presence of one substance decreases the perceived intensity of a mixture of two or more substances.
Psychological factors	
Expectation error	This error arises from a subject's knowledge about a product and is manifested in the expectation for specific attributes or differences based on that knowledge.
Error of habituation	This error results from a tendency to continue to give the same response when a series of slowly increasing or decreasing stimuli are presented.
Stimulus error	This error occurs when subjects have (or think they have) prior knowledge about products in a test, and as a result will assign scores in an atypical manner or will find differences that are unexpected.
Logical error	Logical errors occur when two or more characteristics of the samples are associated in the mind of the assessors.
Halo effect	When more than one attribute of a sample is evaluated, the ratings will tend to influence each other. Simultaneous scoring of various flavour aspects along with overall acceptability can produce different results rather than if each characteristic evaluated separately.
Order of presentation of samples	At least five types of bias may be caused by the order of the presentation: contrast effect, group effect, error of central tendency, pattern effect and positional bias.
Mutual suggestion	The response of a panelist can be influenced by other panelists.
Lack of motivation	The degree of effort a panelist will make to discern a subtle difference, to search for the proper term for a given impression, or to be consistent in assigning scores is of decisive importance for the results.

1.2.2. Sensory thresholds

Thresholds are the limits of sensory capacities. It is convenient to distinguish among the absolute threshold, the recognition threshold, the difference threshold, and the terminal threshold (Table I.3).

Table I.3 – The different thresholds used in sensory analysis (Jounella-Erikson, 1983; Sauvageot, 1990; Meilgaard *et al*., 1991).

Absolute threshold (detection threshold)	It is the lowest stimulus capable of producing a sensation. It corresponds to the minimum concentration detected, with a certain statistical significance, by 50% of the panelists of a group.
Recognition threshold	It is the level of a stimulus at which the specific stimulus can be recognised and identified. The recognition threshold is usually higher than the absolute threshold. It corresponds to the minimum concentration, for which 50% of the panelists identify the nature of the stimulus.
Difference threshold	It is the extent of change in the stimulus necessary to produce a noticeable difference. It is usually determined by presenting a standard stimulus which is then compared to a variable stimulus.
Terminal threshold	It is that magnitude of a stimulus above which there is no increase in the perceived intensity of the appropriate quality for that stimulus; above this level, pain often occurs.

Thresholds of added substances are used with water supplies, foods, beverages, cosmetics, and solvents to determine the point at which known contaminants begin to reduce acceptability. These are the most important uses, and testing may be done with hundreds of panelists in order to map the distribution of relative sensitivity in the population. Thresholds may also be used as a means of selecting panelists, but this should not be the main basis for selection unless the test objective requires detection of the stimulus at very low levels (Meilgaard *et al*., 1991). Furthermore, the concept of the "odour unit" or "aroma value" uses the threshold as a measure of aroma intensity. This concept was first proposed by Rothe and Thomas (1962) with the aim of establishing the olfactory importance of compounds identified in food. The number of odourous unities of a compound in food, U_i, is equal to the ratio between the compound concentration in food (C_i) and its concentration in threshold (L_i).

1.2.3. Psychophysical theory

The major goal of psychophysics is to improve the way we understand responses to sensory stimulus (Meilgaard *et al*., 1991). Over the past century, two forms of the psychophysical function have been in use: Fechner's law and Stevens' law.

Fechner's law

Fechner selected as his measure of the strength of sensation the Just Noticeable Difference (JND). For example, he would regard a sensation of 8 JDNs as twice as strong as one of 4 JNDs. JNDs had just become accessible to measurement through difference testing, which Fechner

learned from Ernst Weber at the University of Leipzig in the mid-1800s. Weber found that difference thresholds increase in proportion to the initial perceived absolute stimulus intensity at which they are measured:

$$\frac{\Delta\phi}{\phi} = k \quad \text{(Weber's law)}$$

where ϕ is the absolute intensity of the stimulus, $\Delta\phi$ is the change in intensity of the stimulus that is necessary for 1 JND, and k is a constant between 0 and 1. Weber's law states, for example, that the amount of an added flavour which is just detectable, depends on the amount of that flavour which is already present; if the k has been determined, we can calculate how much extra flavourant is needed. The actual derivation of Fechner's law,

$$\psi = k \log_{10} \phi$$

is complex and depends on a number of assumptions. Support for Fechner's law is provided by common category scaling. When panelists score a number of samples that vary along one dimension using a scale such as 0 to 9, the results plot out as a logarithmic curve (Meilgaard *et al*., 1991).

Stevens' law

Stevens (1961, 1970) and his collegues at the Phsycho-acoustic laboratory working at Harvard University a century after Fechner, pointed out that if equation resulting from Fechner' law was correct, a tone of 100 dB should only sound twice as loud as one of 50 dB. He then showed, with the aid of Magnitude Estimation Scaling, that subjects found the 100 dB tone to be 40x as loud as the one of 50 dB. Steven's main contention, that sensation magnitude grows as a power function of stimulus intensity, can be expressed mathematically as:

$$\psi = k\phi^n$$

where k is a constant which depends on the units in which we choose to measure ψ, and n is the exponent of the power function, *i.e.*, a measure of the rate of growth of perceived intensity as a function of stimulus intensity. The finding that the exponent for visual length is 1.0, *i.e.*, simple proportionality, has led to the common use of scales for rating sensory intensity. When n is larger than 1, the sensation grows faster than the stimulus. Conversely, when n is smaller than 1, as is for many smells, the sensation grows more slowly than the stimulus. Stevens proposed that only ratio scales be valid for the measurement of sensation, and his magnitude estimation scales are probably the most used in psychophysics today. However, many authors have pointed out that there are serious shortcomings: the exponents vary with the range of stimuli in the test and with the modulus used and the exponents differ greatly among researchers and among individuals (Meilgaard *et al*., 1991).

1.3. AROMA EVALUATION

The aroma evaluation of wine, other beverages or food involves pluridisciplinar studies. The chemical analysis tries to identify and to quantify the volatile compounds which are present in the samples using the separative techniques. On the other hand, the sensory analysis, with resource to different methodologies, tries to determine the odour intensity and/or quality of the individual chemical compounds as well as the sensory characteristics of samples.

1.3.1 Sensory analysis

The sensory analysis can be defined as "a scientific discipline used to evoke, measure, analyse and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch and hearing." This definition, adopted by several authors (Stone and Sidel, 1993; Lawless and Heymann, 1999) and international organizations (Institute of Food Tecnologists, American Society for Testing and Materials), reveals the experimental character of the sensory analysis and, in parallel, points out their perception components and reactive behavior.

1.3.1.1. Descriptive analysis

Descriptive analysis includes the flavour profile, texture profile, quantitative descriptive analysis and attributes rating methods.

Favour profile

The flavour profile method of descriptive analysis provides a record of a product's aroma and flavour components. Similarities and differences may be pinpointed by direct comparison of records. The flavour profile includes an overall impression rating, "amplitude", which reflects the degree of blending of the sensory components. A panel of four to six members is used (ASTM, 1981).

Texture Profile

The texture profile method is a descriptive analysis technique based on the principle of the flavour profile method. It provides a systematic approach to measuring the textural dimensions of food in terms its mechanical, geometrical, fat, and moisture characteristics. The panel is composed of six to eight members (ASTM, 1981).

Quantitative Descriptive Analysis

Quantitative descriptive analysis (QDA), like the flavour profile method, is used to set up a permanent record of the sensory components of a product or ingredient. Quantitative descriptive analysis describes the appearance, aroma, flavour, and texture attributes of a product or sample

under study according to the order of detection. This method makes use of a special scaling technique and statistical analysis of the resultant intensity values to compare the sensory components of several products. In addiction, the method makes use of a multidimensional type of visual portrayal to show similarities and differences. A panel of ten to twelve members is generally recommended, although in certain situations as few as six may be used (ASTM, 1981).

Attribute Rating

The attribute rating method measures intensities of specified characteristics (attributes). The characteristics may be defined through flavour or texture profile analysis or by a person or persons thoroughly familiar with a product's sensory attributes. Trained panelists analytically discriminate intensity differences among the samples presented. The attribute rating method employs either one or two scaling approaches – category scaling or ratio scaling (magnitude estimation). Category scaling may be either structured or unstructured. Structured category scales utilise a series of equidistant scalar intervals, anchored with appropriate descriptive terms at each interval. Unstructured scales have descriptive quantitative points at either end; the anchor points indicate the extremes of the characteristic to be measured. In magnitude estimation, number amounts (ratios or proportions) are assigned to indicate the intensity (magnitude) of a specific characteristic; the technique utilises numbers to express intensity (ASTM, 1981).

1.3.1.2. Normative references

There are several International Standards available around the sensory analysis theme. Table I.4 presents a list of the main normative references which constitute an essential base for the elaboration of sensory analysis.

Table I.4 – Main International Standard normative references.

ISO 3591:1977	Sensory analysis –Apparatus – Wine-tasting glass.
ISO 6564:1985	Sensory analysis – Methodology – Flavour profile methods.
ISO 6658:1985	Sensory analysis – Methodology – General guidance.
ISO 4121:1987	Sensory analysis – Methodology – Evaluation of food products by methods using scales.
ISO 8589:1988	Sensory analysis – General guidance for the design of test rooms.
ISO 5492:1992	Sensory analysis – Vocabulary.
ISO 8586-1:1993	Sensory analysis – General guidance for the selection, training and monitoring of assessors – Part 1: Selected assessors.
ISO 8586-2:1994	Sensory analysis – General guidance for the selection, training and monitoring of assessors – Part 2: Experts.
ISO 11035:1994	Sensory analysis – Identification and selection of descriptors for establishing a sensory profile by a multidimensional approach.

1.4. SENSORY ANALYSIS COUPLED WITH INSTRUMENTAL ANALYSIS

1.4.1. Sniffers in gas chromatography-olfactometry (GC-O) analysis

In GC-O analysis, sniffers judge the olfactory impressions elicited by the volatile compounds immediately after elution from the GC column. Methodological problems may arise from the non-random sequence in which the compounds elute. Not all judgments are similarly affected by variation in the quality of the responses during an experimental session. Results of an experiment can be systematically affected by decreasing alertness. Decrease in alertness will be most important when only a small number of compounds can be perceived, when these compounds show low odour intensity, when the stimulus is brief, when a session is long and when assessors are not motivated (Mackworth, 1948). Sensory and cognitive transfer effects can also affect consecutive judgments. Furthermore, problems can arise due to the varying inter-stimulus intervals; sometimes sniffers have to decide very rapidly (Kleykers, 1995). Therefore, it is not surprising that many authors have shown large variability within and between sniffers.

1.4.2. Gas chromatography-olfactometry (GC-O) methods

Distinguishing between odour-active compounds and the whole range of volatiles present in a particular food product or more specifically in wine is an important task in flavour analysis. An interesting approach is gas chromatography-olfactometry (GC-O). This technique was first used empirically and only to detect the smell of effluents at the exit of a chromatographic column using the human nose as a detector. Qualities were attributed to the various "odourant peaks" which were also characterised by their retention indexes. Experience shows that many odour-active compounds occur at very low concentrations; their sensory relevance is due to low odour thresholds. Therefore, the peak profile obtained by any "chemical" detector does not necessarily reflect the aroma profile of a food.

GC-O was proposed by Fuller *et al.* as early as 1964 (van Ruth, 2001) and was shown to be a valuable method for the selection of odour-active compounds from a complex mixture (Grosch, 1993).

In Portugal, and to our knowledge, the first GC-O analyses of wines and wood extracts were performed by Clímaco *et al.* (1985), Clímaco (1987), Clímaco *et al.* (1988), Clímaco (1993) and Borralho (1994) during which the main odourant zones and the peaks of the chromatograms with odourant relevance were identified.

With the early GC-O devices, reproducibility was a serious problem, which was caused by discomfort from sniffing hot dry effluent gases and the lack of sensitivity of the "chemical detectors" to identify the odour-active compounds. The latter problem is still with us today. Dravnieks and O'Donnell published a GC-O design in 1971, which minimised the discomfort of

sniffing. The hot column effluent was combined with humidified air to reduce nasal dehydration. Nowadays, the same principle is still used in most GC-O equipments.

Acree (1997) summarised the history of GC-O, giving examples of its application in natural product chemistry. He speculated about its future, using it as a method to bridge the gap between sensory science and analytical chemistry. Acree emphasised the fact that none of the detectors used in gas chromatography is as sensitive as the human nose for many of the odourants found in foods.

Several techniques have been developed to collect and process GC-O data and to estimate the sensory contribution of single odour-active compounds which can be classified in the four following categories (Acree and Barnard, 1994):

a) Dilution analysis methods for producing potency values based on stepwise dilution to threshold: CharmAnalysis™ (Acree *et al*., 1984a,b) and Aroma Extract Dilution Analysis (AEDA) (Schieberle and Grosch, 1987a,b; Ullrich and Grosch, 1987).
b) Time-intensity methods for producing estimates of perceived intensity recorded simultaneously with the elution of the chromatographic peak, *e.g.*, OSME analysis (McDaniel *et al*., 1990; Miranda-López *et al*., 1992a,b).
c) Detection frequency methods for recording detected odours over a group of sniffers. The number of sniffers detecting an odour (detection frequency) is used as an estimate of the odour's intensity (Linssen *et al*., 1993).
d) Posterior intensity methods for producing estimates of perceived intensity, which are recorded after a peak has eluted (Petersen *et al*., 1998; Tønder *et al*., 1998).

a) Dilution analysis methods

CharmAnalysis™

A procedure based on the relative odour thresholds of volatile compounds with known GC retention indexes was proposed by Acree *et al*. (1984a,b) transforming the qualitative GC-O to a quantitative method by computerising the observations.

The concept of CharmAnalysis™ was systematically constructed from the idea of OAV "odour activity values". The "odour activity value" or "odour unit" is defined as the ratio of an aroma compound's concentration divided by its odour threshold. The logarithm of the odour threshold (log OAV) is calculated to represent changes in concentration which are significant for olfactory discrimination. Odour activity follows a sigmoidal response curve in which significant aroma responses require order-of-magnitude changes in concentration. Consequently, logarithmic functions more significantly represent meaningful sensory differences. Aroma unit values >1 are indicative of compounds present at a concentration that greatly exceeds their thresholds, and are therefore likely to contribute significantly to aroma impact (Guadagni *et al*., 1966; McGorrin and Gimelfard, 1998).

During CharmAnalysis™, an aroma extract is injected into the gas chromatograph, and during the entire chromatogram, the sniffer presses a button each time he notices an odour and again when he does not detect it anymore. This produces a diagram with square signals. Then, the extract is diluted by a known factor and another run is done. This continues until there is no further response. The different diagrams are then combined.

Aroma extract dilution analysis (AEDA)

Also based on the OAV concept, the aroma extract dilution analysis (AEDA) was developed by Grosch and his research group (Schieberle and Grosch, 1987a,b; Ullrich and Grosch, 1987; Grosch, 1993; Guth and Grosch, 1994; Grosch, 1995). As obviously stated by Grosch (1994), distinguishing between the more potent odourants and those volatiles having low or no odour activity is the first task to be solved in flavour analysis. Like ChamAnalysis, AEDA is based on the progressive dilution of an extract obtained from the food, with each diluted sample being analysed by GC-Olfactometry. In this case, an odourant compound is noted when smelled. The highest dilution at which it is perceivable gives the flavour dilution (FD) factor, that is to say, the ratio of the concentration of the odourant in the initial extract to its concentration in the most diluted extract in which no odour is detected by GC-O. The FD factor is therefore a relative measure and is proportional to the OAV of the compound in air. The graph (log FD *vs* retention indexes), called an aromagram or olfactogram, is represented by bars. Odourants with high FDs are important contributors to the characteristic flavours or off-flavours of foods or beverages. The big advantage of the method is that it does not need sophisticated equipment.

The CharmAnalysis™ and AEDA analyses based on odour detection thresholds (measurement of the odour potency) have been criticised. The conclusions regarding the relative contribution of odourants to a flavour may be limited, as the responses to a given compound dependent on the concentration. The relative intensity of two odourants with the same thresholds does not necessarily correspond to their relative concentrations in a mixture (Abbott *et al*., 1993). For these authors, the contribution of a compound to an odour is probably better determined by CharmAnalysis™ than by AEDA, which does not take into account the duration of the odour. Besides, the major drawbacks of the dilution approach are, first, the difficulty of using more than one panelist, as is advisable in sensory analysis because the method is very time-consuming and, second, the results obtained are based on detection thresholds and not on real intensities (Etiévant *et al*., 1999).

b) Time-intensity methods

OSME method is based on a magnitude estimation of the odour intensity and was initially proposed by McDaniel *et al.* (1990). This method allows a direct estimation of the intensity of the odours using a reasonable number of sniffers (da Silva *et al*., 1994) and was developed

taking into account psychophysical laws (de Maria *et al*., 1994). The odour intensities of the eluted compounds are followed by trained sniffers moving a cursor with a scale (from none = 0 to extreme = 15) and recorded. This gives an aromagram called an "osmegram" (odour intensity *vs* retention indexes) representing odour significance of the compounds in a flavour. At the same time, the qualities are described. The application of this method to wine analysis, was published by Miranda-López *et al*. (1992b), concerning Pinot noir wines of different vintages and maturities. Surprisingly, the authors interpreted the differences between wine OSME aromagrams using the frequency of detection of the odours as proposed by Pollien *et al*., (1997b) and van Ruth *et al*. (1996a,b,c) but not using the estimation of their actual intensities as originally planned. This limitation of the OSME data analysis could be explained by the conjunction of a large discrepancy observed in the number and quality of the substances detected between panelists, as observed by the same authors in previous papers (da Silva *et al*., 1994; McDaniel *et al*., 1989), associated with a reduced number of panelists evaluating the wine extracts.

Etiévant *et al*. (1999) reported a cross-modality matching method with the finger span. They described a prototype for the precise measurement and acquisition of the distance between the thumb and another finger during analysis. Their four-member panel was able to determine most characteristics of the solutions with reference compounds and to create a finger span multidimensional space highly correlated with the theorical intensity space. However, they also showed relatively poor individual performance by the assessors and they recommended the use of several individuals to perform this type of analysis.

Currently, the time-intensity methods have not been very frequently used for GC-O. Methodological aspects should receive more attention before the value of this technique can be fully evaluated. For instance, it is unknown how reproducible the technique is and how parameters of time-intensity generally relate to physical concentration and to posterior intensity measurements (van Ruth, 2001).

The authors Acree and Barnard (1994) and Marin *et al*. (1988) showed a considerable variance in Charm values for individual sniffers as well as between sniffers. Similar results have been published by Etiévant *et al*. (1999) for time-intensity methods. It can be concluded from the studies of these various authors, that a group of sniffers is a prerequisite for reliable GC-O analysis.

Another time-intensity method, GC-SNIF analysis or headspace-GC-sniffing, has been developed (Pollien *et al*., 1997). It is carried out by panels of eight (six as a minimum) to ten members. Only one concentration level is needed. As in CharmAnalysis™, the sniffer presses a button as long as he can perceive the odour of a GC effluent. The time-dependent signal is continuously recorded by the computer. The individual aromagrams (square signals) are

averaged. The mean aromagram is normalised and independent panels generate similar aromagrams. The height of a signal represents the number of sniffers who have detected an odour at the corresponding retention time, not the odour intensity. The peak height is called NIF (nasal impact frequency) and the peak area is SNIF (surface of nasal impact frequency). The method does not require trained sniffers or dilutions, and is therefore quicker and easier than the dilution methods or OSME analysis (Chaintreau, 2001).

c) Detection frequency methods

In detection frequency methods the number of sniffers detecting an odour-active compound at the sniffing port simultaneously (the frequency of detection) is used as a measure for the intensity of a compound. The method proposed by Linssen *et al*. (1993) uses a group of sniffers instead of one or two sniffers. Detection frequency methods overcome the limitations of a small number of sniffers and the use of detection thresholds. Significant correlations have been established between the number of sniffers perceiving odour-active compounds and intensity scores of attributes in sensory analysis (van Ruth and Roozen, 1994; van Ruth *et al*., 1995). Furthermore, the number of sniffers perceiving odour-active compounds was shown to relate very well to the intensity of an odour-active compound, recorded after elution from the column. Despite good correlations between the number of sniffers and intensities at the sniffing port and intensities of sensory attributes for a number of compounds, the fact that the method is not based on real intensities is a drawback (van Ruth, 2001).

The authors Priser (1997) and Priser *et al.* (1997) compared the three techniques, CharmAnalysis™, OSME and detection frequency method, on Champagne wines and concluded that the key compounds contributing mainly to the flavour were identical whatever the method considered. However, the detection frequency method seemed to be a better way to determine key compounds in a minimum of time because it does not require a trained panel. Only one injection by panelist is needed, and the great number of panelists limits the problem of anosmia. However, the detection frequency method needs a group of sniffers (6 to 12) and it just supplies the information regarding the presence or not of a compound present in the aroma extract detected by most of the sniffers, supposedly because the higher the frequency of detection of a compound, the higher its contribution to the aroma of the beverage or food will be (van Ruth and O' Connor, 2001).

d) Posterior intensity methods

Other GC-O methods were developed for intensity evaluation, namely posterior intensity methods which measure the odour intensity of a compound in the GC effluent (Petersen *et al.,* 1998; Tønder *et al*., 1998). The posterior intensity method was used to evaluate the flavour of

Cheddar cheese aroma (Arora *et al*., 1995), the aroma of raw and boiled potatoes (Peterson *et al*., 1998) and the aroma of orange juice (Tønder *et al*., 1998).

The posterior intensity method is quite similar to the OSME method, except that the perceived odour intensity of each odourant compound is rated in a memorised five-point intensity interval scale after a peak has eluted from the olfactory detection port (ODP).

van Ruth (2001) showed that data resulting from the posterior intensity method correlated reasonably well with those of the detection frequency method (r = 0.822). Furthermore, lower correlation coefficients were obtained for posterior intensity and dilution analyses (r = 0.667). Comparing the detection frequency method with the posterior intensity method highlights an evident limitation of the second one, in that it implicates the use of a trained group of sniffers while the first does not involve any previous training.

In comparison with other GC-O methods using dilution techniques, the posterior intensity method is more advantageous because, within the same time span, it is possible to use more sniffers and thereby obtain more representative results that can be evaluated by standard statistical techniques (Tønder *et al*., 1998).

1.5. GAS CHROMATOGRAPHY-OLFACTOMETRY (GC-O) APPLICATION IN WINES AND MUSTS

This section provides a discussion of the major odour-active compounds involved in wine flavour and an overview of the aroma profile of musts and wines from grape varieties *(Vitis vinifera* L.*)* studied around the world. The knowledge of wine flavour has paralleled developments in analytical chemistry. In the nineteenth century, analytical methods focused on the determination of major wine components such as ethanol, organic acids, and sugars. The development of chromatographic techniques in the early 1900s and the particular development of gas chromatography in the early 1950s ushered in a new era of discovery. Currently, more than 1300 volatile compounds have been identified in alcoholic beverages (Ebeler, 2001).

Several papers report the overall identification of odour-active compounds in white and red wines and musts. Gas chromatography-olfactometry (GC-O) technique strongly contributed to most of publications. In fact, using quantitative GC-O made it possible to find out key differences in the odour profiles of three monovarietal young red wines (López *et al*., 1999), in different Spanish aged red wines (Ferreira *et al*., 2001), and in four Madeira wines from Malvazia, Boal, Verdelho and Sercial cultivars (Campo *et al*., 2006). The studies focusing on Chardonnay wines (Moio *et al*., 1994); on white Riesling and some hybrids (Chisholm *et al*., 1994); on aged Vidal blanc (Chisholm *et al*., 1995); on Gewürztraminer (Guth, 1997a,b; Ong and Acree, 1999); on Schreube (Guth, 1997a,b); on Pinot Noir (Moio and Etiévant, 1995); on Grenache wines (Ferreira *et al*., 1998, López *et al*., 1999); on Tempranillo wines (Ferreira *et al*., 1998); on young Cabernet Sauvignon and Merlot juices and wines (López *et al*., 1999; Ferreira *et al*., 2000; Kotseridis and Baumes, 2000); on six Premium Quality Spanish aged red wines (Culleré *et al*., 2004) and on Touriga Nacional clonal wines (Falco, 2004) should be pointed out.

1.5.1. Odour-active compounds in grapes and wines

Several reviews provide detailed information on the chemical components involved in wine aroma and flavour (Nykänen, 1986; Rapp, 1988; Rapp and Pretorius, 1990; Cole and Noble, 1994; Noble, 1994; Waterhouse and Ebeler, 1998; Ebeler, 2001). The main chemical families of odourant compounds, its origin and its contribution to the aroma of wines are described below.

Terpenoid compounds

Terpenoids constitute the largest family of natural plant products with over 30,000 members (Dewick, 2002). Terpenoids are classified by the homologous series of a number of five carbon isoprene units in their structure: hemiterpenes C_5 (1 isoprene unit), monoterpenes C_{10} (2 isoprene units), sesquiterpenes C_{15} (3 isoprene units), diterpenes C_{20} (4 isoprene units), triterpenes C_{30} (6 isoprene units), tetraterpenes C_{40} (8 isoprene units), polyterpenes $(C_5)n$ where

"n" may be 9-30,000 (McGarvey and Croteau, 1995). Terpenoid biosynthetic pathway is schematised in Figure I.9.

Accordingly, the first step originates mevalonic acid from glucose by the acetyl coenzyme A (CoA). This main pathway is generally recognised although another seems to exist through the intermediary of amino acids such as leucine or valine. The second step produces isopentenyl pyrophosphate (IPP) from mevalonic acid. Throughout the enzymes isopentenyl pyrophosphate isomerase, IPP is isomerised into dimethylallyl pyrophosphate (DMAPP). These two isoprenic units play an active role in terpenoid synthesis. One IPP unit condenses with a DMAPP molecule by prenyl transferase (head-tail condensation of the two molecules) to produce a C_{10} molecule, geranylpyrophosphate (GPP), which constitutes an important junction in terpenoid synthesis. From this compound, the biosynthetic pathways can originate either acyclic or cyclic monoterpenoids or more condensed terpenes (McGarvey and Croteau, 1995; Luthra *et al*., 1999).

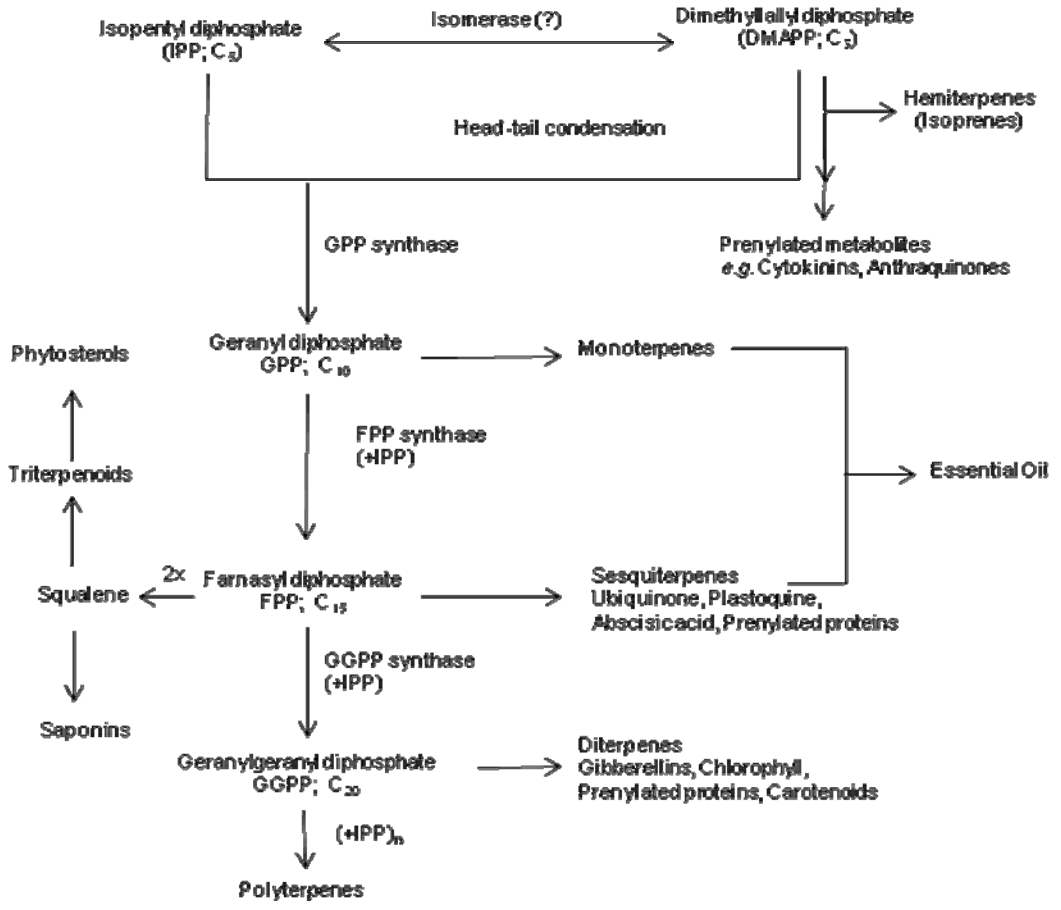


Fig. I.9 - Synthesis of various classes of terpenoids in plants (Dubey *et al*., 2003). The question mark (?) indicates the controversial role of isomerase via non-MVA route in which both IPP and DMAPP are reported to be synthesised independently.

Monoterpenoids, particularly linalool, geraniol, and nerol are responsible for the characteristic floral aroma in grapes and wines of the *Vitis vinifera* L. cultivars Muscat, Gewürztraminer, and Riesling (Marais, 1983). A significant portion (~90%) of the terpenes is present as non-volatile glycosides that can be hydrolysed (enzymatically or chemically) to the free form during fermentation and ageing. Free monoterpene concentrations in grape berries generally range from 0 to <1000 µg/kg (Marais, 1983; Wilson *et al*., 1984, 1986).

The diglycosides are composed of a glucose molecule associated with another sugar, *i.e.*, rhamnose, arabinose or apiose. The hydrolysis of these heterosides requires two sequential enzymatic activities. A α-L-rhamnosidase, a α-L-arabinosidase or a β-D-apiosidase must act on the molecule before the β-D-glucosidase is able to exert its action (Figure I.10).

In practice, this hydrolysis is relatively limited. Grape glucosidases have an optimal activity at a pH between 5 and 6, and they only retain part of this activity at the pH of must. These glucosidases are very specific and are not active in certain terpenic heterosides, notably tertiary alcohol derivates. Moreover, β-glucosidase is strongly inhibited by free glucose (Bayonove, 1993).

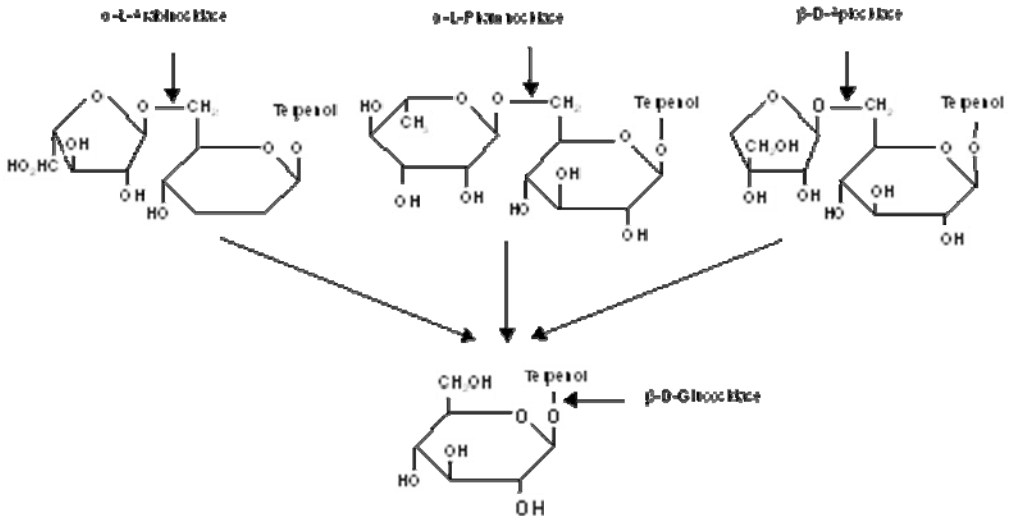


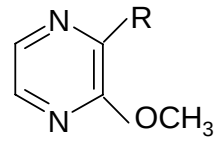
Fig. I.10 – Hydrolysis mechanism of the terpenic glycosides (adapted from Gunata *et al.*, 1990).

Acid-catalysed rearrangements during wine processing and ageing can result in changes in concentration and formation of new compounds that were not present in the original grapes and young wines, *e.g.*, transformation of linalool to α-terpineol, hydroxyl linalool, geraniol, and nerol (Rapp *et al*., 1985).

Pyrazines

Vegetative aroma, characteristic of *Vitis vinifera* L. cultivars, Sauvignon blanc and Cabernet Sauvignon, are generally attributed to the compound 2-methoxy-3-isobutylpyrazine (MIBP). MIBP concentrations in wines are generally present in concentrations of less than < 40 $ng.L^{-1}$;

however, these concentrations are frequently above the odour detection threshold of 2 ng.L^{-1} in water (Buttery *et al*., 1969). Cooler climates and low levels of light exposure in the vine canopy during grape maturation contribute to high levels of MIBP, resulting in *vegetative* aroma in the grapes and in the wines made from these grapes (Heymann and Noble, 1987; Lacey *et al*., 1991). Furthermore, under comparable climatic conditions in the Bordeaux vineyards (Bourbée *et al*., 2000), the MIBP contents of the grapes at véraison and variations during ripening are strongly influenced by the environmental and cultural conditions (type of soil, pruning, training system and density of plantation). Unriped grapes contain a high concentration of methoxypyrazines (a few dozen nanograms per liter) in certain varieties, such as Cabernet Sauvignon (Figure I.11). The concentrations of these compounds drop significantly in the course of grape maturation. Using a sample of 50 red wines (Bordeaux and Loire) from different vintages, made of Cabernet Sauvignon, Cabernet franc and Merlot grapes, Bourbée *et al*. (2000) showed that these wines generally had a marked *green bell pepper* character with an MIBP content of 15 ng.L^{-1}.



R: $CH_2CH(CH_3)_2$ 2-methoxy-3-isobutylpyrazine
R: $CH(CH_3)_2$ 2-methoxy-3-isopropylpyrazine
R: $CH(CH3)CH_2CH_3$ 2-methoxy-3-secbutylpyrazine

Fig. I.11 – Chemical structures of some pyrazines found in grapes.

C_{13}-Norisoprenoids

C_{13}-norisoprenoids are part of another group of important derived compounds present in grapes that arise from carotenoid degradation by enzymatic or chemical pathways (Strauss *et al*., 1987; Winterhalter *et al*., 1990b; Bayonove, 1993; Sefton *et al*., 1993; Masson *et al*., 1996, 1997b). The carotenoid concentrations in grape berries vary from 15 to nearly 2500 µg.kg^{-1} in fresh weight (Razungles, 1985). These substances share the same origin as terpenoids but have a higher molecular weight (Ribéreau-Gayon *et al*., 2000). The most important, in decreasing order, are: lutein, β-carotene, neoxanthyn, and lutein-5,6-epoxyde. These molecules, generally enclosed in cellular organites, are essentially located in the solid parts of the berries: the skin is two to three times richer in carotenoids than the pulp. During maturation, a decrease in the carotenoid concentration and an increase in certain carotenoid-derived molecules such as norisoprenoids are observed. Like the monoterpenes, also norisoprenoids occur in grapes and wines predominately as glycosidically-bound precursors. Megastigmane forms (benzene circle substituted on carbons 1, 5 and 6, and an unsatured aliphatic chain with four carbon atoms

attached to C_6) of these norisoprenoid derivates include compounds like β-damascenone and β-ionone (Figure I.12).

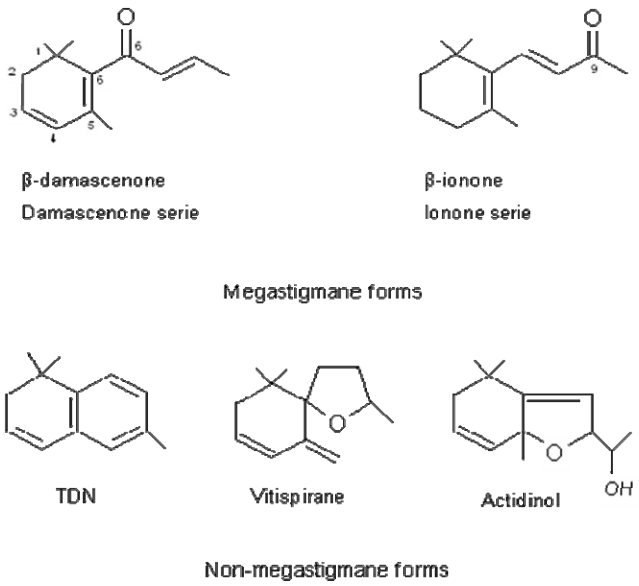
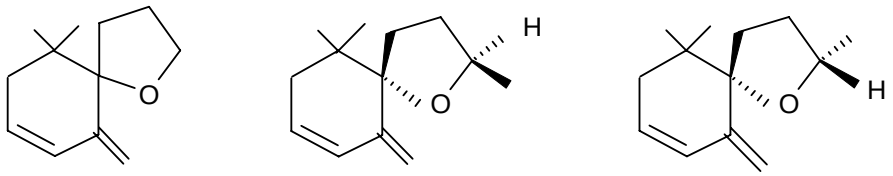


Fig. I.12 - Main families of C_{13}-norisoprenoids derivates in grapes.

Both compounds, together with vitispirane and α-ionone, contribute to the increasing of wine aroma complexity with notes of *tea*, *honey*, *pineapple* and *violet flowers* (Schreier *et al*., 1976; Razungles *et al*., 1988; Sefton *et al*., 1989; Winterhalter *et al*., 1990b; Winterhalter, 1991). Vitispirane has been detected in Riesling wines (Noble *et al*., 1980; Winterhalter *et al*., 1990a,b; Waldmann and Winterhalter, 1992), in Chardonnay grapes (Sefton *et al*., 1993), and in wines made from Merlot and Cabernet Sauvignon (López *et al.*, 1999). However, the amounts found in wines are substantially below the olfactive perception threshold of vitispirane (Simpson, 1978).

In fact, the C_{13}-norisoprenoids contribute to complex aroma, including *grassy*, *tea*, *lime*, *honey*, and *pineapple* of many red and white varieties of *Vitis vinifera*, including Chardonnay, Chenin blanc, Semillon, Sauvignon blanc, Cabernet Sauvignon, and Syrah (Sefton *et al*., 1989; Francis *et al*., 1992; Marais *et al*., 1992a,b,c,d; Sefton *et al*., 1993; Razungles *et al*., 1993; Sefton *et al*., 1994).

Vitispirane and TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) both contribute to the bottle-ageing character of some floral wine varieties, including Riesling (Winterhalter *et al*., 1990a,b). Vitispirane was detected in wines from Riesling (Noble *et al.*, 1980; Winterhalter *et al.*, 1990a,b; Waldmann and Winterhalter, 1992), in grapes from Chardonnay (Sefton *et al.*, 1993) and in red wines from Merlot and Cabernet Sauvignon (López *et al.*, 1999). This volatile compound has two diastereoisomers (Fig. I.13). The (6*S*,9*S*) diastereoisomer is unmistakable different from the (6*R*,9*R*) and has a green odour and a flowery-fruity note (Humpf *et al.*, 1992).



Vitispirane (6*S*,9*S*) (6*S*,9*R*)

Fig. I.13 – Vitispirane and respective diastereoisomers.

The (*6S,9R*) diastereoisomer has been characterised by a heavy scent of exotic flowers with an earthy-woody odour description (Humpf *et al.*, 1992). Vitispirane is considered to be derived from the carotenoid, neoxanthine and has two odourless precursors (Waldmann and Winterhalter, 1992), megastigm-4-en-3,6,9-triol-3-O-β-D-glucopyranoside and megastigm-4-en-3,6,9-triol-9-O-β-D-glucopyranoside; both are stored in the grape (Figure I.14).

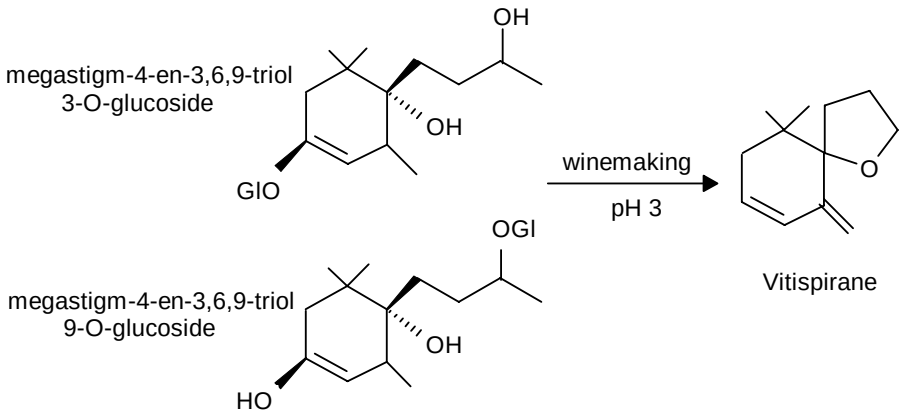


Fig. I.14 – Proposed formation of vitispirane from grape precursors (adapted from Waldmann and Winterhalter, 1992).

TDN occurs in grapes as non-volatile precursors (Versini *et al*., 1996; Winterhalter, 1991; Winterhalter *et al*., 1990a,b) and it is liberated by acid-catalysed hydrolysis in wine during ageing. It has a threshold value of 20 ppb in wine (Simpson, 1978). When present at too high intensities, the *kerosene-like* aroma characteristic of TDN becomes a negative quality of Riesling wine aroma, a phenomenon often observed in warm climate wine-producing countries (Marais, 2002).

β-ionone with an aroma descriptor of *violet* has a low perception threshold and can participate in the wine aroma. This compound can be formed directly by β-carotene degradation (Kanasawud and Crouzet, 1990) or by its sugar precursor hydrolysis (Kotseridis, 1999).

Among C_{13}-norisoprenoids, β-damascenone (1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one) is one of the most important compounds with a low perception olfaction threshold. The origin of this compound is not well established; there are two main possible pathways

(Figure I.15), via acid hydrolysis (Skouroumounis *et al*., 1992) and direct degradation of neoxanthyn (Sefton *et al*., 1993; Skouroumounis *et al*., 1995; Skouroumounis and Sefton, 2002). β-Damascenone is nowadays recognised as an odour-active compound found in grapes and wines. In fact, it was found by GC-O analysis in Cabernet Sauvignon wines (Kotseridis and Baumes, 2000), in red wines from Rioja (Aznar *et al*., 2001). Ferreira *et al*. (2002) attributed the second place in importance to β-Damascenone in the key-odourants of wines from Grenache, using AEDA.

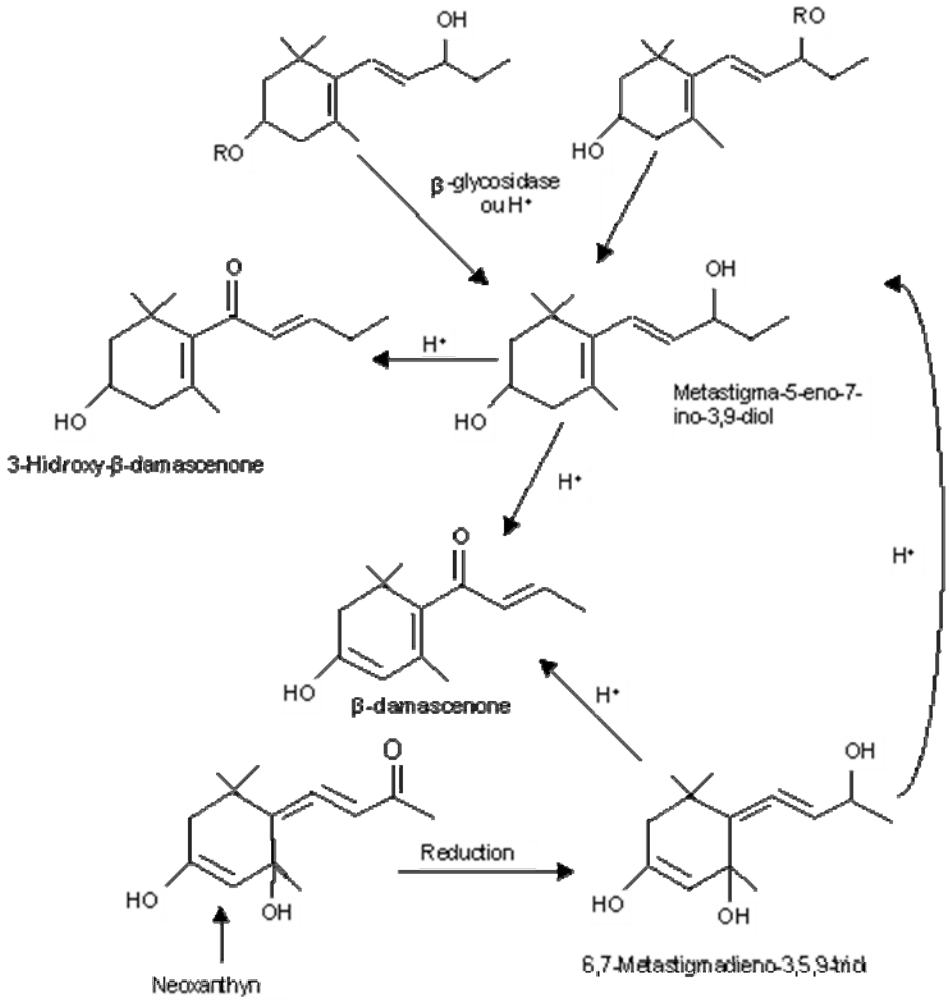


Fig. I.15 - Pathways of β-damascenone in grapes and wine (R = β-D-Glc) (adapted from Skouroumounis *et al*., 1992, Sefton *et al*., 1993; Skouroumounis *et al*., 1995; Skouroumounis and Sefton, 2002).

Lactones

The lactones, just as other chemical groups, can have three possible origins: grapes, the fermentation process or formation during wine ageing. There are several volatile lactones which can contribute for the aroma of wines (Figure I.16).

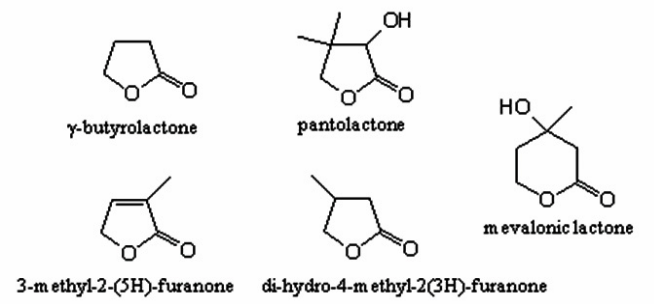


Fig. I.16 - Some lactones identified in wines (adapted from Ribéreau-Gayon *et al.*, 1998).

The best well-known lactone is the γ-butirolactone that results from the lactonization of the γ-hydroxybutiric acid, an unstable molecule that derives from the glutamic acid by deamination and decarbonication, according to the Ehrlich reaction (Figure I.17).

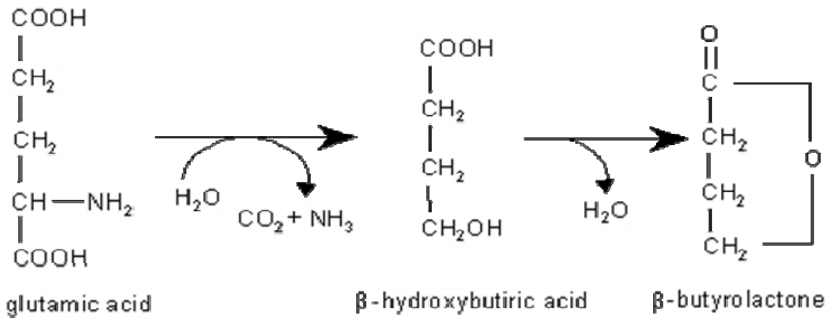


Fig. I.17 - γ-Butyrolactone biosyntesis (adapted from Ribéreau-Gayon *et al.*, 1998).

The 3a,4,5,7a-tetrahydro-3,6-dimethylbenzofuran-2(3H)-one, known as wine lactone, has been identified as an important odourant of the Scheurebe and Gewürztraminer wines (Guth, 1997a,b). This volatile compound has an aroma described as *coconut-like*, *woody* and *sweet*. Winterhalter *et al*. (1998) have suggested that a monoterpenoid acid precursor isolated from Riesling wines, the (*E*)-2,6-dimethyl-6-hydroxyocta-2,7-dienoic acid 7-O-glucopyranoside (Figure I.18) is converted to wine lactone at typical wine pH (pH 3.2).

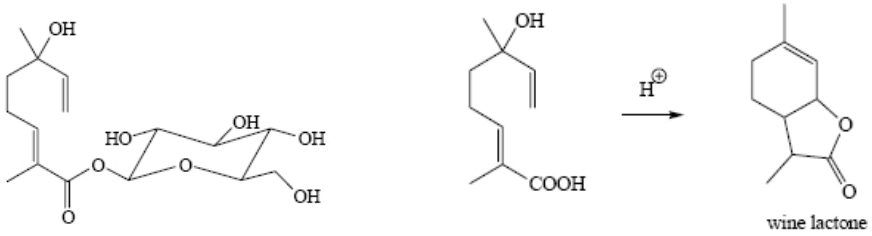


Fig. I.18 – Possible wine lactone formation pathway (adapted from Winterhalter *et al*., 1998).

Several aroma compounds are transferred from the wood to the wine during fermentation and storage in oak barrels. One of the most important is β-methyl-γ-octalactone, commonly known as oak or whiskey lactone. The β-methyl-γ-octalactone is characterised by a particular aroma of *coconut* and *oak wood* (Reazin, 1981; Boidron *et al*., 1988; Clímaco *et al*., 1988; Chatonnet *et al*., 1990; Abbot *et al*., 1995; Chatonnet, 1995; Singleton, 1995; Clímaco and Borralho, 1996;

Masson *et al*., 1997a; Garde Cerdán *et al*., 2002). According to Masuda and Nishimura (1971) and Masson *et al.* (1995, 1997b), from the four possible esterioisomers of β-methyl-γ-octalactone, only two were found in oak wood (Figure I.19).

3*S*,4*R trans* β-methyl-γ-octalactone 3*S*,4*S cis* β-methyl-γ-octalactone

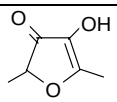
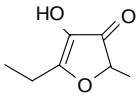
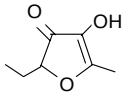
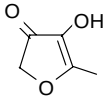
Fig. I.19 – Chemical structure of the esterioisomers of β-methyl-γ-octalactone of oak wood (adapted from Masson *et al*., 1996).

Both esterioisomers have a *woody*, *oaky*, *coconut-like* aroma; however, the aroma threshold for the *cis* isomer has been observed at 92 ppb, compared to 460 ppb for the *trans* isomer (Waterhouse and Towey, 1994).

Furanones

3(2*H*)-Furanones are important compounds contributing to the flavour of many natural products. 4-hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol™, 1), 2(or 5)-ethyl-4-hydroxy-5(or 2)-methyl-3(2H)-furanone (homofuraneol, 2), and 4-hydroxy-5-methyl-3(2H)-furanone (norfuraneol, 3) have been identified in wines (Table I.5) as odour-active compounds (Guth, 1997a; Sarrazin *et al*., 2007). They have been described as important contributors to wine aroma due to their low detection threshold and they have *caramel-like*, *burnt sugar* and *candy-cotton* odour descriptors.

Table I.5 – Main 3(2H)-furanones found in wines (adapted from Fay *et al*., 1997).

Compound number	Compound name	Compound structure	Main daughter ions of the molecular ion m/z (relative intensity)
1	Furaneol™		128(60), 110(25), 100(5), 85(100), 72(60), 57(10), 43(15)
2A	Homofuraneol A		142(10), 127(2), 114(1), 99(1), 86(1), 85(<1), 72(<1), 71(1), 57(100), 43(1)
2B	Homofuraneol B		142(70), 127(100), 114(35), 99(55), 86(10), 85(<1), 72(10), 71(15), 57(50), 43(10)
3	Norfuraneol		114(20), 96(<1), 85(<1), 71(1), 58(35), 57(<1), 43(100)

Alkylated 4-hydroxy-3(2H)-furanones exist in the tautomeric forms I and II (Figure I.20). The tautomers of homofuraneol can be separated by GC on polar stationary phases (Blank and Fay, 1996; Blank *et al*., 1997). In contrast, Furaneol™ tautomers cannot be distinguished due to the symmetry of the molecule (Fay *et al*., 1997).

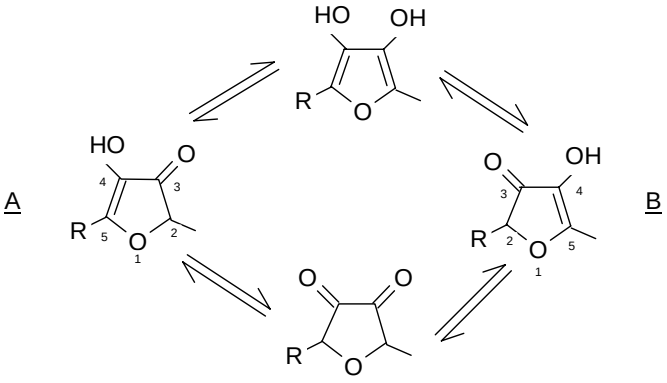


Fig. I.20 – Tautomerization of Alkylated 4-Hydroxy-3(2*H*)-furanones resulting in the Tautomeric forms A and B (R = CH_3 for Furaneol™ and R = C_2H_5 for homofuraneol) (Fay *et al*., 1997).

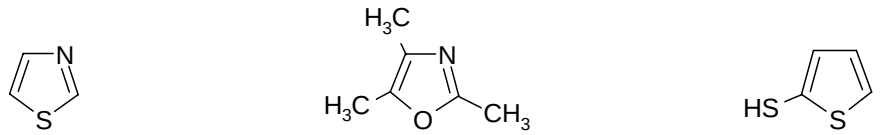
The 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon) has been described as a very potent odourant, with a limiar of perception of 0.02 $ng.L^{-1}$ in air (Blank *et al*., 1996) and 5 $\mu g.L^{-1}$ in wine (Guth *et al*., 1997b). At low levels of concentration, it possesses a *nut* odour descriptor while in high concentration levels it presents a *curry* odour descriptor. *Curry* notes in Porto and *sweet* Grenache fortified wines were attributed to sotolon (Silva Ferreira, 1998; Schneider *et al*., 1998; Cutzach *et al*., 1998a).

Sulfur volatile compounds

Sulfur derivates represent an important family of volatile compounds in wines which have only been highlighted recently due to the low concentrations found. Their role is a paradoxical one in that they can be responsible for organoleptic defects or contribute to the typical characteristics of the varietal aroma of wines.

Sulphur compounds that are responsible for unpleasant odours in wines are sulphides, thiols (or mercaptans, named after their characteristic of being captured or trapped by mercury), thiophenes and thiazoles. The thiols form stable complexes with copper, whereas the sulphides do not react with this metal during the vinification of wines. Most of the volatile sulphur compounds which have been identified in wines belong to one of the two classes, thiols or sulphides (Darriet *et al*., 1999). Ocasionally, some volatile sulphur compounds may belong to other chemical classes: sulphur dioxide (sulfonic acid class), dimethylsulfoxide (sulfoxide class), benzothiazole (thiazole class), and 2-methyl-tetrahydrothiophene (thiophene class). Each one gives its own unique odour to the wine aroma. For example, according to Marchand *et al*. (2000), thiazole contributes with *popcorn* or *peanut* odour, trimethyloxazole contributes with *ripe fruit* odour, thiophene-2-thiol with a *burnt* odour (Figure I.21).

Two groups have been distinguished on the basis of their volatility: the groups of "light", highly-volatile sulphur compounds (boiling points below 90ºC) and the group of "heavy" sulphur compounds of limited volatility (boiling points above 90ºC). The "light" sulphur compounds were long considered solely responsible for reduction defects.



Thiazole Trimethyloxanole Thiophene-2-thiol

Fig. I.21 – Some examples of volatile sulphur compounds: thiazole, trimethyloxanole, thiophene-2-thiol.

They evoke odours which are particularly unpleasant, as *rotten eggs* or *garlic* (Table I.6) and they depreciate the wine aroma even at low concentration levels (around a microgram per liter).

Table I.6 – Some "light" sulphur compounds contributing to defects in wines (adapted from Darriet *et al*., 1991; Etiévant, 1991).

Compounds	Olfactory perception threshold ($\mu g.L^{-1}$)	Descriptors	Normal wine concentrations ($\mu g.L^{-1}$)	"Reduced" wine concentrations ($\mu g.L^{-1}$)	Boiling point (ºC)
carbonyl sulphide[a]	-----	ethereal	0.7	0.4	-50
hydrogen sulphide	0.8	rotten egg	0.3	16.3	-61
methanethiol	0.3	stagnant water	0.7	5.1	6
ethanethiol	0.1	onion	0.0	10.8	35
dimethyl sulphide	5.0	quince, truffe	1.4	2.0	35
carbon dioxide	----	rubber	1.7	2.4	46

[a] determined by ratio peak surface to peak of internal standard

Among the "heavy" sulphur compounds identified in wines, which are included in Table I.7, only a few play a significant role in reduction defects.

Table I.7. – Some "heavy" sulphur compounds contributing to defects in wines (adapted from Darriet *et al*., 1991; Etiévant, 1991).

Compounds	Olfactory perception threshold ($\mu g.L^{-1}$)	Descriptors	Normal wine concentrations ($\mu g.L^{-1}$)	"Reduced" wine concentrations ($\mu g.L^{-1}$)
dimethyl disulphide	2.5	quince, asparagus	0.0	2.0
2-mercaptoethanol	130.0	burnt rubber	72.0	124
2-methyl-tetrahylthiophenone	90.0	"gas"	68.0	276.0
2-methylthio-ethanol	250.0	cauliflower	56.0	80.0
ethyl methionate	300.0	metallic	1.0	2.0
methionyl acetate	50.0	mushroom	1.5	3.0
methionol	1200.0	cabbage	838.0	1776.0
4-methylthio-butanol	80.0	earthy	36.0	35.0
benzothiazole	50.0	caiutchouc	2.0	11.0

Methionol (3-methylthio-1-propanol) is a "heavy" sulphur compound which is formed by yeast from methionine which undergoes successive deamination and decarboxylation (Ehrlich reaction), producing methional and then methionol (Darriet *et al*., 1999).

4-Mercapto-4-methyl-pentan-2-one (4 MMP) contributes to a typical *black currant* note to Scheurebe wines (Guth, 1998). According to the author, it has been found in concentrations up to 0.40 $\mu g.L^{-1}$ in this variety with an estimated aroma threshold of approximately 0.6 $ng.L^{-1}$. This compound has also been identified in Sauvignon blanc, Merlot, Cabernet Sauvignon and Cabernet Franc wines (Darriet *et al*., 1999). The compound 4 MMP is thought to be

enzymatically released from the bound precursor, S-(4-methylpentan-2-one)-1-cysteine, by the enzyme cysteine β-liase during winemaking processes (Tominaga *et al*., 1995). Different thiols (Figure I.22) were described as character impact odourants of Sauvignon blanc wines (Darriet *et al*., 1995; Tominaga *et al*., 1996, 1998a) and Scheurebe wines (Guth, 1997a,b). For example, 3-mercaptohexyl-acetate has been identified in Sauvignon wines, giving a predominant *box-tree* odour at low concentrations, and a *fruit zest* and *passion fruit* notes at high concentrations (Darriet *et al*., 1999). The *exotic fruits* notes in Cabernet Sauvignon and Merlot wines have been attributed to some thiols (Bouchilloux *et al*., 1998a).

It is now well established that 3-mercapto-hexan-1-ol, 4-methyl-4-mercaptopentan-2-one and 4-mercaptopentan-2-ol exist in must in the form of S-conjugates with cysteine: S-3-(hexan-1-ol)-cysteine, S-4-(4-methylpentan-2-one)-cysteine, S-4-(4-methylpentan-2-ol)-cysteine and S-3-(hexan-1-ol)-cysteine (Tominaga *et al.*, 1996, 1998c).

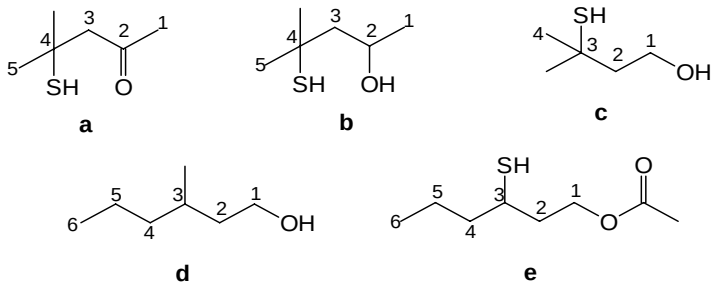


Fig. I.22 - Volatile thiols identified in Sauvignon wines **a**: 4-mercapto-4-methylpentan-2-one (4 MMP); **b**: 4-mercapto-4-methylpentan-2-ol (4 MMPOH); **c**: 3-mercapto-3-methylbutan-1-ol (3 MMB); **d**: 3-mercapto-hexan-1-ol (3 MH); **e**: 3-mercapto-hexanol acetate (A 3MH) (adapted from Ribéreau-Gayon *et al.*, 1998).

These compounds are present in musts in much higher quantities than the aroma compounds they generate in wines. The corresponding compounds are revealed during alcoholic fermentation, through the action of a specific β-lyase exemplified in Figure I.23.

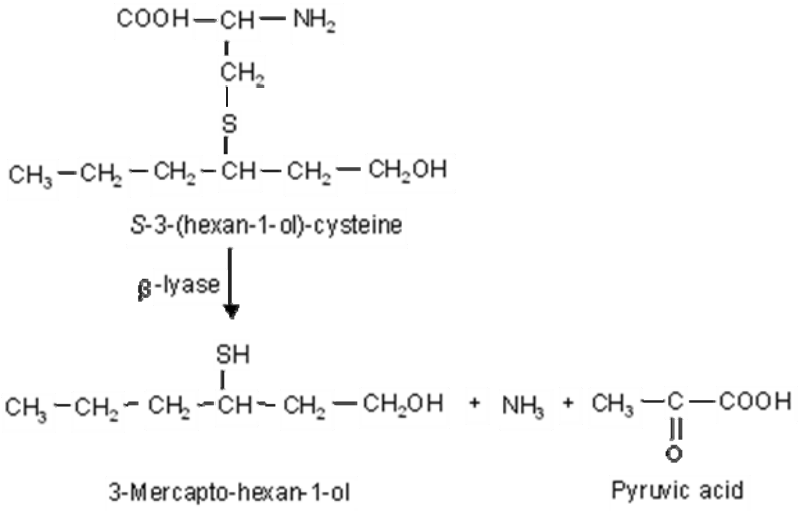


Fig. I.23 - Form of 3-mercapto-hexan-1-ol S-conjugates with cysteine and its appearance by the action of the specific β-lyase (Ribéreau-Gayon *et al*., 1998).

Volatile phenols

The volatile phenols can be generated from grapes, from the metabolic activity of yeasts and lactic bacteria and from the wood of barrels where the wine is stored. Although volatile phenols can contribute positively to the aroma of some wines, they are better known for their contribution to off-flavours such *baryard* or *stable*, which results from high concentrations of ethyl-phenols (Dubois, 1983). Trace amounts of volatile phenols are present in grape must, but they are predominantly produced by yeast during fermentation (Baumes *et al*., 1988). The nonflavonoid hydroxynnamic acids, such as *p*-coumaric acid and ferulic acid (Figure I.24), are decarboxylated in a non-oxidative process by *Saccharomyces cerevisiae* to form the volatile phenols 4-vinylguaiacol and 4-vinylphenol, respectively (Chatonnet *et al*., 1993). The *Brettanomyces/Dekkera* spp. yeasts are well-known for their ability to form volatile phenols in wine (Chatonnet *et al*., 1992; Chatonnet *et al.*, 1995; Licker, 1998; Licker, 1999; du Toit and Pretorius, 2000). These yeasts are associated with the more unpleasant odourous ethylphenols, and are therefore regarded as spoilage organisms resulting in aroma described as *medicinal*, *pharmaceutical*, *barnyard-like*, *horsey*, *sweaty*, *leathery*, *mouse urine*, *wet dog*, *smoky*, *spicy* and *rancid* (Chatonnet *et al*., 1995).

Phenolic acids can also be decarboxylated into volatile phenols, usually first into 4-vinyl derivates and then reduced to 4-ethyl derivates through enzymes called phenolic acid decarboxylases (Cavin *et al*., 1993). Several bacteria and fungi, such as *Bacillus pumilus*, *Lactobacillus plantarum*, *Bacillus subtilis*, *Pediococcus pentosaceus*, have been found to contain the genes encoding phenolic acid decarboxylases (Clausen *et al*., 1994; Zago *et al*., 1995; Cavin *et al*., 1997; Cavin *et al.*, 1998; Barthelmebs *et al*., 2000).

In addition to the metabolic activity of yeast and bacteria, other factors such as oak maturation can also increase the amount of volatile phenols in wine (Pollnitz *et al.*, 2000).

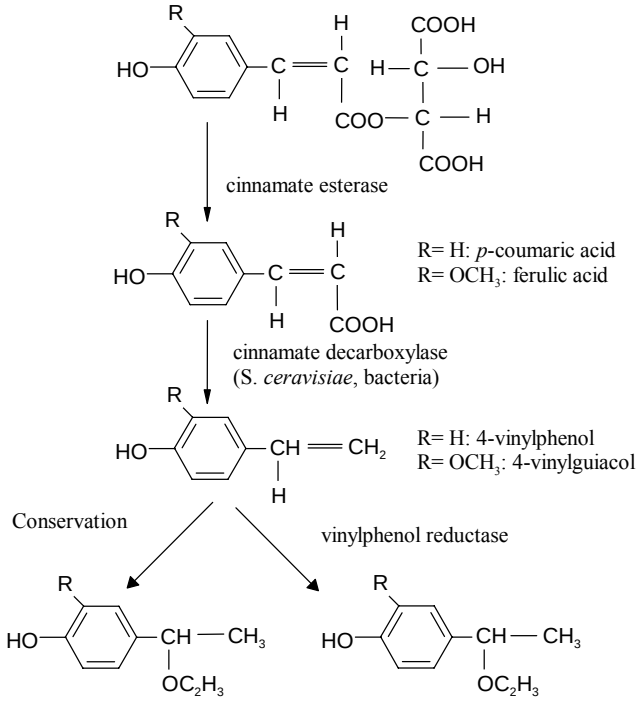


Fig. I.24 – Volatile phenols pathway during vinification and conservation of wines (adapted from Bayonove *et al.,* 1998).

In Table I.8 the main volatile phenols found in wine are presented. Ferreira *et al*. (1998) found that guaiacol, eugenol and 4-vinylguaiacol may play a role in the *liquorice* and *phenolic* notes of the red wines from Grenache, even if not aged in wood.

Table I.8 – Chemical structure of the main volatile phenols found in wine aroma analysis (adapted from Chatonnet *et al*., 1989).

Common name	IUPAC name	R_1	R_2	R_3	R_4	Structure
guaiacol	2-methoxyphenol	OCH_3	H	H	H	
4-methylguaiacol	2-methoxy-4-methylphenol	OCH_3	H	CH_3	H	
4-ethylguaiacol	4-ethyl-2-methoxyphenol	OCH_3	H	CH_2-CH_3	H	
4-propylguaiacol	2-methoxy-4-propylphenol	OCH_3	H	CH_2-CH_2-CH_3	H	
4-vinylguaiacol	2-methoxy-4-vinylphenol	OCH_3	H	CH=CH_2	H	
eugenol	2-methoxy-4-(2-propenyl)phenol or 4-allyl-2-methoxyphenol	OCH_3	H	CH_2-CH=CH_2	H	
(*E*)-isoeugenol	2-methoxy-4-(1-propenyl)phenol	OCH_3	H	CH=CH-CH_3	H	
syringol	2,6-dimethoxyphenol	OCH_3	H	H	OCH_3	

Culleré *et al*. (2004) found (*E*)-isoeugenol, eugenol, vanillin and guaiacol in a study with six Spanish aged red wines aged in wood, being these compounds of great capacity to differentiate those wines.

Furthermore, guaiacol, 4-ethylguaiacol, eugenol, 4-ethylphenol, 4-vinylguaiacol and syringol have been referred to in the literature as important odourants found in red wines (Ferreira *et al*., 2000, 2001; Kotseridis and Baumes, 2000). Guaiacol has been previously reported in flavour precursor fractions of Syrah grapes (Bureau *et al*., 2000), 2,6-dimethoxyphenol in flavour

precursor fractions of Merlot and Cabernet Sauvignon grapes (Francis *et al*., 1999) and 4-vinylphenol in precursors from Chardonnay juices (Sefton *et al*., 1993).

Higher alcohols and Esters

The higher alcohols identified in the wines are mainly of fermentative origin, resulting from the metabolic activity of the yeasts. The main alcohols quantified in wines are 2-methylpropanol, 2-methylbutanol and 3-methylbutanol. The last one is considered as a key-odourant by AEDA analysis in Grenache, Cabernet Sauvignon and Merlot wines (Ferreira *et al*., 1998; Kotseridis and Baumes, 2000). Higher alcohols could be formed during the fermentation by two different pathways: biosynthesis of aminoacids from sugars and degradation of aminoacids by the Ehrlich reaction (Figure I.25.).

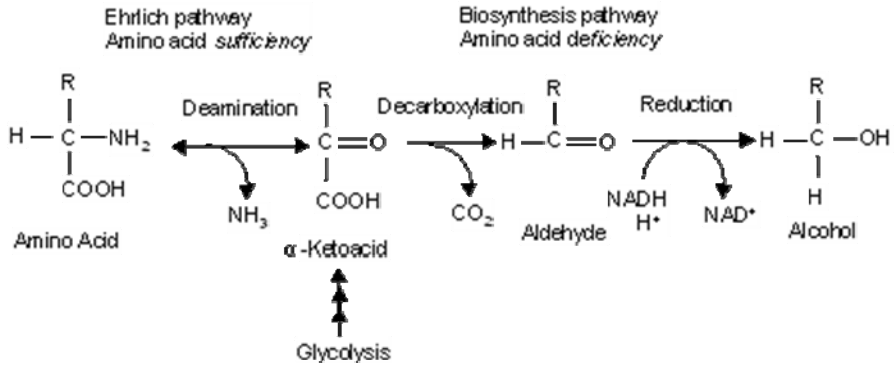


Fig. I.25. - Biosynthesis pathway of higher alcohols by Ehrlich reaction (Bell and Henschke, 2005).

It is assumed that during alcoholic fermentation these alcohols are formed from α-keto acids that come either from carbohydrate metabolism or by transamination of amino acids. When the intracellular pool of α-keto acids in yeast cells is too high, the excess of α-keto acids lead to higher alcohols fermentation (Bell and Henschke, 2005). In Figure I.26 some of the higher alcohols and esters found in wine are presented: 2-phenylethanol and isoamyl alcohol, ethyl esters of fatty acids (ethyl butanoate, ethyl hexanoate) and the acetates (isoamyl acetate, hexyl acetate).

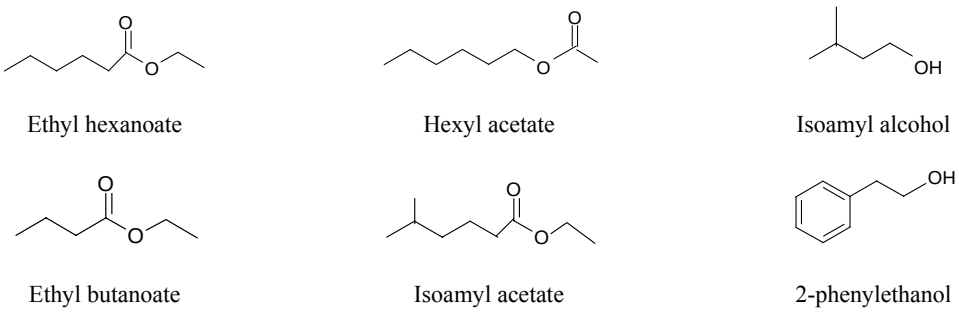


Fig. I.26 – Chemical structures of some fermentation-derivates commonly found in red wines.

2-Phenylethanol is a well known product of yeast metabolism formed during alcoholic fermentation of wines, also referred to as a component of Tempranillo and Grenache juice

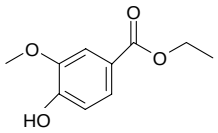
hydrolysates (López *et al*., 2004), being also detected in other type of wines by GC-O (Ferreira *et al*., 1998; Kotseridis and Baumes, 2000; Aznar *et al*., 2001).

Fatty acid ethyl esters like, ethyl butanoate, ethyl hexanoate and ethyl octanoate are synthesised by ethanolyses of acyl-CoA that is formed during fatty acid synthesis or degradation. Acetate esters, isoamyl acetate and hexyl acetate, are the result of the reaction of acetyl-CoA with higher alcohols that are formed from the degradation of amino acids and carbohydrates (Ribéreau-Gayon *et al.*, 1998). Although they can exist in small amounts in grapes and musts, they are formed by the metabolism of the yeasts (Maarse and Visscher, 1989). According to Soles *et al*. (1982), the esters production during alcoholic fermentation is influenced by several factors, such as the level of aeration, fermentation temperature, yeast strain, pH and sulphur dioxide content. Both groups of esters reach a maximum concentration during fermentation; hexyl acetate and the fatty acid ethyl esters at the midpoint and the higher alcohol acetates towards the end (Herraiz and Ough, 1993). Chemical esterification that could occur during storage and ageing might contribute to their formation (Ribéreau-Gayon *et al.*, 1998).

Ethyl 2,3-dihydrocinnamate, ethyl cinamate, methyl anthranilate, and ethyl anthranilate, identified in a Burgundy wine of *Vitis vinifera* L. cv. Pinot Noir as minor components by gas chromatography – mass spectrometry (GC-MS), were suspected of contributing to the typical aroma of Pinot Noir wines, according to the results of a GC-O analysis (Moio and Etiévant, 1995). Ethyl 2,3-dihydrocinnamate was described by Freitas *et al*. (1999) as a minor constituent of Porto wines and also as one of the volatile compounds responsible by "*esteva"* (*Cistus ladaniferus*) aroma descriptor. The ethyl cinamate is particularly abundant in wines resulting from carbonic maceration (Ducruet *et al*., 1983; Ducruet, 1984).

Ethyl vanillate (Table I.9), commonly associated with ageing in oak wood (Boidron *et al*., 1988), is also a derivate from glycosilated precursors present in grapes (Sefton *et al*., 1994; Jarauta *et al*., 2005), being found in Cabernet Sauvignon and Merlot wines (Kotseridis and Baumes, 2000), in Primitivo and Aglianico cultivars (Genovese *et al*., 2005), in wines from Riesling (Guntert *et al.*, 1986), in clonal red wines from Touriga Nacional (Falco, 2004), as well as in Spanish aged red wines from different regions (Aznar *et al*., 2001, 2003; Ferreira *et al*., 2001). In some of these studies the ethyl vanillate was also referred to as an odour-active compound in wines with *pollen* and *flowery* odour descriptors (Ferreira *et al*., 2001).

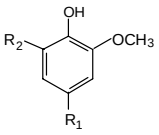
Table I.9 – Chemical structure of ethyl vanillate found in grapes and wines.

Common name	IUPAC name	Structure
ethyl vanillate	ethyl 4-hydroxy-3-methoxybenzoate	

Other volatile compounds

Vanillin has long been associated with the ageing of wine in wood. Moreover, this volatile compound (Table I.10) has been described as an important odour-active compound in several wines from particular cultivars (Guth, 1997a).

Table I.10 – Chemical structure of vanillin found in grapes and wines.

Common name	IUPAC name	R_1	R_2	Structure
vanillin	4-hydroxy-3-methoxy-benzaldehyde	CHO	H	

Vanillin with a *vanilla* odour descriptor (Ferreira *et al*., 2001) is a well-known component of many grape glycosidic fractions (Williams *et al*., 1989; Francis *et al*., 1999; Sefton *et al*., 1993; López *et al*., 2004) and it has been reported as being an odour-active component of young red wines (Ferreira *et al*., 1998; López *et al*., 1999). Nevertheless, in Grenache red wines, vanillin has not been identified or detected by AEDA analysis (Ferreira *et al.*, 1998).

1.6. RECONSTITUTION, ADDITION AND OMISSION SENSORY TESTS

Having determined a list of compounds of likely consequence to a wine aroma by employing the methods discussed above, the decisive test of the importance of these compounds can be determined by reconstitution or spiking sensory experiments (Grosch, 2001), together with omission tests, where odourants are removed from a mixture. These experiments have been carried out to an increasing but still limited extent in the last decade, and have provided new insights into which compounds are the key to wine aroma.

Ferreira *et al*. (2002) working with a Grenache rosé wine, performed a series of reconstitution and omission tests using synthetic aroma models. They concluded that an aroma model prepared by mixing the 24 compounds with OAV > 0.5 in a synthetic wine showed a high qualitative similarity with the aroma of rosé wine. The addition of compounds with OAV < 0.5 did not improve the model, whereas the aroma of a model containing only odourants with OAV > 10 was very different then that of the wine. Moreover, omission tests revealed that the most important odourant of the Grenache rosé wine was 3-mercapto-1-hexanol with a deep impact on the *fruity* and *citric* notes of the wine aroma. The synergic action of Furaneol™ and homofuraneol also had an important impact on the wine aroma, particularly in its *fruity* and *caramel* notes, according to the same authors. Important studies undertaken by Guth (1997a,b) and Ferreira and colleagues (Aznar *et al.*, 2001; Ferreira *et al*., 2002; Escudero *et al*., 2004) have shown that while multiple compounds strongly contribute to a wine's flavour, in many cases a relatively small number is suficient to produce a close similarity to the original wine aroma.

A series of other studies at the University of Bordeaux by the research group led by Dubourdieu (Darriet *et al.*, 1995; Bouchilloux *et al.*, 1998b, 2000; Tominaga *et al*., 1998a,b,c, 2000a,b, 2003a,b; Blanchard *et al*., 2001; Murat *et al*., 2001) have increased the level of understanding of the contribution made by a number of volatile thiols to the aroma properties of wines made from several red and white grape cultivars. This work has determined that these sulphur compounds are at least as important to Sauvignon blanc aroma as the well established and equally potent alkyl methoxypyrazines (Allen *et al.*, 1991), and are equally significant to wines of many other varieties. The Bordeaux researchers have recently combined some sensory studies with quantitative GC-MS data, the results of which suggest that these thiol compounds can be responsible for both *tropical fruit* and *cat's urine* aroma in white wine, and *berry-like* aroma in red wine (Murat *et al*., 2001). Whilst these sulphur-containing volatile compounds are difficult to analyse and quantify, nevertheless, work carried out by several independent groups continues to point to their role in the flavour of wines of many varieties (Guth 1997b; Schneider *et al*., 2003; Escudero *et al*., 2004).

1.7. EXTRACTION METHODS FOR QUANTITATIVE ANALYSIS OF WINES, MUSTS AND GRAPES

Through GC-O and sensory analyses useful information about the aroma composition of wines can be obtained. However, this information should be complemented with quantitative data of each volatile compound. Thus, several isolation and concentration methods have been developed for the quantitative analyses of volatile compounds in wines, musts and grapes.

Wine is one of the most complex alcoholic beverages. Regarding the complexity of wine aroma various factors have been verified:

- Hundreds of volatile compounds have been identified;
- Volatile components have a different chemical nature covering a wide range of polarity, solubility, volatility and pH;
- An important number of the volatile components can be found at a very low concentration. Therefore, the samples need to be highly concentrated;
- Many of the aromatic components are unstable. They may be easily oxidised in contact with air or degraded by heat or extreme pH, giving rise to the appearance of artifacts.

One of the main problems that researchers have to face when studying the compounds responsible for wine, must or grape aroma is the choice of a suitable extraction procedure to qualitatively and quantitatively represent the sample original aroma. That is, to obtain an extract that contains all the volatile compounds contained in the original sample, without them having been altered or degraded or artifacts being formed. Several methods have been developed trying to achieve that goal. All of them present some advantages and disadvantages regarding each other (Blanch *et al*., 1991; Etievant, 1996).

Usually, it is necessary to combine different methods to obtain the complete extraction of all the volatile compounds contained in samples without them being altered (Ortega-Heras *et al.*, 2002).

Classical analytical methods, such as liquid-liquid extraction (Villén *et al.*, 1995; Zhou, *et al.*, 1996; Schneider *et al.*, 1998; Lavigne *et al.*, 1998), static and dynamic headspace (Salinas *et al.*, 1994; García-Jares *et al.*, 1995; Campo *et al*., 2006), simultaneous distillation-solvent extraction (Blanch *et al.*, 1991), and solid-phase extraction (Edwards and Beelman, 1990) have been widely used for the extraction of volatile components from wine. Other methods, for example, involving ultrasound (Hernanz *et al.*, 1999), supercritical fluid extraction (Etievant, 1987; Blanch *et al.*, 1995), purge and cold trapping (Salinas *et al.*, 1994), and solid-phase microextraction (SPME) have also been applied to analysing volatile content of grapes, musts and wines.

The supercritical fluid extraction (SFE) was used in the determination of 2,4,6-trichloroanisole (TCA) in corks (Taylor *et al.*, 2000) and in the extraction of glycosylated precursors of grape aroma (Palma *et al.*, 2000).

SPME was invented by Pawliszyn and co-workers (Arthur and Pawliszyn, 1990; Louch *et al.*, 1992; Zhang *et al.*, 1994). SPME integrates sampling, extraction, concentration and sample introduction into a single solvent-free step. Analytes in the liquid samples or in the headspace are directly extracted and concentrated in the extraction fibre. The method saves preparation time and disposal costs and can improve detection limits (Kataoka *et al.*, 2000). It has been routinely used in combination with gas chromatography (GC) and GC-mass spectrometry (GC-MS) and successfully applied to a wide variety of compounds, especially in the extraction of volatile and semi-volatile organic compounds from environmental, biological and food samples.

Different extraction methods based on solid-phase microextraction (SPME) have been applied in the analysis of certain types of volatile compounds in wines (Evans *et al.*, 1997; Vas *et al.*, 1998; Francioli *et al.*, 1999; Hayasaka and Bartowsky, 1999; Mestres *et al.*, 1999, 2000; Mallouchos *et al.,* 2002*;* Whiton and Zoecklein, 2000). Vas *et al*. (1998) reported the use of SPME for fast screening of different wine types. Whiton and Zoecklein (2000) carried out the optimisation of headspace-SPME for the analysis of ten wine aroma compounds. Câmara (2004) and Câmara *et al*. (2004) used a dynamic headspace-SPME coupled with GC-MS in order to study the free fraction of musts and wines of Boal, Malvasia, Sercial and Verdelho white grape varieties. Falco (2004) developed a method using headspace-SPME coupled with GC-MS to analyse wine aroma components from four different clones of Touriga Nacional cultivar.

A comparative analysis of volatile compounds of "fino" sherry wine by rotator and continuous liquid-liquid extraction and solid-phase microextraction was performed by Castro *et al.* (2004). According to the referred study, SPME presented higher sensitivities while liquid-liquid extraction showed high repeatability and had the possibility of simultaneous extraction of several samples (up to 12). However, the SPME technique is a solvent-free procedure presenting major advantages, such as small sample volume and higher sensitivity and simplicity.

More recently, stir bar sorptive extraction (SBSE) has also been developed (Baltussen *et al*., 1999; Sandra *et al.*, 2001; Bicchi *et al.*, 2002). According to these authors SBSE is more sensitive and can be used in trace analysis, while SPME is ideally appropriate for the analysis of compounds in higher concentrations. SBSE uses a stir bar (typically 10-mm length) incorporated in a glass tube and coated with polydimethylsiloxane (PDMS). This extraction procedure coupled with GC-MS was used in Cabernet Sauvignon aroma wine analysis by Hayasaka *et al.* (2003).

In spite of this great variety of analytical methods, liquid-liquid extraction continues to be the reference technique for the extraction of volatile compounds from wine (Villén *et al*., 1995; Zhou *et al*., 1996; Lavigne *et al*., 1998; Schneider *et al*., 1998).

Several methods have been described to isolate glycosides of volatiles, specifically to analyse the glycosidically-bound fraction of wines, musts and grapes. The great majority of them involve the selective retention of glycosides from aqueous extracts on two hydrophobic adsorbents: the RP-18 reversed-phase resin (Williams *et al.*, 1982a,b; Sefton and Williams, 1991) and the Amberlite XAD-2 resin (Gunata *et al.*, 1985; López *et al.*, 2004; Oliveira, 2000; Oliveira *et al.*, 2004). Besides, a rapid method to assess the glycoconjugates (Glycosyl-Glucose, (G-G)) was developed by Williams *et al*. (1995) and further modified by Iland *et al*. (1996). Also, Schneider *et al*. (2004) developed a fast method using Fourier-transform infrared spectrometry and chemometric techniques for grape aroma glycoconjugates analysis.

1.8. CLONAL WINES CHARACTERISATION AROUND THE WORLD

The clonal selection was created in Germany in 1926 and later, in 1978, the Portuguese Clonal Selection Program was created. According to the definition of the *Office International de la Vigne et du Vin* (OIV) "one clone is the certified vegetative descent of one vine chosen for its identity, its phenotypic characteristics and its sanitary condition". Nowadays, in our country, the "Direcção Geral de Agricultura e Desenvolvimento Rural (DGADR)" based on the orientations of the "Comissão Nacional para o Exame das Variedades de Videira (CNEVV)" is the official organism which decides the admission to the certification of the different grapevine clones.

There is few research works published on the variability of the responsible compounds for the aroma in clones of different varieties. The aroma of grapes and wines from different Chardonnay clones were studied by several group researchers such as Versini *et al*., 1988, 1989a,b; Scienza *et al*., 1989; Villa *et al*., 1993; Scienza *et al*., 1994; Battistutta *et al*., 1996; Bettiga 2003a,b.

The terpenic fraction of grapes from different clones of Traminer and Riesling was also studied (Scienza *et al*., 1990; Versini *et al*., 1990; Scienza *et al*., 1994). The authors Schoeffling and Faas studied the clones of the grape varieties Kerner, Gewürztraminer, Riesling and Mueller-Thurgau and concluded that the chromatographic analysis of grapes and wines should be included in the clonal selection programs (Schoeffling and Faas, 1990).

Clones selection of Gewürztraminer based on the concentration of terpenes were recommended by Marais (1990). The study of free and bound terpenes present in clones of Gewürztraminer and Weisser Riesling also permitted the selection of clones which could lead to wines with greater aroma tipicity (Marais and Rapp, 1991).

Aroma of grapes of four clones of Merlot during five vintages was studied by Bettiga (2003a,b) and the aroma of other four clonal wines from Merlot noir was studied by Kotseridis *et al*. (1998) and the last authors found differences among them.

In Portugal, Rodrigues (1996) and Rodrigues *et al*. (1996) carried out the characterisation of the aroma of six clones from white Fernão Pires cultivar. More recently, Falco (2004) studied four clonal red wines from Touriga Nacional cultivar during three consecutive vintages and found that there was a great similarity among their aroma. Furthermore, using CharmAnalysis™, a group of twenty odourants was found as the most potent odourants in those clonal wines.

CHAPTER 2

Gas chromatography-olfactometry method selection

2. GAS CHROMATOGRAPHY-OLFACTOMETRY METHOD SELECTION

2.1. INTRODUCTION

Gas chromatography-olfactometry (GC-O) is a unique analytical technique which associates the resolution power of the capillary column in GC with the selectivity and sensitivity of the human nose. This latter sometimes detects odourants that occur in extremely low amounts, much below the detection limit of any physical system. GC-O is limited to screening for odour-active compounds, unless any quantification of the chemical stimuli and of the sniffers' responses is performed. At present, GC-O methods quantifying the odour's potency or intensity can be classified in four categories: dilution analysis, time-intensity methods, detection frequency methods and posterior intensity methods. The present study deals with the comparison of two GC-O methods, the detection frequency method and the posterior intensity method. The comparison was carried out with the main aim of selecting the more useful and adequate method to study clonal red wines and musts.

2.2. MATERIALS AND METHODS

2.2.1. Samples

Three certified clones of grapes (*Vitis vinifera* L. *cv*. Aragonez: 3AE1 = Aragonez T 54 EAN (PT), 3AE2 = Aragonez T 56 EAN (PT), 3AE4 = Aragonez T 58 EAN (PT)) were obtained from vineyards in Portugal's Estremadura Denomination of Controlled Origin, in the 2003 vintage. About 60 Kg of healthy grapes of each clone from Aragonez variety were hand-harvested, crushed and destemmed. The winemaking was performed using 60 Kg-capacity stainless steel cubes in the experimental cellar of Estação Vitivinícola Nacional. The finished wines, after malolactic fermentation, were bottled and stored at cellar temperature until analysis.

2.2.2. Sample preparation

Volatile compounds were extracted from wine samples (50 mL), spiked with an aliquot of 400 μL of 2-octanol (IS, 81.9 $mg.L^{-1}$, 50% ethanol solution) for quantification (chapter 6). The extraction was performed using discontinuous ultrasound liquid-liquid extraction with redistilled dichloromethane, dried over sodium sulphate anhydrous and then concentrated to 0.30 mL (Cocito *et al*., 1995; Ribeiro-Corrêa, 1996). The wine extraction was performed in duplicate and the extracts were stored at -20 ºC until analysis.

2.2.3. Reagents

Dichloromethane and sodium sulphate anhydrous, both analytical grade, were purchased from Merck (Darmstadt, Germany). The dichloromethane was redistilled in a Vigreux column. The GC-O and GC-MS standards: ethyl butanoate, diacetyl, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, ethyl hexanoate, ethyl octanoate, benzaldehyde, 2-methylpropanoic acid, γ-butyrolactone, butanoic acid, 3-methylbutanoic acid, hexanoic acid, guaiacol, 2-phenylethanol, 2,5-dimethyl-4-hydroxy-3(2H)-furanone (Furaneol™, registered trademark of Firmenich S.A., Geneva, Switzerland), eugenol, 4-ethylphenol, syringol and vanillin were purchased from Fluka Chemie (Buchs, Switzerland); ethyl isobutyrate, isoamyl acetate, 3-(methylthio)-1-propanol, 4-vinylguaiacol, ethyl vanillate and acetovanillone from Aldrich Chem, Co (Gillingham-Dorset); (*Z*)-hexen-3-ol, 4-ethylguaiacol and 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone (homofuraneol) from TCI (Tokyo Chemical Industry Co., Ltd); β-damascenone was kindly supplied by Symrise (Holzminden, Germany).

2.2.4. GC-O analysis

The GC-O system consisted of an Agilent Technologies 6890 *Series* chromatograph (Wilmington, DE, USA) equipped with a flame ionization detector (FID) and an Olfactory Detection Port (ODP, Gerstel, Germany). GC effluent was split 1:3 between the FID and the ODP. Each sample (0.6 μL) was injected using the splitless mode into a capillary column (INNOWAX, 30 m length x 0.25 mm i.d. x 0.25 μm film thickness, J&W Scientific, Folsom, CA). Operating conditions were as follows: injector and FID, 250 ºC; ODP, 220 ºC; carrier gas hydrogen, 2.0 ml min^{-1}; the oven temperature was held at 45 ºC for 5 min and increased to 210 ºC at 3.5 ºC min^{-1} and held at 210º C for 20 min. The linear retention indices (LRI) of the compounds (FID and the olfactometry peaks) were calculated from the retention time of n-alkanes (C9-C26, C28 and C30) by linear interpolation (Philips, 1989). Each wine sample was analysed by the eight sniffers and no odour descriptions were given in advance. Furthermore, they were asked to describe the quality of the odour detected, which was recorded.

The area where the GC-O equipment is installed (Figure II.1) was maintained at 20º C and had an air exhaustion system to maintain the air free from odours that could interfere with the analysis.

(a) **(b)** **(c)**

Fig. II.1 – **(a)**: general aspect of the GC-O equipment; **(b)**: aspect of an analysis session; **(c)**: moment of the written record of the information vocally supplied by the sniffer during a session (photos by M.L. Avelar).

2.2.5. Detection frequency method

A panel of eight experienced sniffers (3 male and 5 female, aged 26-63) in GC-Olfactometry (Caldeira, 2004) was selected. The sniffers smelled the effluent of the column during 50 min and pressed a joystick whenever they detected an odour. The number of sniffers detecting an odour-active compound at the olfactory detection port (detection frequency) is used as an estimate of the odour's intensity (van Ruth and O'Connor, 2001; Pollien *et al*., 1997). The detection frequency of odours having the same retention time was calculated. The odours detected by less than 3 sniffers, considered as odour noise, were eliminated.

2.2.6. Posterior intensity method

The same 8 sniffers were previously trained to use a memorised five-point intensity interval scale (1 - very mild; 2 - mild; 3 - moderate; 4 - strong; 5 - very strong) for intensity evaluation, before the analysis. This training consisted of a period of familiarisation with the scale during three months with standard solutions and wine extracts. During this period the sniffers were asked to rate the intensity of the eluted odour using the proposed scale. After the training period, they were instructed to rate the intensity of the eluted odours using the same five-point intensity interval scale during the wine GC-O analysis. The panel average intensity scores were calculated. The intensity of odours not detected by a sniffer was set to 0 (zero).

2.2.7. GC-MS analysis

Equipment 1

A Finnigan MAT (San Jose, CA, USA) GC-MS equipment (Magnum) was used to analyse the wine extracts. An aliquot of 0.6 μL was injected and volatile compounds were separated using a fused silica capillary column of polyethylene glycol (DB-WAX, 30 m length x 0.25 mm i.d. x 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). Operating conditions were as follows: injector and interface temperature, 250 ºC; carrier gas helium (inlet pressure 12 psi and split ratio 1:60); the temperature gradient used began at 50 ºC for 2 min, and was raised to 180

ºC at 3.5 ºC min^{-1} and held at this temperature for 25 min. The mass spectrometer was operated in the electron impact mode at 70 eV, scanning the range m/z 39-340. Identification of volatile compounds was systematically confirmed with the retention indices of the available pure standard compounds (determined in the same analysis conditions) and with the comparison between the mass spectra of the volatile compounds and of the pure standard compounds. All mass spectra were also compared with those of the data system libraries (NIST and Wiley).

Equipment 2

An Hewlett Packard 6890 (USA) GC equipment furnished with a spectrometer 5973 MSD (USA) was used to analyse the wine extracts. An aliquot of 1.0 µL was injected and volatile compounds were separated using a fused silica capillary column of polyethylene glycol (HP Innowax, 30 m length x 0.25 mm i.d. x 0.50 µm film thickness, J&W Scientific, Folsom, CA, USA). Operating conditions were as follows: injector and interface temperature, 250 and 300ºC, respectively; carrier gas helium (30 cm/s); the temperature gradient used began at 45 ºC for 5 min, and was raised to 180 ºC at 3.5 ºC min^{-1} and held at this temperature for 25 min. The mass spectrometer was operated in the electron impact mode at 70 eV, scanning the range m/z 39-340. The injector was of the splitless type (opening of valves after 30 seconds). The acquisition was done using SIM mode (Selected Ion Monitoring).

In order to increase the sensitivity of GC-MS analysis for correct identification of benzaldehyde, β-damascenone, Furaneol™ and homofuraneol, their most characteristic ions (bold type) were used for SIM mode analysis (Table II.1).

Table II.1 – Mass fragment ions [ion (relative intensity % of the base fragment)] of compounds identified by SIM mode.

Compound	Molecular Ion	Mass spectrum, most important fragments for identification
benzaldehyde	106	**106**(100), **105**(95), **77**(81), 51(31), 50(20), 78(16), 74(9), 107(8)
β-damascenone	69	**190**(5), **121**(43), 105(18), 91(10), 79(7), 77(8), **69**(100)
Furaneol™	128	**128**(100), 43(88), **57**(60), **85**(27), 55(11), 129(5)
homofuraneol	142	**142**(100), **57**(70), 43(50), **71**(15); 127(10), 72(10)

Acquisition with three ions makes it possible to prove the presence of these compounds. In fact, the relative abundance of the three ions is a fingerprint of the molecule and must always have the same abundance report of the compound reference (Pinho and Bertrand, 1995).

2.2.8. Statistical Analysis

Statistical treatment was performed using SPSS software version 14.0 for Windows (SPSS Inc. Chicago, IL, USA). Spearman's ranked correlation test was used to establish the repeatability of the sniffers in the posterior intensity method and to compare the GC-O data obtained by detection frequency and posterior intensity methods.

2.3. RESULTS AND DISCUSSION

2.3.1. Sniffers panel evaluation in detection frequency method

A simultaneous output of a chromatogram of the 3AE1 clonal wine extract by GC-FID and respective aromagram are presented in Figure II.2.

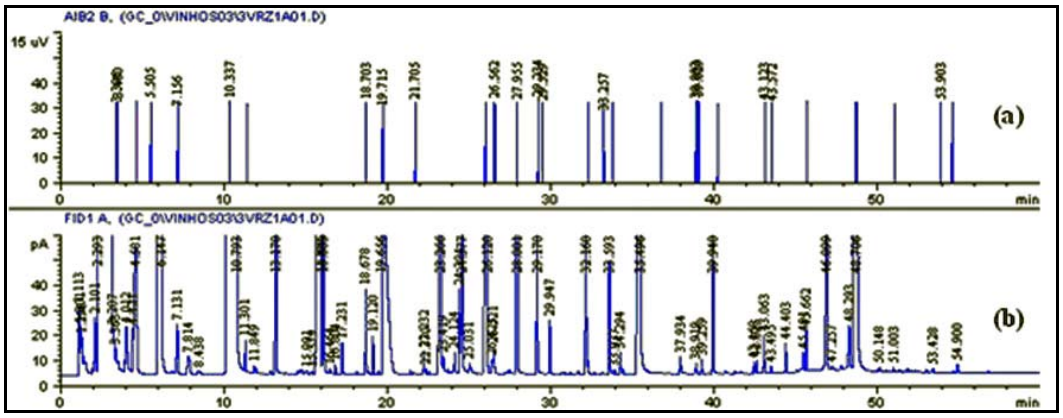


Fig. II.2 - A simultaneous output of a chromatogram of 3AE1 clonal wine extract by GC-FID **(b)** and respective aromagram **(a)**, obtained by one of the sniffers, in the Olfactory Detection Port (ODP).

In order to evaluate the group of eight sniffers, two replicates (Rep 1 and Rep 2) of the same wine extract (A1) were evaluated by each sniffer. The sniffers were not informed about the existence of duplicates of the same sample. Thirty-five different odourants were detected by the sniffers. The number of detected odourant peaks for each replication and the list of sniffers (A-H) are shown in Table II.2. The number of odourant peaks detected is very similar across the two replicates of each sniffer, whereas a discrepancy across the sniffers was found. The former results indicate a good performance and the repeatability of each sniffer and the latest results could be explained by inter-individual variability in olfactory thresholds (Stevens *et al*., 1988; Walker *et al*., 2003).

Table II.2 - Number of detected odourant peaks in the wine extract (A1) for two replicate samples in the frequency detection method.

Sniffers	Rep 1	Rep 2
A	33	32
B	27	27
C	31	32
D	28	27
E	26	26
F	25	24
G	29	29
H	23	23

These results are in agreement with previous studies, which underline a considerable variation between sniffers (Le Guen *et al*., 2000; Marin *et al*., 1988; Etiévant *et al*., 1999), and emphasize the importance of using a group of sniffers in the GC-O methods (van Ruth and O'Connor, 2001).

2.3.2. Sniffers panel evaluation in posterior intensity method

In order to study the consistency of each individual sniffer, two replicates of the same wine extract (A1) were also analysed by all the eight sniffers using a five-point intensity interval scale. The intensity values of all odourants detected in two replications by each sniffer (A-H) were used to perform the Spearman's ranked correlation test. The results (Table II.3) demonstrated that they all had a good consistency in the use of that scale. In fact, according to Spearman's ranked test ($p \leq 0.01$), no statistical significant differences between replicates were found by all sniffers.

Table II.3 - Results of Spearman's ranked correlation for the eight sniffers evaluation in the posterior intensity method (2 replicates).

Sniffers	A	B	C	D	E	F	G	H
Spearman correlation coefficient	0.969**	0.917**	0.826**	0.924**	0.915**	0.877**	0.923**	0.980**

**. Correlation is significant at the $p < 0.01$ level

2.3.3. Comparison of detection frequency and posterior intensity methods

Three different Aragonez clonal wines (A1-A3) were evaluated by detection frequency and posterior intensity methods. In Table II.4 the number attributed to the detected odourant peaks, the linear retention indices (LRI), the compounds identity, the main odour descriptors, detection frequency and average intensity scores obtained by detection frequency and posterior intensity methods are presented. Thirty-seven odourant peaks were perceived by at least 3 sniffers in both methods.

In order to study the correlation between scores from the posterior intensity method and the frequency from the detection frequency method the Spearman's ranked correlation test ($p \leq 0.01$) was performed and the results obtained on the three Aragonez clonal wines are presented in Table II.5.

The two GC-O methods used in this study allowed the determination of the odour's intensity in the clonal wines and a high significant correlation between the results generated by both methods was found.

Similarly, a high correlation (Spearman's ranked correlation, $r = 0.920$) has been found by van Ruth and O'Connor (2001) between detection frequencies and posterior intensity scores of individual compounds in a reference mixture. However, for some odourants, the maximum detection frequency and the maximum intensity scores were not coincident, namely for the compounds 2+3-methyl-1-butanol (P8), 3-methylbutanoic acid (P16), Furaneol™ (P26) and 4-vinylguaiacol (P31) detected in all extracts (Table II.4).

Table II.4 - Odourant compounds found in three Aragonez clonal wines: peak number, gas chromatographic retention data, compound identification, olfactory description, detection frequencies and average scores.

				Aragonez clonal wines					
				3AE1		3AE2		3AE4	
Peak no.	LRI[a]	Compound	odour descriptor	freq.[b]	score[c]	freq.	score	freq.	score
P1	971	ethyl isobutyrate[d]	fruity	8	2.4	8	2.4	7	2.0
P2	975	diacetyl[d]	caramel, butter	7	2.5	8	2.8	8	2.1
P3	1028	ethyl butanoate[d]	fruity	7	1.4	6	1.1	7	1.1
P4	1048	ethyl 2-methylbutanoate[d]	fruity	7	1.1	7	1.6	8	1.5
P5	1064	ethyl 3-methylbutanoate[d]	fruity	7	1.4	7	1.5	8	1.5
P6	1086	2-methyl-1-propanol[d]	pungent, herbaceous	n.d.[f]	0.0	n.d.[f]	0.0	3	0.6
P7	1121	isoamyl acetate[d]	fruity, banana	6	1.5	5	0.9	6	1.1
P8	1217	2+3-methyl-1-butanol[d]	pungent	8	2.6	8	2.3	8	2.5
P9	1232	ethyl hexanoate[d]	fruity	7	1.6	6	1.3	7	1.5
P10	1383	*(Z)*-hex-3-enol[d]	herbaceous, cut grass	4	0.8	n.d.[f]	0.0	4	0.6
P11	1433	ethyl octanoate[d]	fruity, floral	3	0.8	5	1.1	5	1.3
P12	1502	benzaldehyde[d]	plastic	6	1.6	4	1.1	5	1.3
P13	1581	2-methylpropanoic acid[d]	cheese	5	1.3	3	0.9	5	1.4
P14	1626	γ-butyrolactone[d]	smoky, hot	4	0.6	n.d.[f]	0.0	3	0.6
P15	1637	butanoic acid[d]	rancid butter, cheese	7	2.9	7	2.6	7	2.6
P16	1680	3-methylbutanoic acid[d]	stinky, cheese	8	4.0	8	3.9	8	3.8
P17	1690	unknown[e]	onion, sweat	n.d.[f]	0.0	n.d.[f]	0.0	5	1.0
P18	1715	3-(methylthio)-1-propanol[d]	raw potatoes	7	2.9	7	2.4	7	2.5
P19	1731	unknown[e]	onion	n.d.[f]	0.0	n.d.[f]	0.0	3	0.6
P20	1814	β-damascenone[d]	floral, fruity, cooked apple	7	2.4	6	1.9	7	2.0
P21	1839	unknown[e]	floral	7	2.4	7	2.3	7	2.4
P22	1854	hexanoic acid[d]	musty, wet cloth	4	1.1	4	1.1	n.d.[f]	0.0
P23	1862	guaiacol[d]	smoky, medicinal-like	8	2.6	7	1.8	8	1.9
P24	1915	2-phenylethanol[d]	floral, roses	7	2.9	7	3.4	7	3.4
P25	2033	4-ethylguaiacol[d]	floral, carnation, clove	7	2.1	5	1.1	6	1.3
P26	2037	Furaneol™[d]	burnt sugar, candy cotton	8	3.9	8	3.6	8	3.6
P27	2078	homofuraneol[d]	burnt sugar, candy cotton	3	1.0	7	2.1	7	1.8
P28	2128	unknown[e]	fruity, floral	5	0.9	n.d.[f]	0.0	n.d.[f]	0.0
P29	2167	eugenol[d]	floral, spicy	5	1.0	4	0.9	7	1.3
P30	2183	4-ethylphenol[d]	animal, horse stable	8	2.6	6	1.6	8	2.5
P31	2203	4-vinylguaiacol[d]	burnt, curry	8	3.9	8	3.8	8	3.4
P32	2269	syringol[d]	medicinal-like, smoky	6	1.4	7	1.6	6	1.5
P33	2282	unknown[e]	floral, burnt	n.d.[f]	0.0	n.d.[f]	0.0	3	0.8
P34	2494	unknown[e]	burnt, unpleasant	n.d.[f]	0.0	4	0.6	n.d.[f]	0.0
P35	2566	vanillin[d]	vanilla	4	0.8	3	0.8	4	0.9
P36	2576	ethyl vanillate[d] + acetovanillone[d]	vanilla, floral	6	2.8	6	2.9	7	3.0
P37	>2600	unknown[e]	burnt, unpleasant	6	1.6	4	1.0	4	1.5

[a]Linear retention index on INNOWAX capillary column (30 m x 0.25 mm x 0.25 µm); [b]Detection frequency method; [c]Posterior intensity method; [d]Identification based on coincidence of gas chromatographic retention indices and mass spectrometric data with those of the pure standards available in the lab; [e]Not identified compound; [f]Not detected.

Table II.5 - Spearman's ranked correlation between detection frequency data and posterior intensity data from Aragonez (A) clonal wines.

Clonal wines	Spearman correlation coefficient
3AE1	0.888**
3AE2	0.933**
3AE4	0.844**

**Correlation is significant at the $p < 0.01$ level.

In fact, a detection frequency of 8 (the maximum value) was found while the average values of intensity (posterior intensity method) ranged from 2.3 to 4.0. These data suggest that the

posterior intensity method is more reliable in odour intensity measurement. Furthermore, the posterior intensity method is highly sensitive for discovering differences between odourant levels in different samples (López *et al*., 2003; Ferreira *et al*., 2003). In agreement with these results the GC-O posterior intensity method will be selected for further GC-O analysis in the current work.

CHAPTER 3

Characterisation of Aragonez and Trincadeira clonal wines by GC-O posterior intensity method

3. CHARACTERISATION OF ARAGONEZ AND TRINCADEIRA CLONAL WINES BY GC-O POSTERIOR INTENSITY METHOD

3.1. INTRODUCTION

Gas chromatography-olfactometry is a commonly used technique for analysis of odour-active compounds in food and wines. From the comparison of two GC-O methods, the detection frequency method and the posterior intensity method, presented in chapter 2, the last one was considered more advantageous in the study of odour-active compounds from clonal red wines. In fact, this method is highly sensitive to discover differences between odourant levels in different wine samples. For this reason, the Trincadeira and Aragonez clonal wines were studied by posterior intensity method in order to identify and characterise, as completely as possible, the odourant compounds detected.

3.2. MATERIALS AND METHODS

3.2.1. Vineyard characterisation

Three different vineyards belonging to three distinct viticultural Denominations of Controlled Origin (DCO), Estremadura DCO, Alentejo DCO and Ribatejo DCO, were selected. The characteristics of the vineyards from which the clonal grapes were collected for the present study, are described in Table III.1.

Table III.1 – Main characteristics of the vineyards.

Characteristics of the vineyards	Estremadura - Arruda (Aragonez, AE)	Alentejo - Estremoz (Aragonez, AA)	Ribatejo – Cartaxo (Trincadeira, T)
Plantation year	1997	1998	1994
Rootstock	99 R[a]	99 R	99 R
Training system	bilateral cordon	bilateral cordon	bilateral cordon
Height to the soil of lowest wire (m)	0.65	0,55	0,50
Lines orientation	N/S	S/E	S/E
Plantation compass (m)	2.60 x 1.00	2.80 x 1.10	2.50 x 1.10

[a] 99 R = 99 Richter

3.2.2. Samples

Clonal red wines selected from the three distinct vineyards were obtained under similar and controlled winemaking conditions as possible. Table III.2 presents the codification of the clonal wines from Aragonez and Trincadeira varieties, which were chosen to simplify the identification of each clonal wine in the current work.

About 60 Kg of healthy grapes of each clone were hand-harvested and transported to the experimental winery of the Estação Vitivinícola Nacional. The winemaking was performed using 60 Kg-capacity stainless steel cubes. The grapes were crushed and destemmed. A 6% solution containing sulfur dioxide was added to the musts prior to alcoholic fermentation (30 mg L^{-1}).

Table III.2 – Codes of Aragonez and Trincadeira clonal red wines.

ARAGONEZ			TRINCADEIRA		
Certified clone	**2001 Vintage Alentejo**	**2003 Vintage Estremadura**	**Certified clone**	**2001 Vintage Ribatejo**	**2003 Vintage Ribatejo**
T 54 EAN (PT)	1AA1	3AE1	T 11 EAN (PT)	1T2	3T2
T 56 EAN (PT)	1AA2	3AE2	T 12 EAN (PT)	1T3	3T3
T 57 EAN (PT)	1AA3		T 13 EAN (PT)	1T4	3T4
T 58 EAN (PT)	1AA4	3AE4	T 14 EAN (PT)	1T5	3T5
T 60 EAN (PT)	1AA5		T 15 EAN (PT)	1T6	3T6

All the alcoholic fermentations were completed by the metabolism of spontaneous yeasts. Wines were transferred to 20 L glass carboys equipped with fermentation locks, and kept at 24 ºC until dry and through malolactic fermentation. Afterwards, wines were racked, and transferred to clean 10 L glass carboys, and the free SO_2 was adjusted to 30 $mg.L^{-1}$. Two weeks after the final rack and SO_2 adjustment, wines were bottled and stored at cellar temperature.

Like all clonal wines from Aragonez, also the five Trincadeira clonal wines from the 2001 and 2003 vintages were analysed after equal time of bottling in order to avoid the influence of the time bottling in the obtaining of the analytical and sensory data. Thus, all these wines were kept approximately for eighteen months in bottle before the extraction for further analyses.

3.2.3. Sample preparation

As described in chapter 2 (section 2.2.2), discontinuous liquid-liquid extraction with ultrasound (Cocito *et al*., 1995; Ribeiro-Corrêa, 1996) was the basis of all obtained wine extracts. This methodology was chosen because a liquid extract was very useful for subsequent analysis by GC-Olfactometry by several sniffers, and for both GC-MS and GC-FID analyses, which guarantee the equal representativeness of the extracts. All the clonal wines were analysed in duplicate.

3.2.4. FTIR analysis

All the clonal wines were analysed by FTIR spectrophotometry, in a WineScan FT120 (Foss, Hillerød, Denmark) equipment, by the Analysis Service of the Enological Chemistry Department of the Estação Vitivinícola Nacional. The infrared measurement range was 926 to 5012 cm. The following analytical parameters were determined: density ($g.mL^{-1}$), alcohol degree (% vol.), titratable acidity (TA, expressed as $g.L^{-1}$ tartaric acid), and pH.

3.2.5. Reagents

The analytical reagents were described in the section 2.2.3. of chapter 2.

3.2.6. GC-O and GC-MS analyses

GC-O and GC-MS analyses were described in chapter 2, in the sections 2.2.4. and 2.2.7., respectively.

3.2.7. Statistical analysis

The software package SPSS release 14.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for analysis of variance (one-way ANOVA), *post hoc* LSD test, principal component analysis (PCA) and hierarchical cluster analysis (HCA). Since the analysis of variance test only suggested that a difference existed among populations, a multiple comparison test was used to rank the means and to identify the means that were different. The Fisher LSD multiple comparison test was applied to the means because it is the least conservative test in comparison with the Tukey and Duncans test and should produce the highest difference (Maroco, 2003).

The multivariate data analysis PCA, based on a correlation matrix, was computed using the SPSS factor reduction procedure with Varimax rotation for the GC-O posterior intensity method average scores of all odourant compounds detected. The Varimax rotation is an orthogonal rotation method which simplifies the factor interpretation (Pardo and Ruiz, 2001). The first principal components (PCs) were retained by the Kaiser criteria and the scree test (Pardo and Ruiz, 2001; Maroco, 2003). Significant loadings with an absolute value >0.700 represented a strong influence (Siebert, 1999).

Stepwise linear discriminant analysis (SLDA) is a supervised method used for classification purposes. SLDA renders a number of orthogonal linear discriminant functions equal to the number of categories minus one. This method minimises the variance within categories and maximises the variance between categories. The variables included in the analysis are determined with a SLDA using Wilk's Lambda as a selection criterion and an *F*-statistic factor to establish the significance of the changes in Lambda when a new variable is tested (Maroco, 2003).

3.3. RESULTS AND DISCUSSION

3.3.1. Aragonez clonal wines from the Alentejo DCO

3.3.1.1. Analytical evaluation of clonal wines

Five clonal wines from the Alentejo Denomination of Controlled Origin (DCO) vintage were characterised by FTIR analysis, obtaining four main analytical results: volumic mass, alcohol degree, TA and pH. As can be seen in Table III.3, the five clonal wines were statistically significantly different considering the four analytical parameters. The clonal wine 1AA3 presented the highest alcohol degree (13.85 % vol.) and in opposition, the 1AA1 showed the lowest average value (12.85 % vol.). The pH values of all clonal wines varied from 3.76 to 4.08.

Table III.3 – Analytical results of the five Aragonez clonal wines (n=4) by FTIR analysis.

Clonal wines		Volumic mass ($g.mL^{-1}$)	Alcohol degree (% vol.)	TA ($g.L^{-1}$ tartaric acid)	pH
1AA1	*x*	0.9911d	12.85a	4.25a	4.08e
	SD	*0.00*	*0.07*	*0.07*	*0.01*
1AA2	*x*	0.9903b	13.45b	4.55b	3.90c
	SD	*0.00*	*0.07*	*0.07*	*0.01*
1AA3	*x*	0.9901a	13.85c	4.95d	3.76a
	SD	*0.00*	*0.07*	*0.07*	*0.01*
1AA4	*x*	0.9912d	13.35b	4.45bc	3.87b
	SD	*0.00*	*0.07*	*0.07*	*0.01*
1AA5	*x*	0.9908c	13.75c	4.35ac	4.06d
	SD	*0.00*	*0.07*	*0.07*	*0.01*
Clonal effect		***	***	**	***

x : average; *SD: standard deviation; ns*: not significant; * Significant ($p < 0.05$); ** Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); average values followed by the same letter, in the same column, are not significantly different (LSD, 0.05).

3.3.1.2. GC-O evaluation of clonal wines

The results of the olfactometric experiments are given in Table III.4.

Table III.4- Odourant compounds found in five Aragonez clonal wines: peak number, gas chromatographic retention data, compound identification, olfactory description, average scores and significance level.

Peak no.	LRI[a]	Compound	odour descriptor	1AA1	1AA2	1AA3	1AA4	1AA5	*Sig.*
P1	971	ethyl isobutyrate[b]	fruity	2.3	1.8	1.6	2.0	1.9	*ns*
P2	975	diacetyl[b]	caramel, butter	0.0a	1.8b	2.3b	2.3b	1.9b	***
P3	1028	ethyl butanoate[b]	fruity	0.9	0.5	0.8	0.4	0.9	*ns*
P4	1048	ethyl 2-methylbutanoate[b]	fruity	0.6	1.3	1.1	0.9	1.4	*ns*
P5	1064	ethyl 3-methylbutanoate[b]	fruity	1.6	1.1	1.3	1.3	1.1	*ns*
P6	1121	isoamyl acetate[b]	fruity, banana	0.0	0.5	1.0	0.8	1.0	*ns*
P7	1217	2+3-methyl-1-butanol[b]	pungent	2.6	2.5	2.3	1.6	2.1	*ns*
P8	1232	ethyl hexanoate[b]	fruity	1.6	1.5	1.4	0.6	1.5	*ns*
P9	1433	ethyl octanoate[b]	fruity, floral	1.1	1.3	1.5	0.0	1.0	*ns*
P10	1502	benzaldehyde[b]	plastic	1.3	1.4	2.0	1.3	1.4	*ns*
P11	1581	2-methylpropanoic acid[b]	cheese	1.1	0.9	1.3	1.3	1.6	*ns*
P12	1626	γ-butyrolactone[b]	smoky, hot, burnt	0.6b	0.0a	0.5b	0.0a	0.0a	*
P13	1637	butanoic acid[b]	rancid butter, cheese	3.4	3.0	3.0	2.9	3.4	*ns*
P14	1680	3-methylbutanoic acid[b]	stinky, cheese	4.3	4.3	3.9	4.3	4.3	*ns*
P15	1715	3-(methylthio)-1-propanol[b]	raw potatoes	3.0	2.1	2.8	1.9	2.5	*ns*
P16	1731	unknown[c]	onion, burnt	0.0a	1.0b	0.0a	0.0a	0.0a	*
P17	1814	β-damascenone[b]	floral, fruity, cooked apple	1.6	0.9	1.9	2.0	1.9	*ns*
P18	1839	unknown[c]	floral	2.8	1.8	2.4	2.5	3.0	*ns*
P19	1862	guaiacol[b]	smoky, medicinal-like	3.1	2.4	2.1	2.8	3.0	*ns*
P20	1915	2-phenylethanol[b]	floral, roses	4.3	4.1	4.1	3.5	4.1	*ns*
P21	2033	4-ethylguaiacol[b]	floral, carnation, clove	0.0a	1.6b	0.0a	0.0a	0.0a	*
P22	2037	Furaneol™[b]	burnt sugar, candy cotton	4.1	3.3	4.3	3.8	4.1	*ns*
P23	2078	homofuraneol[b]	burnt sugar, candy cotton	3.5	3.3	3.0	3.0	3.3	*ns*
P24	2128	unknown[c]	fruity, floral	0.0a	1.0b	0.0a	0.0a	0.9b	**
P25	2167	eugenol[b]	floral, spicy	1.4b	0.0a	0.9bc	0.6ac	0.6ac	**
P26	2183	4-ethylphenol[b]	animal, horse stable	1.3	2.0	0.9	1.0	1.1	*ns*
P27	2203	4-vinylguaiacol[b]	burnt, curry	4.3	3.8	4.0	3.3	4.3	*ns*
P27	2269	syringol[b]	medicinal-like, smoky	3.0	2.8	2.9	2.4	2.4	*ns*
P29	2494	unknown[c]	burnt, unpleasant	0.9	0.6	2.6	0.5	0.0	*ns*
P30	2566	vanillin[b]	vanilla	1.5b	0.0a	0.8ab	1.3b	0.0a	**
P31	2576	ethyl vanillate[b] + acetovanillone[b]	vanilla, floral	2.3	2.5	3.1	2.8	3.0	*ns*
P32	>2600	unknown[c]	burnt, unpleasant	0.0a	1.0b	1.5b	0.0a	0.0a	***

[a]Linear retention index on INNOWAX capillary column (30 m x 0.25 mm x 0.25 μm); [b]Identification based on coincidence of gas chromatographic retention indices and mass spectrometric data with those of the pure standards available in the lab; [c]Not identified compound; *ns* – not significant; * Significant ($p < 0.05$); ** Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, $p < 0.05$).

This table presents the number attributed to the detected odourant peaks, the linear retention indices (LRI), the identity of the compounds, the reliability of identification, the main odour descriptors, the average intensity scores obtained by the posterior intensity method, and the clonal wine effect on average intensity score differences among the wines.

Thirty-two odourant peaks were perceived by the sniffers in at least one of the five clonal wine extracts according to the posterior intensity method and twenty-nine odourant compounds were identified by GC-MS. Accordingly to the results presented in Table III.4, 3-methylbutanoic acid (P14), 2-phenylethanol (P20), Furaneol™ (P22), homofuraneol (P23), and 4-vinylguaiacol (P27), were the highest average intensities odourant compounds in all clonal wines.

Each clonal wine showed a few differences, particularly in the number of odourant peaks detected. In fact, the clonal wines 1AA2 and 1AA3 presented the highest number of odourant peaks, twenty-nine, while the other three 1AA1, 1AA4, and 1AA5, showed twenty-six odourant peaks. Figure III.1 shows the odourant profile of the five Aragonez clonal wines from the 2001 vintage. As can be seen, the five profiles are very similar to what as been previously confirmed by LSD test (Table III.4). In fact, only in 25% of the odourant peaks, were statistically significant differences found, regarding the average intensities.

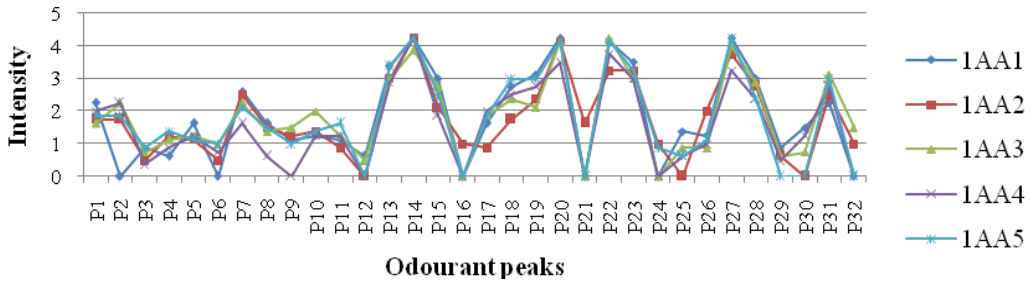


Fig. III.1 – Odourant profiles of the five Aragonez clonal wines from the 2001 vintage.

The principal component analysis (PCA) was applied to the posterior intensity method (GC-O) data of the five clonal wines from the 2001 vintage in order to verify if it could or not be possible to clearly differentiate the wines. This multivariate analysis permitted the establishment of a relationship between the different odourant compounds variables and the wines, and the finding of the most important factors of variability. The four principal components (PCs) explained 100% of the total variance observed. Figure III.2 shows in a two dimensional plot of PC1 against PC2 the locations of the thirty-two GC-O peaks and the five wines. The percentage value corresponding to each PC, presented in Figure III.2, indicates the percentage of variation in the data explained by the PC's.

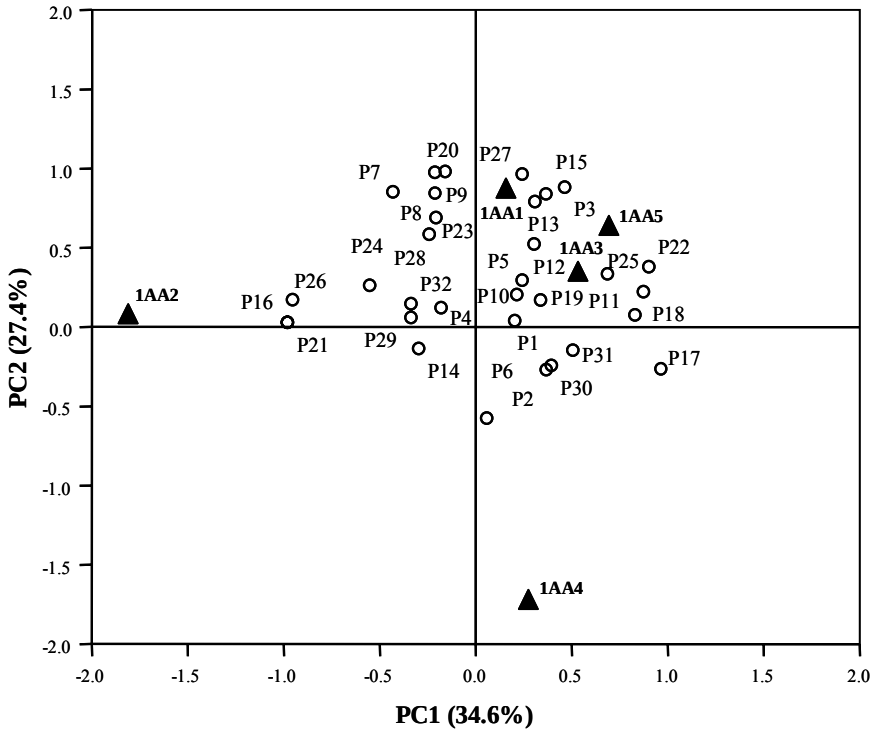


Fig. III.2 - Plot of the first and second principal components (PCs) of the GC-O data and the five Aragonez clonal wines. The percentage of variation explained by each PC is indicated between brackets.

The wines 1AA1, 1AA3 and 1AA5, are closely located on the positive side of PC1 and PC2, which might indicate their similarity previously demonstrated by the LSD results, as shown in the Table III.4, in which only six statistically significant differences were found among the average intensity of the odourant compounds diacetyl (P2), γ-butirolactone (P12), homofuraneol (P23), unknown (P24), vanillin (P30) and unknown (P32). The 1AA4 wine is located on the PC1 positive side and PC2 negative side, while the 1AA2 wine is located on the negative side of PC1 and positive side of PC2. These two wines are distant from one another and both are distant from the group of the other three wines previously referred.

3.3.2. Aragonez clonal wines from the Estremadura DCO

3.3.2.1. Analytical evaluation of clonal wines

Aragonez clonal wines from the Estremadura Denomination of Controlled Origin (DCO) and from the 2003 vintage were characterised by FTIR analysis (Table III.5).

As can be seen in Table III.5, the three clonal wines (3AE1, 3AE2 and 3AE4) were not significantly different regarding volumic mass and TA while statistically significant differences were detected in ethanol and pH (ranging from 3.36 to 3.64) among clones. The clonal wine 3AE2 presented the highest alcohol degree (11.70 % vol.) and the 3AE1 showed the lowest average value (10.45 % vol.).

Table III.5 – Analytical results of the five Aragonez clonal wines (n=4) by FTIR analysis.

Clonal wines		Volumic mass ($g.mL^{-1}$)	Alcohol degree (% vol.)	TA ($g.L^{-1}$ tartaric acid)	pH
3AE1	*x*	0.9927	10.45a	4.60	3.64c
	SD	*0.00*	*0.07*	*0.14*	*0.03*
3AE2	*x*	0.9915	11.70c	5.75	3.36a
	SD	*0.00*	*0.14*	*0.35*	*0.02*
3AE4	*x*	0.9925	11.05b	5.40	3.51b
	SD	*0.00*	*0.07*	*0.42*	*0.06*
Clonal effect		*ns*	**	*ns*	*

x: average; *SD*: standard deviation; *ns* – not significant; * Significant ($p < 0.05$); ** Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same column, are not significantly different (LSD, 0.05).

3.3.2.2. GC-O evaluation of clonal wines

The results of the GC-Olfactometry analysis are given in Table III.6.

Table III.6 - Odourant compounds found in three Aragonez clonal wines: peak number, gas chromatographic retention data, compound identification, olfactory description, average scores and significance level.

Peak no.	LRI[a]	Compound	odour descriptor	3AE1	3AE2	3AE4	*Sig.*
P1	971	ethyl isobutyrate[b]	fruity	2.4	2.4	2.0	*ns*
P2	975	diacetyl[b]	caramel, butter	2.5	2.8	2.1	*ns*
P3	1028	ethyl butanoate[b]	fruity	1.4	1.1	1.1	*ns*
P4	1048	ethyl 2-methylbutanoate[b]	fruity	1.1	1.6	1.5	*ns*
P5	1064	ethyl 3-methylbutanoate[b]	fruity	1.4	1.5	1.5	*ns*
P6	1086	2-methyl-1-propanol[b]	pungent, herbaceous	0.0	0.0	0.6	*ns*
P7	1121	isoamyl acetate[b]	fruity, banana	1.5	0.9	1.1	*ns*
P8	1217	2+3-methyl-1-butanol[b]	pungent	2.6	2.3	2.5	*ns*
P9	1232	ethyl hexanoate[b]	fruity	1.6	1.3	1.5	*ns*
P10	1383	*(Z)*-hex-3-enol[b]	herbaceous, cut grass	0.8	0.0	0.6	*ns*
P11	1433	ethyl octanoate[b]	fruity, floral	0.8	1.1	1.3	*ns*
P12	1502	benzaldehyde[b]	plastic	1.6	1.1	1.3	*ns*
P13	1581	2-methylpropanoic acid[b]	cheese	1.3	0.9	1.4	*ns*
P14	1626	γ-butyrolactone[b]	smoky, hot	0.6	0.0	0.6	*ns*
P15	1637	butanoic acid[b]	rancid butter, cheese	2.9	2.6	2.6	*ns*
P16	1680	3-methylbutanoic acid[b]	stinky, cheese	4.0	3.9	3.8	*ns*
P17	1690	unknown[c]	onion, sweat	0.0a	0.0a	1.0b	*
P18	1715	3-(methylthio)-1-propanol[b]	raw potatoes	2.9	2.4	2.5	*ns*
P19	1731	unknown[c]	onion	0.0	0.0	0.6	*ns*
P20	1814	β-damascenone[b]	floral, fruity, cooked apple	2.4	1.9	2.0	*ns*
P21	1839	unknown[c]	floral	2.4	2.3	2.4	*ns*
P22	1854	hexanoic acid[b]	musty, wet cloth	1.1	1.1	0.0	*ns*
P23	1862	guaiacol[b]	smoky, medicinal-like	2.6	1.8	1.9	*ns*
P24	1915	2-phenylethanol[b]	floral, roses	2.9	3.4	3.4	*ns*
P25	2033	4-ethylguaiacol[b]	floral, carnation, clove	2.1	1.1	1.3	*ns*
P26	2037	Furaneol™[b]	burnt sugar, candy cotton	3.9	3.6	3.6	*ns*
P27	2078	homofuraneol[b]	burnt sugar, candy cotton	1.0	2.1	1.8	*ns*
P28	2128	unknown[c]	fruity, floral	0.9a	0.0b	0.0b	**
P29	2167	eugenol[b]	floral, spicy	1.0	0.9	1.3	*ns*
P30	2183	4-ethylphenol[b]	animal, horse stable	2.6	1.6	2.5	*ns*
P31	2203	4-vinylguaiacol[b]	burnt, curry	3.9	3.8	3.4	*ns*
P32	2269	syringol[b]	medicinal-like, smoky	1.4	1.6	1.5	*ns*
P33	2282	unknown[c]	floral, burnt	0.0	0.0	0.8	*ns*
P34	2494	unknown[c]	burnt, unpleasant	0.0a	0.6b	0.0a	*
P35	2566	vanillin[b]	vanilla	0.8	0.8	0.9	*ns*
P36	2576	ethyl vanillate[b] + acetovanillone[b]	vanilla, floral	2.8	2.9	3.0	*ns*
P37	>2600	unknown[c]	burnt, unpleasant	1.6	1.0	1.5	*ns*

[a]Linear retention index on INNOWAX capillary column (30 m x 0.25 mm x 0.25 µm); [b]Identification based on coincidence of gas chromatographic retention indices and mass spectrometric data with those of the pure standards available in the lab; [c]Not identified compound; *ns* – not significant; * Significant ($p < 0.05$); ** Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, $p < 0.05$).

Thirty-seven odourant peaks were perceived by the sniffers in at least one of the three clonal wine extracts according to the posterior intensity method and thirty-two odourant compounds were identified by GC-MS. Six odourant compounds with the highest average intensities in all clonal wines, 3-methylbutanoic acid (P16), 2-phenylethanol (P24), Furaneol™ (P26), 4-vinylguaiacol (P31), ethyl vanillate and acetovanillone (P36) were found. Analysing each clonal wine, there are some differences among them in the number of odourant peaks detected. In fact, the clonal wine 1AE4 presented the highest number of odourant peaks, thirty-four. The other two clonal wines 1AE1 and 1AE2, showed thirty-two and thirty odourant peaks, respectively.

In Figure III.3 the odourant profile of the three Aragonez clonal wines from the 2003 vintage are shown. The three profiles are strongly similar as previously confirmed by LSD test (Table III.6). In fact, only 8% of the odourant peaks showed statistically significant differences in average intensities.

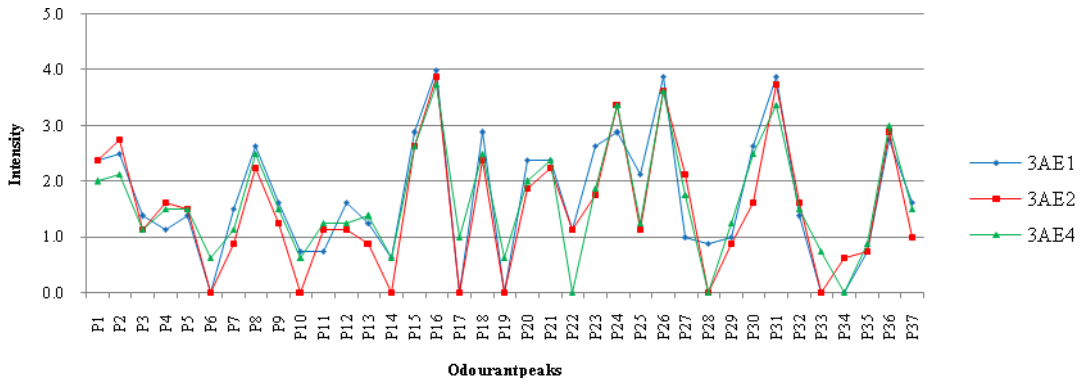


Fig. III.3 – Odourant profiles of the three Aragonez clonal wines from the 2003 vintage.

The principal component analysis (PCA) applied to the posterior intensity method (GC-O) data of the three clonal wines of the 2003 vintage permitted the establishment of a relationship between the different odourant compound variables and the wines as well as to find out the most important factors of variability.

The two principal components (PCs) found explained 100% of the total variance. Figure III.4 shows in the two dimensional plot of PC1 against PC2 the locations of the thirty-seven GC-O peaks and the clonal wines.

The percentage value corresponding to each PC, presented in Figure III.4, indicates the percentage of variation in the data explained by the PC's. The wines 3AE2 and 3AE4 are located on the negative side of PC1 and on opposite quadrants in PC2. The 3AE2 is on the negative side of PC2 and on the opposite, the 3AE4 is located on the positive side. The 3AE1 is located on the positive side of PC1 and on the negative side of PC2. According to the PCA plot, the 3AE1 appears to be stongly correlated with the odourant compounds ethyl butanoate (P3),

benzaldehyde (P12), butanoic acid (P15), 3-methylbutanoic acid (P16), β-damascenone (P20), guaiacol (P23), 4-ethylguaiacol (P25), Furaneol™ (P26) and an unknown compound (P28).

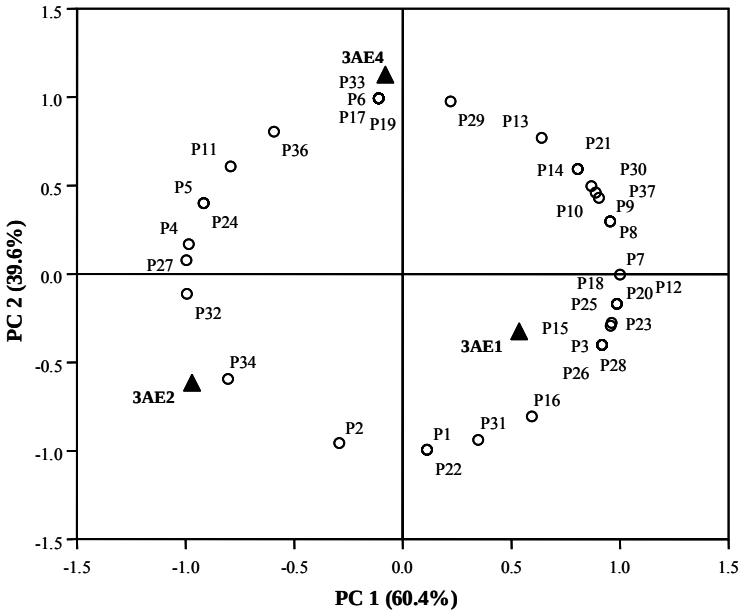


Fig. III.4 - Plot of the first and second principal components (PCs) of the GC-O data and the three Aragonez clonal wines. The percentage of variation explained by each PC is indicated between brackets.

The 3AE4, on the other hand, is strongly correlated with three unknown odourant compounds (P17, P19 and P33), as well as with 2-methyl-1-propanol (P6). The PCA plot also shows that 3AE2 is highly correlated with an unknown odourant compound (P34).

3.3.3. Characterisation and differentiation of Trincadeira clonal red wines by their GC-O profiles

3.3.3.1. Analytical evaluation of clonal wines

Five Trincadeira clonal wines from the Ribatejo Denomination of Controlled Origin and from the 2001 and 2003 vintages were analysed by FTIR analysis (Table III.7). The wines from the 2001 vintage showed high similarity and no significant differences in any analytical parameters were detected. Among the wines from the 2003 vintage, statistically significant differences were detected in two parameters, volumic mass and alcohol degree. The clonal wine 3T5 presented the highest alcohol degree (13.30 % vol.) and, the 3T4 showed the lowest average value (12.75 % vol.). Statistically significant differences were detected in volumic mass and pH. The pH values varied between 3.36 and 3.42, and between 3.53 and 3.69, in the 2001 and the 2003 vintage, respectively.

Table III.7 – Analytical results of the five Trincadeira clonal wines (n=4) from the two vintages by FTIR analysis.

Clonal wines		Volumic mass ($g.mL^{-1}$)	Alcohol degree (% vol.)	TA ($g.L^{-1}$ tartaric acid)	pH
VINTAGE 2001					
1T2	*x*	0.9910	13.35	5.75	3.42
	SD	*0.00*	*0.64*	*0.49*	*0.05*
1T3	*x*	0.9912	13.35	5.70	3.42
	SD	*0.00*	*0.49*	*0.71*	*0.05*
1T4	*x*	0.9903	14.30	6.00	3.36
	SD	*0.00*	*0.14*	*0.14*	*0.01*
1T5	*x*	0.9927	13.60	5.55	3.39
	SD	*0.00*	*0.85*	*0.07*	*0.10*
1T6	*x*	0.9926	13.60	5.50	3.41
	SD	*0.00*	*0.57*	*0.42*	*0.04*
Clonal effect		*ns*	*ns*	*ns*	*ns*
VINTAGE 2003					
3T2	*x*	0.9941b	12.70a	5.40	3.59
	SD	*0.00*	*0.00*	*0.28*	*0.06*
3T3	*x*	0.9916a	13.10d	5.20	3.53
	SD	*0.00*	*0.00*	*0.14*	*0.04*
3T4	*x*	0.9923a	12.55b	4.75	3.69
	SD	*0.00*	*0.07*	*0.07*	*0.00*
3T5	*x*	0.9920a	13.30e	5.00	3.62
	SD	*0.00*	*0.00*	*0.14*	*0.03*
3T6	*x*	0.9922a	13.00c	5.05	3.56
	SD	*0.00*	*0.00*	*0.07*	*0.04*
Clonal effect		**	***	*ns*	*ns*
Vintage effect		*	*ns*	*ns*	***

x: Average; *SD*: standard deviation; *ns* – not significant; * Significant ($p < 0.05$); ** Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same column, are not significantly different (LSD, $p < 0.05$).

3.3.3.2. GC-O evaluation of clonal wines

The results of the olfactometric experiments are given in Table III.8: number attributed to the detected odourant peaks, the linear retention indices (LRI), the compounds identity, the reliability of identification, the main odour descriptors, the average intensity scores obtained by the posterior intensity method, the clonal wine effect for each vintage and the vintage effect on average intensity scores differences among the vintages.

Forty-one odourant peaks were perceived by the sniffers in at least one of the five clonal wine extracts from the two vintages according to the posterior intensity method, and thirty-one odourant compounds were identified by GC-MS. All the odourant compounds identified in Trincadeira clonal wines, similar to the Aragonez wines previously reffered to in this chapter, are common to other wines from *Vitis vinifera* L. as previously reported in literature (Williams *et al*., 1982a,b; Rapp and Mandery, 1986; Rapp, 1988; Etievant, 1991; Bayonove *et al*., 1998; Ferreira *et al*., 1998; Ferreira *et* al., 2000; Ebeler, 2001; Campo *et al*., 2006).

Ethyl esters of fatty acids produced by the yeasts like ethyl isobutyrate, ethyl butanoate, ethyl 2-methylbutanoate and ethyl 3-methylbutanoate are commonly found in red wines, with a joint

contribution to the *fruity* notes because their concentrations are frequently above the perception threshold (Nykänen, 1986). According to Table III.8, 3-methylbutanoic acid (P15), 2-phenylethanol (P22), Furaneol™ (P27), and 4-vinylguaiacol (P35) presented the highest average intensities in the five Trincadeira clonal wines from the two vintages.

During the analysis of each clonal wine from the 2001 vintage some differences in the number of odourant peaks were detected. In fact, the clonal wines 1T2 and 1T5 presented the highest number of odourant peaks, twenty-seven, while the other three 1T3, 1T4 and 1T6, showed twenty-six, twenty-three and twenty-five odourant peaks, respectively. Regarding the wines from 2003 vintage, 3T4 showed the highest number of odourant peaks, thirty-six. The other four 3T2, 3T3, 3T5 and 3T6, showed thirty-four, thirty-three, thirty and twenty-nine odourant peaks, respectively. Seven esters, well-known as important constituents of young wine aroma and referred to as key compounds in the *fruity* flavours of wines (Nykänen, 1986; Herraiz *et al*., 1991), were detected in GC-O experiments in this study, like ethyl isobutyrate (P1), ethyl butanoate (P3), ethyl 2-methylbutanoate (P4), ethyl 3-methylbutanoate (P5), isoamyl acetate (P7), ethyl hexanoate (P9), and ethyl octanoate (P10). Among these esters, all produced by yeast during alcoholic fermentation, isoamyl acetate had an important role in the vintage differentiation among clonal wines ($p < 0.001$). In fact, in the five wines from the 2001 vintage, the average intensity of this volatile compound was zero, while in all wines from the 2003 vintage, the average intensity of isoamyl acetate varied from 0.8 to 1.0. The odourant compound 2-phenylethanol (P22) reached a high intensity average score in all Trincadeira clonal wines. This compound has been detected in wines from different proveniences by GC-O (Kotseridis and Baumes, 2000; López *et al.,* 2004) and, despite its presence in grapes and precursor hydrolysates, it has been reported in GC-O experiments by other authors (López *et al*., 2004; Genovés *et al.*, 2005), and is mostly produced by yeasts during alcoholic fermentation.

Among C_{13}-norisoprenoid compounds, only β-damascenone (P17), an important odour-active compound found in musts (López *et al*., 2004) and wines (Kotseridis and Baumes, 2000; Ferreira *et al*., 2001), was detected in both vintages. The wine 1T6 was the only one with an average intensity score of zero. In all other wines, the average intensity ranged between 0.9 and 2.0. Monoterpenic compounds were not detected either by GC-O or GC-MS in all wines analysed in this study.

Table III.8 - Odourant compound intensity scores determined by GC-O posterior intensity method in Trincadeira clonal wines. Clonal and vintage effects on average intensity score differences of odourant compounds among clonal wines.

				2001 Vintage					Clonal	2003 Vintage					Clonal	Vintage
Peak no.	LRI[a]	Odourant compound	Odour description	1T2	1T3	1T4	1T5	1T6	effect	3T2	3T3	3T4	3T5	3T6	effect	effect
P1	971	ethyl isobutyrate[b]	fruity	2.1	2.4	2.1	2.4	2.8	*ns*	2.0	2.4	2.1	2.5	2.3	*ns*	*ns*
P2	975	diacetyl[b]	caramel, butter	2.6	2.6	2.8	2.4	2.8	*ns*	2.0	2.6	2.8	2.1	2.9	*ns*	*ns*
P3	1028	ethyl butanoate[b]	fruity	0.4ab	0.6b	0.0a	0.5b	0.0a	**	1.3	0.0	1.0	1.4	0.8	*ns*	**
P4	1048	ethyl 2-methylbutanoate[b]	fruity	1.4	1.8	1.3	1.6	1.6	*ns*	0.8	1.0	1.1	1.3	1.4	*ns*	*ns*
P5	1064	ethyl 3-methylbutanoate[b]	fruity	1.5	2.0	1.6	1.6	1.6	*ns*	1.3	1.9	1.9	1.6	1.4	*ns*	*ns*
P6	1086	2-methyl-1-propanol[b]	pungent, herbaceous	0.0	0.0	0.0	0.0	0.0	*ns*	0.0a	0.8b	0.0a	0.0a	0.0a	*	*ns*
P7	1121	isoamyl acetate[b]	fruity, banana	0.0	0.0	0.0	0.0	0.0	*ns*	0.9	0.8	1.0	1.0	0.9	*ns*	***
P8	1217	2+3-methyl-1-butanol[b]	stinky	2.5	2.8	2.6	2.5	2.3	*ns*	2.6	2.6	2.1	2.4	2.6	*ns*	*ns*
P9	1232	ethyl hexanoate[b]	fruity	0.8	0.9	0.0	1.0	1.1	*ns*	0.8	0.6	1.4	1.0	0.9	*ns*	*ns*
P10	1433	ethyl octanoate[b]	fruity, floral	0.9ac	1.3bc	1.0ab	0.9a	0.0a	*	1.3	0.8	0.8	1.0	1.1	*ns*	*ns*
P11	1502	benzaldehyde[b]	plastic	2.3	2.3	2.0	1.8	2.1	*ns*	1.0	1.9	1.8	1.9	1.6	*ns*	*ns*
P12	1581	2-methylpropanoic acid[b]	cheese	1.1	1.3	1.0	1.1	1.4	*ns*	1.0ab	0.9ab	1.9b	0.0a	0.0a	**	*ns*
P13	1626	γ-butyrolactone[b]	smoky, hot	0.5	0.0	0.0	0.6	0.9	*ns*	0.8	0.6	0.5	0.0	0.0	*ns*	*ns*
P14	1637	butanoic acid[b]	rancid butter, cheese	2.8	2.6	2.0	2.1	2.3	*ns*	2.9	2.5	2.8	2.0	2.9	*ns*	*ns*
P15	1680	3-methylbutanoic acid[b]	stinky, cheese	3.9	4.5	4.0	3.8	4.0	*ns*	3.9	4.0	3.8	3.8	3.8	*ns*	*ns*
P16	1715	3-(methylthio)propanol[b]	raw potatoes	2.5	2.1	1.5	2.3	1.8	*ns*	2.3	2.5	2.3	1.4	2.4	*ns*	*ns*
P17	1814	β-damascenone[b]	floral, fruity, cooked apple	1.4b	1.3b	1.0b	0.9ab	0.0a	*	1.8	1.4	2.0	1.4	1.8	*ns*	**
P18	1839	unknown[c]	floral	2.6	2.5	1.6	2.8	2.6	*ns*	2.4	2.1	2.5	2.1	2.1	*ns*	*ns*
P19	1854	hexanoic acid[b]	musty, wet cloth	0.0	0.0	0.0	0.0	0.0	*ns*	0.0a	0.0a	0.6b	0.0a	0.0a	*	*ns*
P20	1862	guaiacol[b]	smoky, medicinal	2.6	3.0	2.6	1.9	2.5	*ns*	2.6	2.4	2.3	2.8	2.4	*ns*	*ns*
P21	1882	unknown[c]	floral	0.0	0.0	0.0	0.0	0.0	*ns*	1.1b	0.0a	1.4b	0.0a	0.0a	**	**
P22	1915	2-phenylethanol[b]	floral, roses	3.4	3.9	3.9	3.9	3.9	*ns*	3.6	3.3	3.4	3.4	3.3	*ns*	*ns*
P23	1959	unknown[c]	floral, medicinal	0.0	0.0	0.0	0.0	0.0	*ns*	0.9b	0.0a	0.0a	0.0a	0.0a	***	*
P24	1998	unknown[c]	spicy	0.0	0.0	0.0	0.0	0.0	*ns*	1.0	0.5	0.8	0.0	0.8	*ns*	***
P25	2023	unknown[c]	sweet, burnt	2.0b	1.3ab	0.9a	2.1b	2.0b	***	0.0a	1.3b	1.1b	0.0a	0.8ab	*	**
P26	2033	4-ethylguaiacol[b]	floral, carnation, clove	0.0	0.0	0.0	0.0	0.0	*ns*	1.3b	1.6b	0.0a	1.1b	0.0a	***	***
P27	2037	Furaneol™[b]	burnt sugar, candy cotton	3.4b	3.8b	2.0a	3.1b	3.6b	**	4.0	3.3	4.0	4.0	3.6	*ns*	**
P28	2078	homofuraneol[b]	burnt sugar, candy cotton	1.8	1.5	0.8	0.9	1.4	*ns*	2.9c	1.3b	1.8b	0.0a	0.0a	***	*ns*
P29	2084	unknown[c]	floral, medicinal	0.0	0.0	0.0	0.0	0.0	*ns*	0.0a	0.0a	0.0a	0.9b	0.0a	*	*ns*
P30	2091	unknown[c]	burnt, spicy	0.0	0.0	0.0	0.0	0.0	*ns*	0.0a	0.0a	0.8b	0.0a	0.0a	**	*ns*
P31	2113	unknown[c]	horse stable, horse sweaty	0.0	0.0	0.0	0.0	0.0	*ns*	0.0a	1.0b	0.0a	0.9b	0.8ab	*	***
P32	2128	unknown[c]	fruity, floral	0.0a	0.0a	0.0a	0.6b	0.8b	*	1.0b	0.0a	0.9ab	0.0a	1.3b	*	*ns*
P33	2167	eugenol[b]	floral, spicy	1.0	1.0	0.9	0.0	1.0	*ns*	1.9b	1.0ab	1.9b	0.6a	1.8b	*	**
P34	2183	4-ethylphenol[b]	animal, horse stable	1.0b	1.1b	2.6c	0.5a	1.6b	**	0.0a	2.0c	2.3c	0.5ab	1.1b	***	*ns*
P35	2203	4-vinylguaiacol[b]	burnt, curry	3.6	4.0	3.8	3.8	3.8	*ns*	4.0	3.5	3.8	3.9	3.5	*ns*	*ns*
P36	2257	unknown[c]	spicy	0.0	0.0	0.0	0.0	0.0	*ns*	1.1	1.3	1.4	1.3	0.0	*ns*	***
P37	2269	syringol[b]	medicinal, smoky	1.8	1.4	2.1	1.6	1.6	*ns*	1.8	2.0	1.8	1.6	1.9	*ns*	*ns*
P38	2352	unknown[c]	floral	0.0	0.0	0.0	0.0	0.0	*ns*	1.6	0.0	1.3	1.0	1.1	*ns*	***
P39	2566	vanillin[b]	vanilla	0.0	0.0	0.0	0.0	0.0	*ns*	1.1b	0.9b	0.9b	0.8b	0.0a	*	***
P40	2576	ethyl vanillate[b]+acetovanillone[b]	vanilla, floral	2.8	2.5	3.0	2.9	2.9	*ns*	2.6	2.5	1.6	2.6	3.3	*ns*	*ns*
P41	>2600	unknown[c]	burnt, unpleasant	1.5	1.8	1.8	0.9	1.1	*ns*	1.6	1.9	1.3	1.5	1.1	*ns*	*ns*

[a] Linear retention index on INNOWAX capillary column (30 m x 0.25 mm x 0.25 µm); [b] Identification based on coincidence of gas chromatographic retention indices and mass spectrometric data with those of the pure standards available in the lab; [c] Not identified compound; *ns*: not significant; * Significant ($p < 0.05$); ** Highly significant ($p < 0.01$); *** Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, $p < 0.05$).

Among the lactones family, only the γ-butyrolactone (P13) was detected by GC-O analysis, with an average intensity ranging from 0.0 to 0.9, which shows its very low odour intensity when compared to the other compounds found in Trincadeira wines. Furaneol™ (P27) and homofuraneol (P28), both described with the odour descriptors, *burnt sugar* (*caramel-like*) and *candy cotton* were detected by GC-O analysis. The average intensity scores of the first compound were always higher in all clonal wines than those of the second one. Furaneol™, identified in juice and wines from *Vitis labrusca* hybrid grapes (Rapp *et al.*, 1980; Baek *et al.*, 1997), has also been recently detected in *Vitis vinifera* wines (Guth, 1997a,b; Kotseridis and Baumes, 2000; Aznar *et al.,* 2001). Homofuraneol was firstly reported in *Vitis vinifera* wines by Guth (1997a) and as since then been considered as an odour-active compound in wines (Kotseridis and Baumes, 2000; Aznar *et al*., 2001; Ferreira *et al*., 2001).

Three volatile acids, butanoic acid (P14), 3-methylbutanoic acid (P15) and hexanoic acid (P19), were also determined by GC-O analysis. The last one had basically no odourant importance since it was detected only in 3T4 clonal wine extract. Six volatile phenols guaiacol (P20), 4-ethylguaiacol (P26), eugenol (P33), 4-ethylphenol (P34), 4-vinylguaiacol (P35) and syringol (P37) that have been considered odour-active compounds of red wines (Kotseridis and Baumes, 2000; Ferreira *et al*., 2001; Culleré *et al.*, 2004), have all been identified in the odourant fraction of Trincadeira, except 4-ethylguaiacol, which was not detected in the wines from the 2001 vintage revealing the high statistical effect of vintage ($p < 0.001$) on the occurrence of this volatile phenol in these wines. Vanillin (P39), ethyl vanillate and acetovanillone (P40) were also detected in the GC-O analysis. The first volatile compound was not detected in all wines from the 2001 vintage, which indicates the effect of the vintage ($p < 0.001$) on vanillin detection in Trincadeira wines. Figure III.5 shows the odourant profile of the five Trincadeira clonal wines from the 2001 vintage. Accordingly, the five profiles showed high similarity, which has been confirmed before by LSD test (Table III.8). Only in 17.1% of the odourant peaks were statistically significant differences found among the average intensities of the clonal wines.

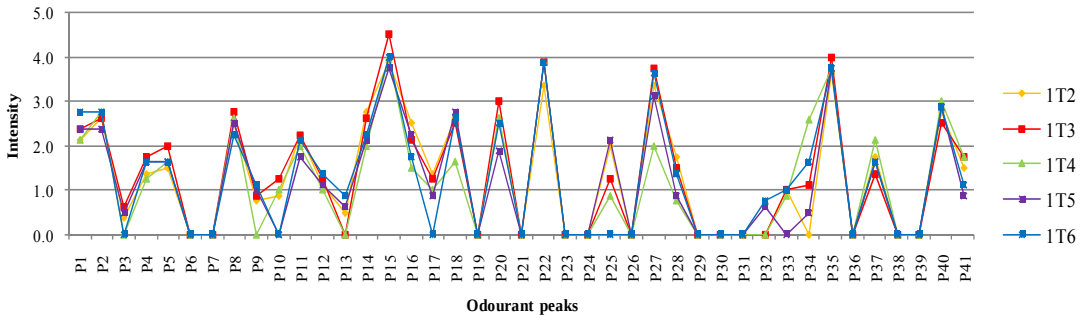


Fig. III.5 – Odourant profiles of the five Trincadeira clonal wines from the 2001 vintage.

Figure III.6 represents the odourant profile of the Trincadeira clonal wines from the 2003 vintage. In opposition to the 2001 vintage, the five profiles are very different from each other, as previously confirmed by LSD test (Table III.8). In fact, in 36.6% of the odourant peaks statistically significant differences among the average intensities were found.

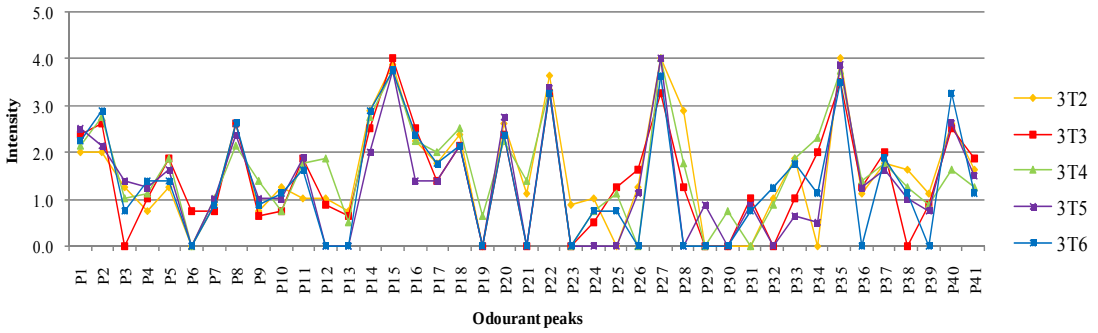


Fig. III.6 – Odourant profiles of the five Trincadeira clonal wines from the 2003 vintage.

Principal component analysis (PCA) was applied to the posterior intensity method (GC-O) data of the ten clonal wines from the 2001 and 2003 vintages in order to verify if it could be possible to clearly differentiate the wines. Figure III.7 shows in the two dimensional plot of PC1 against PC2 the locations of the forty-one GC-O peaks and the ten wines. The percentage value corresponding to each PC, presented in Figure III.7 indicates the percentage of variation in the data explained by the PC's. The wines from the 2001 vintage (1T2 to 1T6) are located as a well defined group on the negative side of PC2, and show a great proximity among them. This similarity was previously demonstrated by the LSD test results (as shown in Table III.8). In relation to the wines from the 2003 vintage, 3T3 and 3T5, both are located on the negative side of PC1 and positive side of PC2 which indicates their high similarity. The other three wines located on the positive side of PC1 are far from them.

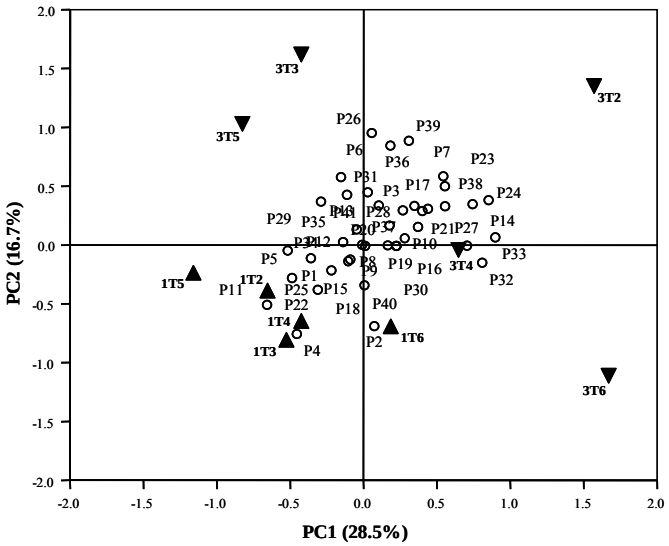


Fig. III.7 - Plot of the first and second principal components (PCs) of the GC-O data and the ten Trincadeira clonal wines. The percentage of variation explained by each PC is indicated between brackets.

For a better visualisation of similarities or dissimilarities among the five Trincadeira wines from both vintages, a hierarchical cluster analysis (HCA) was done using the same data. Figure III.8 shows the dendogram obtained using the Ward method. The dendogram displays three clusters: the one with more elements includes all the five Trincadeira clonal wines from the 2001 vintage. The wines from the 2003 vintage were grouped together in two distinct clusters. 3T2 and 3T4 wines represent one cluster, while 3T3, 3T5 and 3T6 compose the other cluster. As long as the HCA demonstrated that there was a clear and well defined separation of clonal wines between the 2001 and 2003 vintages, a stepwise linear discriminant analysis (SLDA) will be presented in the next section, in order to show the discriminating variables which are responsible for this wine discrimination by vintage.

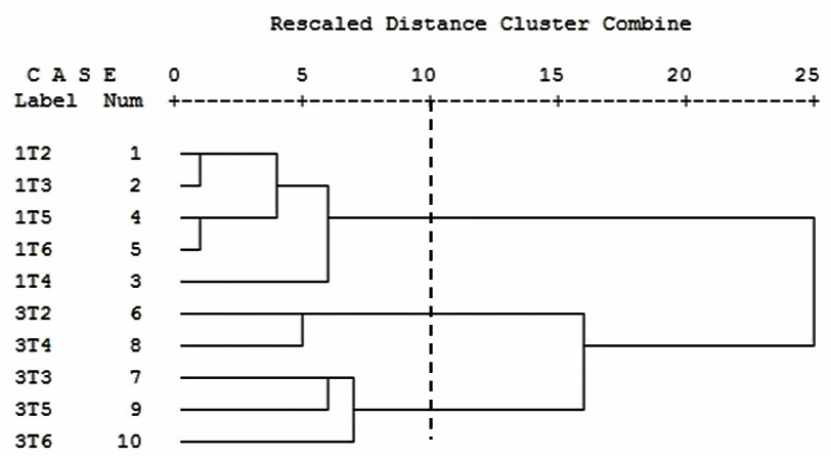


Fig. III.8 – Dendogram of Trincadeira clonal wines using the Ward method.

3.3.3.3. Discrimination of the five Trincadeira clonal wines between the two vintages

After the PCA analysis of the Trincadeira clonal wines, a SLDA using the odourant compounds data was performed in order to discriminate the five clonal wines under study. Table III.9 presents the number of steps, the selected variables, the value of F-to-remove of selected variable, the significance level (*Sig.*), and the standardised coefficients of discriminant functions (DFs).

Table III.9 – Stepwise linear discriminant analysis according to vintage (years, 2001 and 2003).

Step	Selected variable	F-to-remove of selected variable	Standardised coefficients of DF
1	P7	370.286	31.588
2	P6	13.984	27.498
3			
4	P24	5.053	19.027
5	P12	26.846	-9.150
6			
7	P19	17.671	-7.768
8	P9	4.934	1.591
Eigenvalues of DFs			62474.692
p-values of DFs			0.000

According to these results, six variables, isoamyl acetate (P7), 2-methyl-1-propanol (P6), unknown (P24), 2-methylpropanoic acid (P12), hexanoic acid (P19) and ethyl hexanoate (P9), were found to be discriminating variables. Table III.10 presents the percentage of correctly classified clonal wines and shows that 100.0% of the original grouped cases were correctly classified.

Table III.10 - Percentage of correctly classified Trincadeira clonal wines.

			Predicted group membership		
		Wine year	**2001**	**2003**	**Total**
Original	Count	2001	5	0	5
		2003	0	5	5
	%	2001	100.0	0.0	100.0
		2003	0.0	100.0	100.0

The discriminant function obtained allowed the classification of all the wines of both vintages in their correct groups. Consequently, the SLDA achieved a good clonal wine separation regarding vintage year.

CHAPTER 4

Characterisation of free and glycosidically-bound odourant compounds of Aragonez clonal musts by GC-O

4. CHARACTERISATION OF FREE AND GLYCOSIDICALLY-BOUND ODOURANT COMPOUNDS OF ARAGONEZ CLONAL MUSTS BY GC-O

4.1. INTRODUCTION

Grapes have several compounds in both free and glycosidically-bound forms. In general, grapes and musts contain an interesting pool of potential volatile aroma compounds that are mainly constituted as odourless, non-volatile glycoconjugates. These compounds have the potential to make an important contribution to the varietal sensory properties of wines. When key components, responsible for specific aroma, are known and quantified, they can be utilised as a tool to optimise viticultural and oenological practices to obtain maximum grape and wine quality. Thus, the posterior intensity method was applied in the study of free and bound fractions of Aragonez musts in order to establish their odourant profiles.

4.2. MATERIALS AND METHODS

4.2.1. Samples

Grapes of three certified clones of *Vitis vinifera* L. *cv*. Aragonez were collected in one vineyard of the Estremadura Denomination of Controlled Origin, in the 2003 vintage (Table IV.1). It must be underlined that the musts obtained from these grapes were used in the winemaking of their respective clonal wines previously referred to in chapters 2 and 3.

Table IV.1 – Codes of Aragonez clonal red musts from the 2001 vintage selected for the study.

Certified clone	Free fractions	Bound fractions
Aragonez 54 T EAN (PT)	3MAE1F	3MAE1B
Aragonez 57 T EAN (PT)	3MAE2F	3MAE2B
Aragonez 59 T EAN (PT)	3MAE4F	3MAE4B

Replicate samples of the musts were taken in 500 mL glass bottles and stored at -30 ºC until analysis.

4.2.2. Reagents

Analytical grade solvents and reagents were used. Water used was deionised (conductivity < 0.1 mS/cm obtained through a Seralpur Pro 90 CN from SERAL (Water Purification Systems, Ransbach-Baumbach, Germany)). LiChroprep RP-18 (40-63 μm), anhydrous sodium sulphate (99%), perchloric acid, ethanol LiChrosolv, methanol and dichloromethane were purchased from Merck (Darmstadt, Germany). The last one was purified by redistillation before use. The GC standards hexanal, benzyl alcohol and vanillin were purchased from Fluka Chemie (Buchs,

Switzerland); *trans*-2-hexenal and 4-nonanol (IS, internal standard) were purchased from TCI Europe nv (Zwijndrecht, Belgium) and β-damascenone was kindly supplied by Symrise (Holzminden, Germany).

4.2.3. Sample preparation

The bottles were taken out at a temperature of -30 ºC, and they were thawed at 4 ºC just before analysis. The liquid must was then centrifuged at 4 ºC (15000 rpm, Sorvall RC-5B, Newtown, USA) for 15 min.

4.2.3.1. Extraction of free fractions

A sample of 100 mL clarified must was spiked with an aliquot of 100 µL of 4-nonanol (IS, 82.7 $mg.L^{-1}$, 50% ethanol solution) for quantification. The free volatile compounds were extracted with the successive addition of 30, 10 and 10 mL of dichloromethane by ultrasonification (P Selecta, model 3000515, 40 KHz, Barcelone, Spain) for 10 min, for each extraction. Between extractions, centrifugation (10000 rpm, 4 ºC, 5 min Sorvall RC-5B, Newtown, USA) was done to help the phase separation process. The organic phases obtained were pooled, dried over anhydrous sodium sulphate and concentrated to approximately 200 µL, on a rotary evaporator at 42 ± 0.5 ºC (Büchi rotavapor R-114 and Büchi heating bath, B-480, Switzerland), without vacuum. The extracts were stored at -20 ºC until analysis by GC-O, GC-MS and GC-FID.

4.2.3.2. Extraction of glycosidically-bound fractions

Must samples were fractionated by solid-liquid chromatography using LiChroprep RP-18 non ionic resin in a glass column (30 x 3 cm i.d.). The LiChroprep RP-18 (40 g) was purified with methanol for 4 h before poured into the glass column. A peristaltic pump (Masterflex, Barrington, USA) was used to help the elution of all solvents. The LiChroprep RP-18 on the column was first pre-conditioned with 100 mL of methanol, then with 200 mL of ultrapure water. A sample of 200 mL clarified must was passed through the column and it was washed with ultrapure water (400 mL) following the adsorption step. During this step, free sugars and other polar constituents are removed while the less polar glycosides are retained (Williams *et al*., 1982b). The free fraction was eluted with dichloromethane (100 mL) to avoid the presence of free volatile compounds in the methanolic extract. This free extract was then discarded. Glycosides were recovered by elution with methanol (100 mL) and this fraction was collected in a separate 150 mL volumetric flask in ice-water bath. Afterwards, the eluate was concentrated to a final volume of 2 mL, in a rotary evaporator (Büchi rotavapor R-200 and Büchi heating bath, B-490, Switzerland) at 32 ºC (±0.5 ºC) under vacuum (Büchi vacuum system, Büchi B-169, Switzerland). The main steps are described in Figure IV.1.

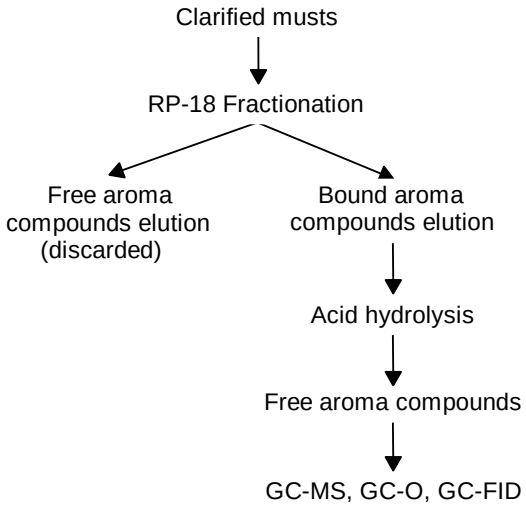


Fig. IV.1 - Experimental scheme of the bound fractions extraction and analysis.

After each sample extraction, the resin was thoroughly rinsed with methanol acidified with perchloric acid (0.1%) and was left in methanol between each use.

4.2.3.3. Acidic hydrolysis of bound fractions

Considering the acidic hydrolysis of bound fractions, experiments were done at pH 3.0 (perchloric acid 1%). Hydrolysis was carried out by heating the acidic extract into a glass vial, at 100 ºC for 20 min. Then the extract was cooled to room temperature (20 ºC). An aliquot of 100 µL of 4-nonanol (IS, 8.27 $mg.L^{-1}$, 50% ethanol solution) was added for quantification. The free volatile compounds generated were extracted with the successive addition of 15, 5 and 5 mL of dichloromethane by ultrasonification (P Selecta, model 3000515, 40 KHz, Barcelona, Spain) for 10 min, for each extraction. Between extractions, centrifugation (10000 rpm, 4 ºC, 5 min Sorvall RC-5B, Newtown, USA) was done to help the phase separation process. The organic phases obtained were pooled, dried over anhydrous sodium sulphate and concentrated to approximately 100 µL, on a rotary evaporator at 42 ± 0.5 ºC (Büchi rotavapor R-114 and Büchi heating bath, B-480, Switzerland), without vacuum. The extracts were stored at -20 ºC until analysis by GC-O, GC-MS and GC-FID.

4.2.4. GC-O and GC-MS analyses

Both GC-MS and GC-O analyses of free and bound fractions extracts were done as described previously in chapter 2 (sections 2.2.4., 2.2.6. and 2.2.7). The only modification was the volume of injection. In fact, the volume of injection of the extracts from free must extractions was 0.6 µL, while the volume of injection of extracts from bound fractions was 1.2 µL, in both chromatographic systems.

4.3. RESULTS AND DISCUSSION

4.3.1. Free odourant compounds in Aragonez musts

An example of the GC-O posterior intensity method application for free odourant compounds analysis, present in one free fraction of Aragonez must, 3MAE2F, is presented in Figure IV.2.

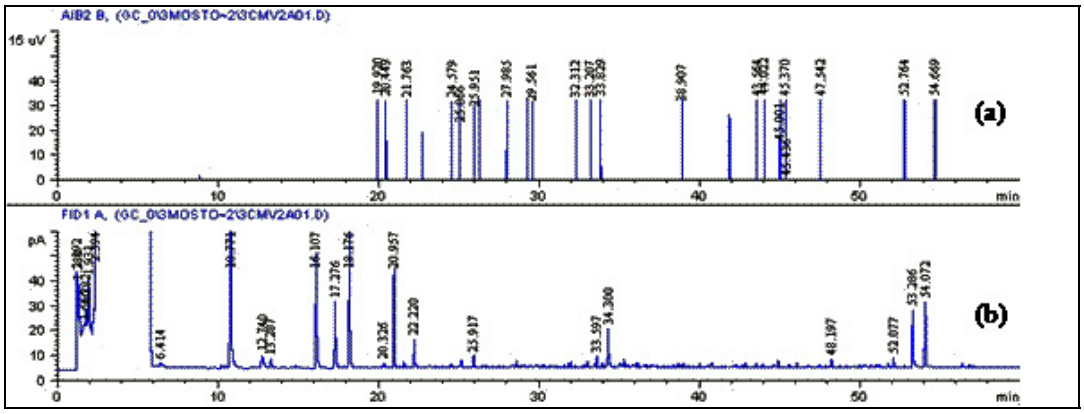


Fig. IV.2 - Simultaneous output of a chromatogram of the free fraction extract, 3MAE2F, from Aragonez must by GC-FID **(b)** and respective aromagram **(a)**, obtained by one of the sniffers, in the Olfactory Detection Port (ODP).

As done before for clonal wines, a table with the odourant compounds found in the three Aragonez clonal must free fractions was built (Table IV.2).

Forty-three odourant peaks were perceived by the sniffers in at least one of the three clonal must free fraction extracts according to the GC-O posterior intensity method and twelve odourant compounds were identified by GC-MS. Analysing each clonal must free fraction in separate, it can be verified that the 3MAE1F fraction has the highest number of odourant compounds detected (34). The other two fractions, 3MAE2F and 3MAE4F, presented a very similar number of odourant compounds detected, 28 and 29, respectively.

Figure IV.3 shows the odourant profile of the three Aragonez clonal musts free fractions. As can be seen, the three profiles are very different among them, which was confirmed before by LSD analysis (Table IV.2). In 37.2% of the odourant peaks, statistically significant differences were found regarding the average score intensity of the clonal musts.

Table IV.2 - Odourant compounds found in three Aragonez clonal must free fractions: peak number, gas chromatographic retention data, compound identification, olfactory description, average scores and significance level.

Peak no.	LRI[a]	Compound	odour descriptor	3MAE1F	3MAE2F	3MAE4F	*Sig.*
1	<1100	hexanal[b]	herbaceous, cut grass	0.8b	0.0a	0.0a	*
2	1144	unknown[c]	herbaceous	1.4	0.8	1.2	*ns*
3	1216	unknown[c]	herbaceous	1.0	1.0	1.0	*ns*
4	1300	unknown[c]	mould, soil	1.4b	0.0a	0.0a	***
5	1383	(*Z*)-hex-3-enol[b]	herbaceous	1.2	1.4	1.8	*ns*
6	1453	unknown[c]	raw potatoes	1.8	1.8	2.2	*ns*
7	1502	benzaldehyde[b]	plastic	3.2a	3.0	2.4	*ns*
8	1531	unknown[c]	wood extract, hot	1.4b	0.8ab	0.0a	*
9	1582	unknown[c]	fruity, melon	1.8	2.2	2.4	*ns*
10	1608	unknown[c]	plastic	1.6b	0.0a	0.0a	**
11	1619	unknown[c]	smoke, hot	2.2	1.4	1.4	*ns*
12	1630	unknown[c]	floral, herbaceous	0.0a	1.6b	0.8ab	**
13	1638	unknown[c]	floral	1.2	0.0	0.0	*ns*
14	1677	3-methylbutanoic acid[b]	stinky, cheese	1.6	1.6	2.4	*ns*
15	1695	unknown[c]	sweet	2.4c	0.8b	0.0a	***
16	1717	unknown[c]	sweet, floral	2.0	2.0	2.2	*ns*
17	1746	unknown[c]	sweet, floral	0.6	1.0	1.2	*ns*
18	1771	unknown[c]	fruity, floral	1.2	0.8	2.0	*ns*
19	1784	unknown[c]	sweet, cheese	0.0	0.0	0.4	*ns*
20	1805	unknown[c]	sweet	2.8b	0.0a	0.0a	***
21	1810	β-damascenone[b]	fruity, floral	0.0	0.8	1.4	*ns*
22	1843	unknown[c]	floral	2.2	1.8	0.0	*ns*
23	1862	guaiacol[b]	smoky, medicinal-like	2.2	2.2	2.4	*ns*
24	1912	2-phenylethanol[b]	floral, roses	2.4	2.2	2.6	*ns*
25	1969	unknown[c]	musty, mouldy	0.0a	0.0a	1.8b	***
26	2004	unknown[c]	vegetal, chemical	1.6b	0.0a	0.0a	*
27	2023	unknown[c]	sweet	0.6	0.0	0.0	
28	2038	Furaneol™[b]	burnt sugar, candy cotton	2.0b	0.0a	3.4c	***
29	2072	unknown[c]	musty, mouldy	1.4b	0.0a	1.0ab	*
30	2092	unknown[c]	animal, horse stable	0.0a	0.0a	0.8b	*
31	2118	unknown[c]	smoky, medicinal-like	0.0a	1.2b	0.8ab	*
32	2172	eugenol[b]	floral, sweet	2.0	1.0	0.8	*ns*
33	2193	unknown[c]	spicy	0.0a	0.0a	0.8b	*
34	2200	4-vinylguaiacol[b]	spicy, curry, smoky	2.4a	2.2	2.4	*ns*
35	2216	unknown[c]	herbaceous, fennel-like	0.0	1.2	1.2	*ns*
36	2244	unknown[c]	floral, fruity	1.2	1.6	0.0	*ns*
37	2256	unknown[c]	sweet, spicy	1.0b	0.0a	0.0a	*
38	2266	syringol[b]	medicinal-like, smoky	2.4	2.2	2.0	*ns*
39	2351	unknown[c]	floral	1.6	1.8	1.4	*ns*
40	2496	unknown[c]	soap-like	0.0	1.0	0.0	*ns*
41	2554	unknown[c]	plastic	1.2b	0.0a	0.0a	*
42	2563	vanillin[b]	vanilla, sweet	2.6	2.6	2.0	*ns*
43	2575	acetovanillone[b]	floral, sweet	1.4	1.4	1.8	*ns*

[a]Linear retention index on INNOWAX capillary column (30 m x 0.25 mm x 0.25 μm); [b]Identification based on coincidence of gas chromatographic retention indices and mass spectrometric data with those of the pure standards available in the lab; [c]Not identified compound; *ns* – not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, $p < 0.05$).

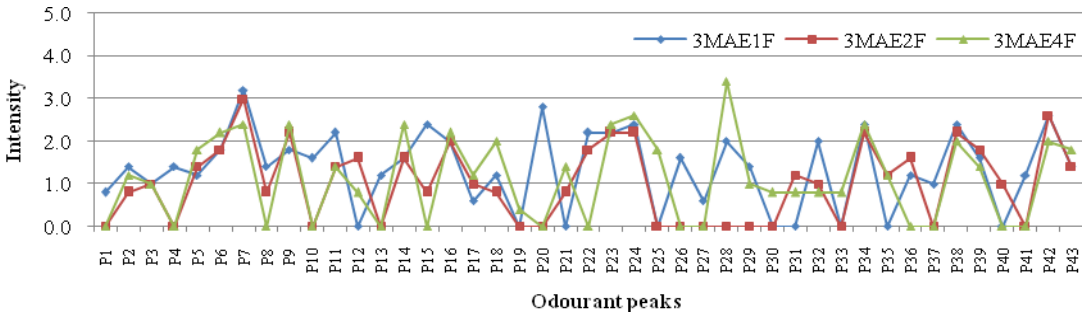


Fig. IV.3 – Odourant profiles of the three Aragonez clonal musts free fractions.

Principal component analysis (PCA) was applied to the posterior intensity method (GC-O) data of the three free fractions of Aragonez musts from the 2003 vintage in order to verify if it could be possible to clearly differentiate the free fractions. This multivariate analysis permitted the establishment of a relationship between the different odourant compound variables and the free fractions, as well as to find the most important factors of variability. The two principal components (PCs) found explained 100% of the total variance. The unknown odourant compound P3 was not considered for PCA analysis, because its variance was zero. Figure IV.4 shows in the two dimensional plot of PC1 against PC2 the locations of the forty-three GC-O peaks and the three free fractions of musts. The percentage value corresponding to each PC, presented in the figure, indicates the percentage of variation in the data explained by the PC's.

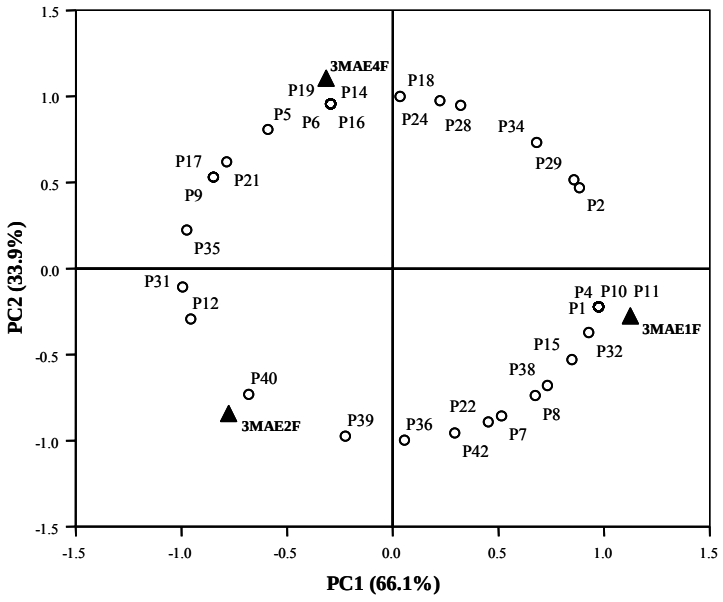


Fig. IV.4 - Plot of the first and second principal components (PCs) of the GC-O data and the three Aragonez must free fractions. The percentage of variation explained by each PC is indicated between brackets.

The free fractions 3MAE2F and 3MAE4F are located on the negative side of PC1 and on opposite quadrants in PC2. The 3MAE2F is on the negative side of PC2 and, in opposition, the

3MAE4F is located on the positive side. The 3MAE1F is located on the positive side of PC1 and negative side of PC2. According to the PCA plot, the 3MAE1F is strongly correlated with the unknown odourant compounds (P4, P10, P11, and P15), hexanal (P1), and eugenol (P32). On the other hand, the 3MAE4F is strongly correlated with three unknown odourant compounds (P6, P16 and P19), (*Z*)-hexen-3-ol (P5) and 3-methylbutanoic acid (P14). Finally, the PCA plot shows that 3MAE2F is highly correlated with an unknown odourant compound (P40).

4.3.2. Glycosidically-bound compounds in Aragonez musts

An example of the GC-O posterior intensity method application for bound odourant compound analysis, present in one bound fraction of Aragonez must, 3MAE1B, is presented in Figure IV.5.

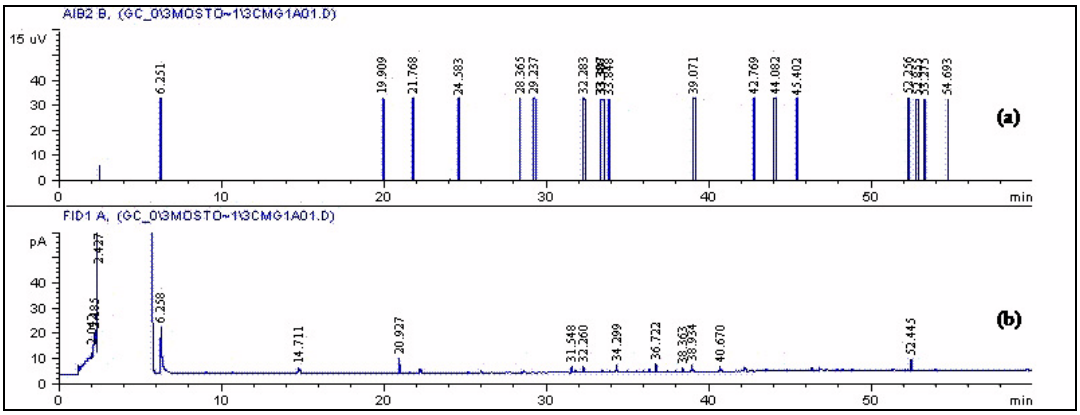


Fig. IV.5 - Simultaneous output of a chromatogram of the bound fraction extract, 3MAE1B, from Aragonez must by GC-FID **(b)** and respective aromagram **(a)**, obtained by one of the sniffers, in the Olfactory Detection Port (ODP).

Table IV.3 presents the odourant compounds found in the three Aragonez clonal musts bound fractions.

Twenty-two odourant peaks were perceived by the sniffers in at least one of the three clonal must bound fraction extracts according to the posterior intensity method and thirteen odourant compounds were identified by GC-MS. Analysing each clonal must bound fraction in separate, can be verified that the 3MAE4B fraction has the highest number of odourant compounds detected (19). The other two fractions, 3MAE1B and 3MAE2B, present a very similar number of odourant compounds detected, 17 and 16, respectively.

Table IV.3 - Odourant compounds found in three Aragonez clonal musts bound fractions: peak number, gas chromatographic retention data, compound identification, olfactory description, average scores and significance level.

Peak no.	LRI[a]	Compound	odour descriptor	3MAE1B	3MAE2B	3MAE4B	*Sig.*
1	<1100	hexanal[b]	herbaceous, cut grass	1.5	1.5	1.5a	*ns*
2	1452	unknown[c]	raw potatoes	2.8	1.5	2.0	*ns*
3	1501	unknown[c]	plastic	0.8	1.0	1.0	*ns*
4	1580	unknown[c]	fruity, melon	1.5	1.0	1.3	*ns*
5	1691	unknown[c]	vegetal	1.0	0.8	2.0	*ns*
6	1696	unknown[c]	sweet	0.0	0.0	0.8	*ns*
7	1728	unknown[c]	vegetal	1.5	0.5	0.0	*ns*
8	1752	TDN[d]	floral, sweet	0.0	0.0	1.0	*ns*
9	1811	β-damascenone[b]	floral, fruity, cooked apple	3.5	3.3	3.3	*ns*
10	1845	unknown[c]	floral	2.0	0.0	1.8	*ns*
11	1862	guaiacol[b]	smoky, medicinal-like	1.3	2.3	2.0	*ns*
12	1909	2-phenylethanol[b]	floral, roses	0.0	0.0	0.8	*ns*
13	1995	4-ethylguaiacol[b]	floral, carnation, clove	0.0	0.0	1.3	*ns*
14	2038	Furaneol™[b]	burnt sugar, candy cotton	3.3	3.5	3.8	*ns*
15	2138	unknown[c]	sweet	0.8	0.0	0.0	*ns*
16	2172	eugenol[b]	floral, spicy	1.3	0.8	1.8	*ns*
17	2200	4-vinylguaiacol[b]	smoky, burnt, curry	0.0a	2.3b	2.5b	**
18	2216	unknown[c]	sweet, floral	3.0	3.5	3.8	*ns*
19	2270	syringol[b]	medicinal-like, smoky	1.5	1.3	1.3	*ns*
20	2546	phenylacetic acid[b]	floral, sweet	1.0	0.5	0.8	*ns*
21	2564	vanillin[b]	vanilla	2.5	2.8	2.0	*ns*
22	2585	acetovanillone[b]	vanilla, sweet	0.8	1.5	0.0	*ns*

[a]Linear retention index on INNOWAX capillary column (30 m x 0.25 mm x 0.25 µm); [b]Identification based on coincidence of gas chromatographic retention indices and mass spectrometric data with those of the pure standards available in the lab; [c]Not identified compound; [d]Tentatively identified; *ns* – not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, $p < 0.05$).

Figure IV.6 shows the odourant profile of the three Aragonez clonal musts bound fractions. As can be seen, the three profiles are strongly similar among them, which was confirmed before by LSD test (Table IV.3). In fact, in only one odourant compound, 4-vinylguaiacol (P17) was a statistically significant difference found among the average score intensities of the clonal must fractions.

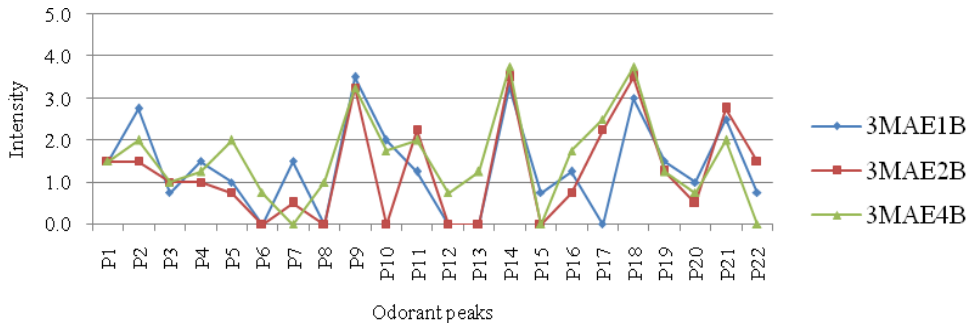


Fig. IV.6 – Odourant profiles of the three Aragonez clonal musts bound fractions.

Principal component analysis (PCA) was applied to the posterior intensity method (GC-O) data of the three bound fractions of Aragonez musts from the 2003 vintage in order to verify if it could be possible to clearly differentiate the bound fractions. This multivariate analysis allowed the establishment of a relationship between the different odourant compound variables and the bound fractions, and enabled the finding of the most important factors of variability.

The two principal components (PCs) found explained 100% of the total variance. The variable hexanal (P1) was not considered for PCA analysis because its variance was zero. Figure IV.7 shows in the two dimensional plot of PC1 against PC2 the locations of the twenty-one GC-O peaks and the three bound fractions of musts.

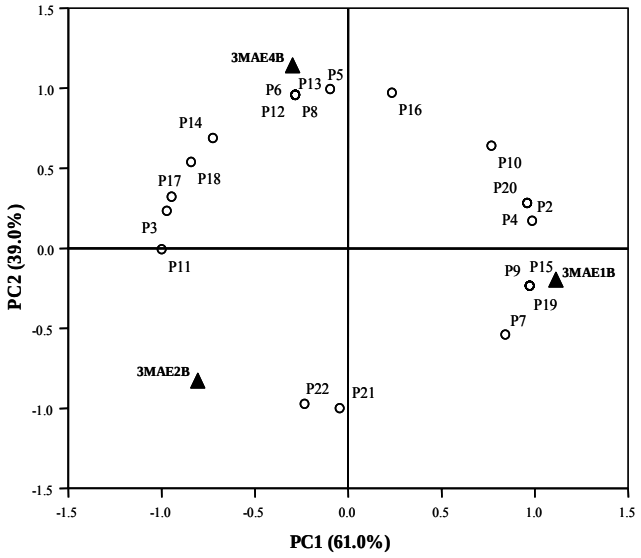


Fig. IV.7 - Plot of the first and second principal components (PCs) of the GC-O data and the three Aragonez musts bound fractions. The percentage of variation explained by each PC is indicated between brackets.

The bound fractions of musts 3MAE2B and 3MAE4B are located on the negative side of PC1 and on opposite quadrants in PC2. The 3MAE2B is on the negative side of PC2 and, in opposition, the 3MAE4B is located on the positive side. The 3MAE1B is located on the positive side of PC1 and negative side of PC2. According to the PCA plot, the 3MAE1B is strongly correlated with the unknown odourant compound (P7), β-damascenone (P9), unknown (P15) and syringol (P19). On the other hand, the 3MAE4B is strongly correlated with three unknown odourant compounds (P5, P6 and P8), 2-phenylethanol (P12) and 4-ethylguaiacol (P13). The PCA plot does not show any clear correlation between the 3MAE2B and the detected odourant compounds. Hexanal, found in our GC-O experiments with free and bound fractions of Aragonez musts, was not detected in GC-O analysis of Aragonez clonal wines (chapter 2) made by these musts. C_6 chain compounds were reported before in GC-O analysis of musts by Kotseridis and Baumes (2000). Besides, as in the study undertaken for this thesis, also López *et al*. (2004) found this odour-active compound in their experiments with Tempranillo juice hydrolysates. Five volatile phenols, among the identified odourants, were found and all of them are important odourants of red wines (Ferreira *et al*., 2000; Kotseridis and Baumes, 2000; Ferreira *et al*., 2001). For example, guaiacol (P11) has been previously reported in flavour precursor fractions of Syrah grapes (Bureau *et al*., 2000) and syringol (P19) in flavour precursor fractions of Merlot and Cabernet Sauvignon grapes (Francis *et al*., 1999). The authors López *et al*. (2004) suggested that some part of the odourant 4-ethylphenol, not detected by GC-O in the

free or bound fractions of Aragonez musts, derives directly from precursors present in the grapes. Vanillin was found in both free and glycosidically-bound form in the GC-O experiments. It is a well known constituent of flavour precursor fractions (Williams *et al*., 1989). This odourant compound was also found in the GC-O analysis of Aragonez and Trincadeira clonal wines which seems to indicate that vanillin as a free or glycosidically-bound compound derives from grapes.

From the shikimic acid-derivates group, 2-phenylethanol, benzaldehyde and phenylacetic acid were detected in our GC-O experiments. These odourant compounds were previously referred to by López *et al*. (2004) as components of Tempranillo and Grenache juice hydrolysates. 2-Phenylethanol has been detected in wine by GC-O (Kotseridis and Baumes, 2000; Aznar *et al*., 2001). With respect to C_{13}-norisoprenoids, only β-damascenone was found as odourant aglycon in our GC-O analysis of musts. This compound is a well-known component of many grape glycosidic fractions (Sefton *et al*., 1993; Francis *et al*., 1999; López *et al*., 2004) and it has been reported as an odour-active component of red young wines (Ferreira *et al*., 1998; López *et al*., 1999).

Monoterpenes were not found in our GC-O experiments, which is in agreement with the non-floral character of the Aragonez grapes. These results are also in accordance with those published by López *et al*. (2004), in which the poorness of monoterpenes is evident: only three odour-active terpenes were found in Tempranillo (syn. Aragonez) juice hydrolysates, citronellyl acetate, 3,7-dimethyloct-1-ene-3,7-diol and farnesol.

CHAPTER 5

Descriptive sensory analysis of the aroma of Aragonez and Trincadeira clonal wines

5. DESCRIPTIVE SENSORY ANALYSES OF THE AROMA OF ARAGONEZ AND TRINCADEIRA CLONAL WINES

5.1. INTRODUCTION

Wine is a product which contains numerous chemical compounds depending on the grape variety and maturity, climate and soil of the viticultural area, viticultural and vinification techniques. This causes a very large variation in quality and aroma profiles. Instrumental methods are precise and reproducible, but are not sufficient for a quality evaluation of wines considering their aroma. Descriptive sensory analysis is a useful and complementary tool for describing aroma profiles as well as for finding differences between wines. The purpose of this study was to obtain sufficient information from descriptive analysis of aroma of clonal wines to be able to differentiate clonal wines within Aragonez and Trincadeira cultivars.

5.2. MATERIALS AND METHODS

5.2.1. Samples

The Aragonez and Trincadeira clonal wine samples analysed by the sensory panel are described and codified in chapter 3 (section 3.2.2.).

5.2.2. Sensory panel

The sensory panel was composed by a group of 10 trained judges (4 males, 6 females; aged 25-62 years old) experienced in red wine taste analysis, referred to as letters A, B, C, D, E, F, G, H, I, and J.

5.2.3. Sensory attributes

The sensory panel scored the aroma quality, between 0 and 5 as well as the overall quality of the clonal wines from 0 (without quality) to 20 (maximum quality). This overall quality evaluation intends to represent the taster's opinion about the wine, considering three sensory components: colour, aroma and gustatory perception. Seven olfactory attributes were used: *sweet*, *herbaceous*, *animal*, *dried fruits*, *red fruits*, *spicy* and *woody*. The judge panel is familiarised with these descriptors which have been frequently applied by them in red wines tasting. The tasters were asked to score these attributes on a structured scale (0: no perception to 5: highest perception). According to the ISO 5492:1992, a descriptor is a term referring the assessor to an element of the perception of the product. The properties of the descriptor (relevance of the product, monodimensional) should be such that it could be used to produce an evaluation on a scale of intensity.

5.2.4. Procedure

The training of the judge panel consisted of descriptive sensory analysis of several red wines, during three months. After the training period, six wines were tasted per session, and they were presented to the judges in random order to eliminate first order carry-over effects (Williams, 1949). An amount of 30 mL of wine samples was given to each panel judge in wine tasting glasses at 20 ºC, under white natural lighting (ISO 3591:1977). All wines were evaluated twice in different sessions to assess panel and taster performance. For in-mouth evaluation, subjects sipped the samples and were required not to swallow it after determination of the attributes' intensities. Water was provided for mouth rinsing between samples.

5.2.5. Statistical analysis

The reliability of the panel was evaluated based on the calculation of Pearson correlation coefficients from the multi-judge correlation matrix (Brien *et al*., 1987; Lima *et al*., 1988; Caldeira *et al*., 2002; Caldeira, 2004). Pearson correlation coefficient calculations were performed using a Statgraphics statistical system, *v.* 5.0 (USA). The software package SPSS *v.* 14.0 for Windows (USA) was used for univariate (one-way ANOVA and LSD test) and multivariate (PCA, HCA and SLDA) statistical data treatment.

5.3. RESULTS AND DISCUSSION

5.3.1. Tasters' reliability

The reliability of the judge panel was assessed in two replicate sessions that took place per taster and per wine. The pairs of scores obtained by each taster for the 18 wines analysed were submitted to Pearson correlation coefficients calculations. The results obtained with the replicates presented at different sessions, for the aroma attributes, are presented in Table V.1.

Table V.1 - Correlation coefficients (*r*) of ten judges calculated from the scores obtained with 18 replicates at different sessions, considering aroma attributes.

Judge	Aroma attributes	
	r	***α***
A	0.96	0.000
B	0.95	0.000
C	0.96	0.000
D	0.96	0.000
E	0.97	0.000
F	0.97	0.000
G	0.98	0.000
H	0.95	0.000
I	0.96	0.000
J	0.96	0.000

α – Significance level of correlation coefficients

All the tasters presented high and significant correlation coefficients. A high similarity of the results of the aroma attributes was found. Hence, the scores given by the ten tasters were considered in the subsequent calculations.

5.3.2. Correlation between aroma attributes and wine aroma quality

In order to understand the relationship between aroma attributes and the aroma quality, the linear correlations between the scores of the attributes, averaged across tasters and the aroma quality of all clonal wines studied in the current work, were calculated. Table V.2 shows that the aroma descriptors *sweet*, *dried fruits*, *red fruits*, *spicy* ($p < 0.01$) and *woody* ($p < 0.05$) present significant positive correlation coefficients with the aroma quality of wines. On the other hand, the *herbaceous* and *animal* descriptors show negative correlation coefficients. However, only the last one was highly significant ($p < 0.01$).

Table V.2 – Linear correlation coefficients (Pearson correlation) between the attribute scores and the aroma quality of the clonal wines (data set used for calculation: scores from all wines averaged per wine across panelists, n=18 clonal wines).

Sensory attributes	Correlation Coefficient	Significance (2-tailed)
sweet	0.315**	0.000
herbaceous	-0.033	0.575
animal	-0.244**	0.000
dried fruits	0.471**	0.000
red fruits	0.356**	0.000
spicy	0.240**	0.000
woody	0.152*	0.010

*Correlation is significant at the $p < 0.05$ level; **Correlation is significant at the $p < 0.01$ level.

5.3.3. Correlation between aroma attributes and overall wine quality

The linear correlations between the attribute scores, averaged across tasters and the overall quality (colour, aroma and taste evaluation) of all clonal wines were also studied (Table V.3), in order to understand how the aroma attributes are related to the overall wine quality.

Table V.3 – Linear correlation coefficients (Pearson correlation) between the attribute scores and the overall quality of the clonal wines (data set used for calculation: scores from all wines averaged per wine across panelists, n=18 clonal wines).

Sensory attributes	Correlation Coefficient	Significance (2-tailed)
sweet	0.473*	0.048
herbaceous	-0.361	0.141
animal	-0.456	0.057
dried fruits	0.715**	0.001
red fruits	0.575*	0.013
spicy	0.710**	0.001
woody	0.536*	0.022

*Correlation is significant at the $p < 0.05$ level; **Correlation is significant at the $p < 0.01$ level.

As Table V.3 shows, five aroma attributes presented a positive correlation with the overall quality of the 18 clonal wines. The highest coefficients were found for *dried fruits and spicy* ($p < 0.01$). The other three attributes *sweet*, *red fruits* and *woody* were positively correlated with

the overall quality ($p < 0.05$). The negative correlation coefficients corresponded to *herbaceous* and *animal* attributes.

The study of all these correlations will be useful to explain the subsequent PCA analysis, in which the distribution and differentiation of clonal wines according to their average intensity aroma descriptors is shown.

5.3.4. Aroma quality appreciation of Aragonez and Trincadeira clonal wines

Figure V.1 shows that among the Aragonez clonal wines, the wines with the highest average intensity scores of aroma quality appreciation were 1AA4 (3.6) and 1AA3 (3.4). In opposition, the wine with the lowest average intensity scores of aroma quality appreciation was the 3AE4 (2.1). Regarding the Trincadeira clonal wines, the wines with the highest average intensity scores of aroma quality appreciation were 3T6 (3.4) and 3T5 (3.3). On the other hand, the wine with the lowest average intensity scores of aroma quality appreciation was 1T4 (2.1).

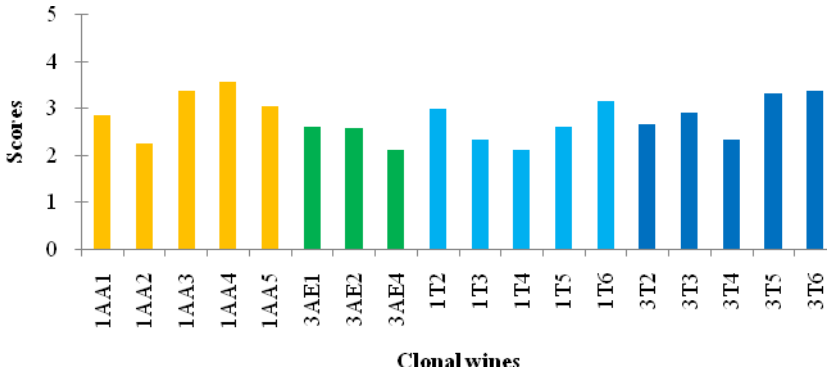


Fig. V.1 – Average scores of aroma quality appreciation of the Aragonez and Trincadeira clonal wines.

5.3.5. Overall quality appreciation of Aragonez and Trincadeira clonal wines

As can be seen in Figure V.2, among the Aragonez clonal wines, the wines with the highest average intensity scores of overall quality appreciation were 1AA4 (13.6) and 1AA3 (13.4). On the other hand, the wine with the lowest average intensity scores of aroma quality appreciation was 3AE4 (10.4).

Regarding the Trincadeira clonal wines, the wines with the highest average intensity scores of aroma quality appreciation were 1T6 (13.2) and 3T6 (13.1). On the other hand, the wine with the lowest average intensity score of aroma quality appreciation was 1T4 (10.0).

For the great majority of clonal wines, the results obtained for aroma quality and overall quality perception were coincident, which seems to demonstrate the importance of aroma quality in the definition of the overall wine quality.

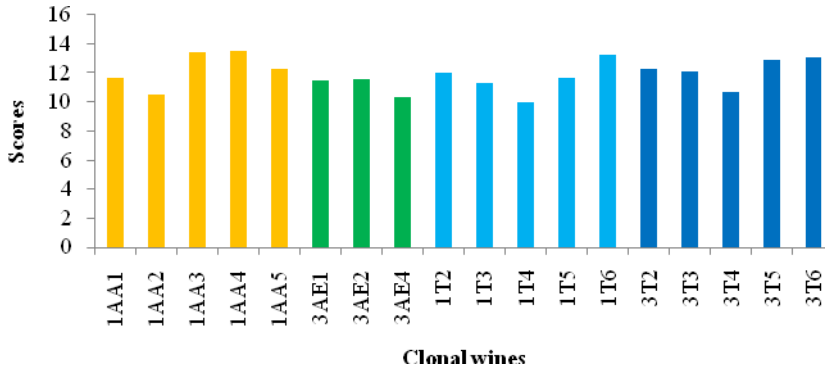


Fig. V.2 – Average scores of overall quality appreciation of the Aragonez and Trincadeira clonal wines.

5.3.6. Alentejo Aragonez clonal wines aroma evaluation

5.3.6.1. Aroma profiles of the five Aragonez clonal wines from the Alentejo DCO

The seven aroma attributes evaluated by the panelists, as described in section 5.2.3., were used for aroma profile characterisation of the five Aragonez clonal wines from the Alentejo Denomination of Controlled Origin and from the 2001 vintage (Table V.4). Statistically significant differences in the four aroma descriptors average of the five Aragonez clonal wines were found: *sweet*, *herbaceous*, *animal* and *dried fruits*. The clonal wines 1AA3 and 1AA4 presented the highest average values for the *sweet* descriptor. Relatively to the *herbaceous* descriptor, the wine 1AA5 had the highest score (1.4). The average scores of the *animal* and *woody* descriptors were low (below 1.0) for all the clonal wines. The *animal* descriptor is commonly associated to aroma depreciation of wines when its intensity value is considerable. In relation to the *red fruits* descriptor, it can be underlined that this descriptor presented the highest average score among overall descriptors. The clonal wine 1AA3 differs from some other wines due to its high average score (2.4) of *red fruits* descriptor. Moreover, the 1AA3 wine showed the highest average score of *sweet* descriptor. Both descriptors are greatly associated with the high aroma quality of wine.

Table V.4 – Average intensities of the aroma attributes and identification of statistically significant differences among clones by one-way ANOVA and LSD test analyses.

Descriptors	1AA1	1AA2	1AA3	1AA4	1AA5	*Sig.*
sweet	0.3a	0.5a	1.2b	1.1b	0.6a	*
herbaceous	0.6b	0.7b	0.0ac	0.5bc	1.4d	***
animal	0.2ab	0.6b	0.0a	0.1a	0.1a	*
dried fruits	0.7ab	0.3b	0.9a	1.3a	0.8ab	**
red fruits	1.8	1.4	2.4	1.8	1.6	*ns*
spicy	0.6	0.2	0.5	0.7	0.5	*ns*
woody	0.1	0.0	0.1	0.2	0.0	*ns*

ns: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

In order to better visualise the aroma profiles of the five Aragonez clonal wines, Figure V.3 is presented.

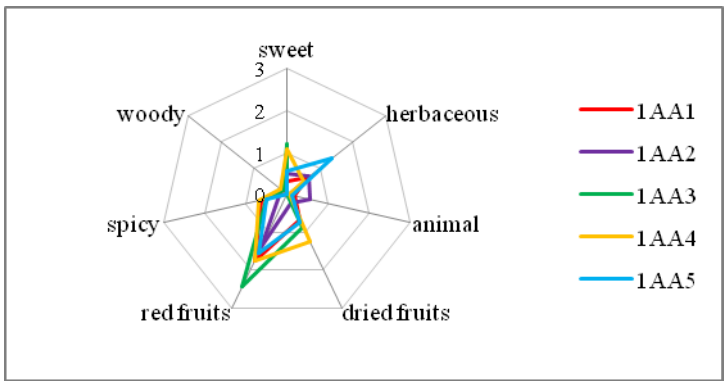


Fig. V.3 – Spider web plot showing the five Aragonez clonal wines. Average scores of attribute intensity are shown as the distance from the center.

5.3.6.2. Differentiation of Aragonez clonal wines from the Alentejo DCO

Principal component analysis (PCA) was applied to the average attribute scores of each wine regarding aroma attributes, in order to describe the group of sensory data, to establish the relationships between the different sensory variables and wines, and to detect the most important factors of variability. As summarised in Table V.5, two principal components were found accounting for 85.6% of the total variance. The Varimax rotated factor loadings are shown in Table V.6; these are the correlations between the PCs and the original data. Loadings with an absolute value greater than 0.700 (shown in bold type) represent a strong influence (Siebert, 1999). Some authors applied the principal component analysis to study the aroma attributes of wines. For example, Presa-Owens and Noble (1995), using eight aroma attributes to characterise three Spanish white wines, performed a PCA analysis and found three principal components that accounted for 70% of the total variance.

Table V.5 - Principal component analysis (PCA) and eigenvalues associated with the factor analysis.

PC	Percentage of each PC (%)	Cumulative variance (%)	Eigenvalue
1	65.4	65.4	4.577
2	20.2	85.6	1.416

Table V.6 - Varimax rotated principal component factor loadings for aroma attributes of Aragonez clonal wines.

Aroma attributes	PC1	PC2
sweet	0.372	**0.777**
herbaceous	-0.018	**-0.947**
animal	**-0.862**[a]	-0.344
dried fruits	**0.930**	0.267
red fruits	0.346	**0.843**
spicy	**0.975**	0.036
woody	**0.714**	0.516

[a]Loadings with an absolute value greater than **0.700** are shown in bold type.

Figure V.4 shows in the two dimensional plot of PC1 against PC2, the locations of the seven aroma attributes and the five clonal wine samples. The first principal component (PC1) was

characterised by the contrast of *spicy*, *dried fruits* and *woody* attributes having a positive loading, while the *animal* attribute displayed a negative loading. In fact, *animal* could be an undesirable aroma attribute of wine aroma if present in considerable intensity. As to the second PC, the attributes *red fruits* and *sweet* showed a positive loading, while *herbaceous* attribute was loading negatively on PC2. Moreover, the PCA analysis showed that the positive side PC1 and PC2 of the PCA plot was characterised by the high aroma quality descriptors. For this reason, the wines located in this quadrant presented higher aroma quality than the others, due to their positive correlation with those descriptors. Besides, the negative side of PC1 and PC2 of the PCA plot was characterised by the *animal* descriptor which is positively correlated with low aroma quality of wines. Thus, when a wine is located in that quadrant, it means that the wine has a high average intensity in *animal* descriptor.

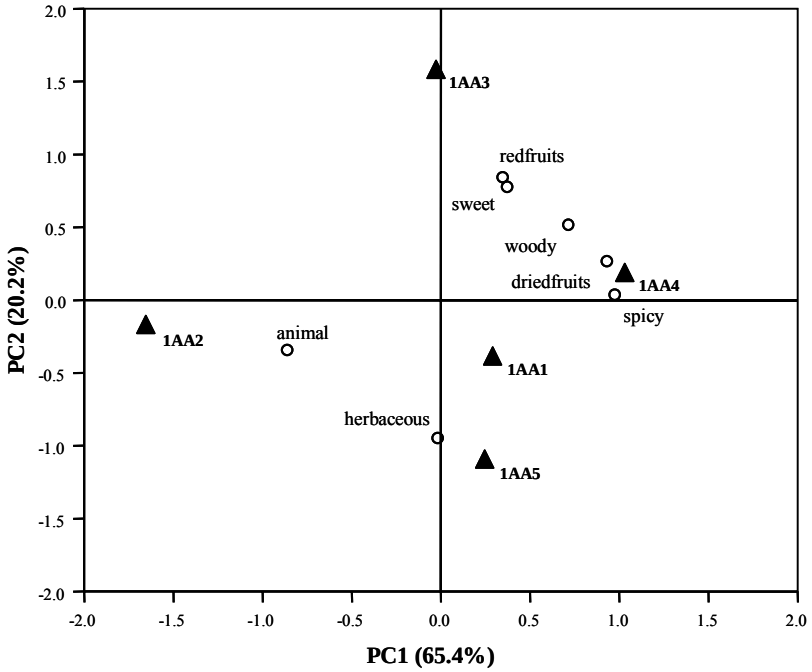


Fig. V.4 - Plot of the first and second principal components (PCs) of the aroma descriptors data and the five Aragonez clonal wines. The percentage of variation explained by each PC is indicated between brackets.

According to the distribution of clonal wines from the 2001 vintage, in the product space (PC1 X PC2), wines 1AA1 and 1AA5 are close to one another and are located on the positive side of PC1 and on the negative side of PC2. In fact, the aroma profile of these wines is very similar, as demonstrated by the LSD test (Table V.4) in which only the *herbaceous* descriptor showed statistically significant differences between both wines. The wine 1AA4 is also on the positive side of PC1 but on the positive side of PC2. The wine 1AA2 is located on the negative side of PC1 and negative side of PC2.

Finally, wine 1AA3 is on the PC1 axis and on the positive side of PC2. The wines 1AA3 and 1AA4 present high positive correlation with the descriptors *red fruits*, *sweet*, *woody*, *dried fruits* and *spicy* which seems to indicate that both wines have higher aroma quality than the others.

Furthermore, wine 1AA2 is positively correlated to the *animal* descriptor and 1AA5 is positively correlated with the *herbaceous* descriptor. These two last correlations indicate that both wines have a lower aroma quality. In fact, according to the Table V.4, they have the highest average intensity for the descriptors *animal* and *herbaceous*, 0.6 and 1.4, respectively.

5.3.7. Estremadura Aragonez clonal wines aroma evaluation

5.3.7.1. Aroma profiles of the three Aragonez clonal wines from the Estremadura DCO

Aroma profile characterisation of the three Aragonez clonal wines from the Estremadura DCO and from the 2003 vintage was done using the seven aroma attributes described before (section 5.2.3.).

Table V.7 presents the average intensities of these seven attributes and the results of its statistical analysis to find differences among clones. Statistically significant differences among the three clonal wines were found only for the *animal* descriptor. The three Aragonez clonal wines were very similar, as the average of the other six aroma descriptors was not statistically different.

Table V.7 – Average intensities of the aroma attributes and identification of statistically significant differences among clones by one-way ANOVA and LSD test analyses.

Descriptors	3AE1	3AE2	3AE4	*Sig.*
sweet	0.8	0.7	0.4	*ns*
herbaceous	0.8	0.5	0.5	*ns*
animal	0.1a	0.0a	0.5b	*
dried fruits	0.3	0.1	0.0	*ns*
red fruits	1.7	1.5	0.8	*ns*
spicy	0.4	0.2	0.1	*ns*
woody	0.0	0.0	0.0	*ns*

ns: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

Regarding the *woody* descriptor, the average intensity was set to zero (0.0) for all wines which indicate that none of the wines reveal *woody* character in their aroma profile. In relation to the *red fruits* descriptor, it can be emphasised that this descriptor presented the highest average score among overall descriptors. The clonal wine 3AE1 differs statistically from wine 3AE3 due to its high average score (1.7). Furthermore, wine 3AE1 showed the highest average value of *sweet* descriptor. Finally, it should be underlined that wine 3AE4 showed average scores less than 1.0 for all aroma descriptors.

Figure V.5 shows the aroma profiles of the three Aragonez clonal wines.

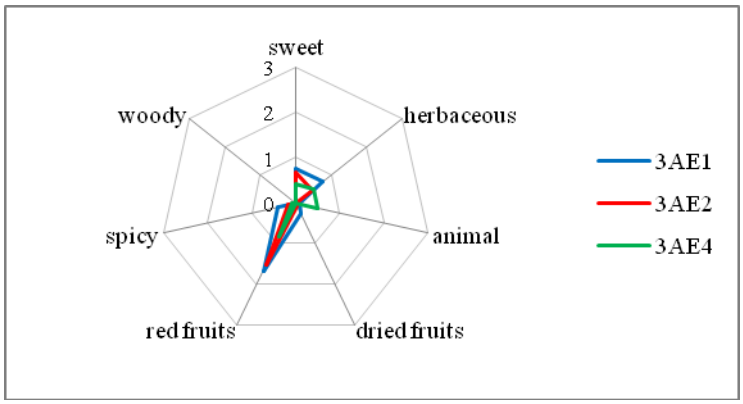


Fig. V.5 – Spider web plot showing the three Aragonez clonal wines. Average scores of attribute intensity are shown as the distance from the center.

5.3.7.2. Differentiation of Aragonez clonal wines from the Estremadura DCO

As summarised in Table V.8 two principal components were found accounting for 100.0% of the total variance. The Varimax rotated factor loadings are shown in Table V.9.

Table V.8 - Principal component analysis (PCA) and eigenvalues associated with the factor analysis.

PC	Percentage of each PC (%)	Cumulative variance (%)	Eigenvalue
1	82.7	82.7	4.964
2	17.3	100.0	1.036

Table V.9 - Varimax rotated principal component factor loadings for aroma attributes of Aragonez clonal wines.

Aroma attributes	PC1	PC2
sweet	**0.863**[a]	0.504
herbaceous	0.129	**0.992**
animal	**- 0.992**	-0.130
dried fruits	0.540	**0.841**
red fruits	**0.889**	0.459
spicy	0.462	**0.887**

[a]Loadings with an absolute value greater than **0.700** are shown in bold type.

Figure V.6 shows in the two dimensional plot of PC1 against PC2, the locations of the seven aroma attributes and the three clonal wine samples. The first principal component (PC1) was characterised by the contrast of *red fruits* and *sweet* attributes, having a positive loading with the *animal* attribute displaying a negative loading. As to the second PC, the attributes *herbaceous*, *spicy* and *dried fruits* showed a positive loading. Moreover, the PCA analysis showed that the positive quadrant (positive PC1 and positive PC2) of PCA plot was characterised by the high aroma quality descriptors. For this reason, the wines located in this quadrant presented a higher aroma quality than the others, because of their positive correlation with those descriptors. Moreover, the negative quadrant (negative PC1 and negative PC2) of the PCA plot was characterised by the *animal* descriptor which is positively correlated with low aroma quality of wines. Thus, when a wine is located in that quadrant it means that the wine has a high average intensity in *animal* descriptor.

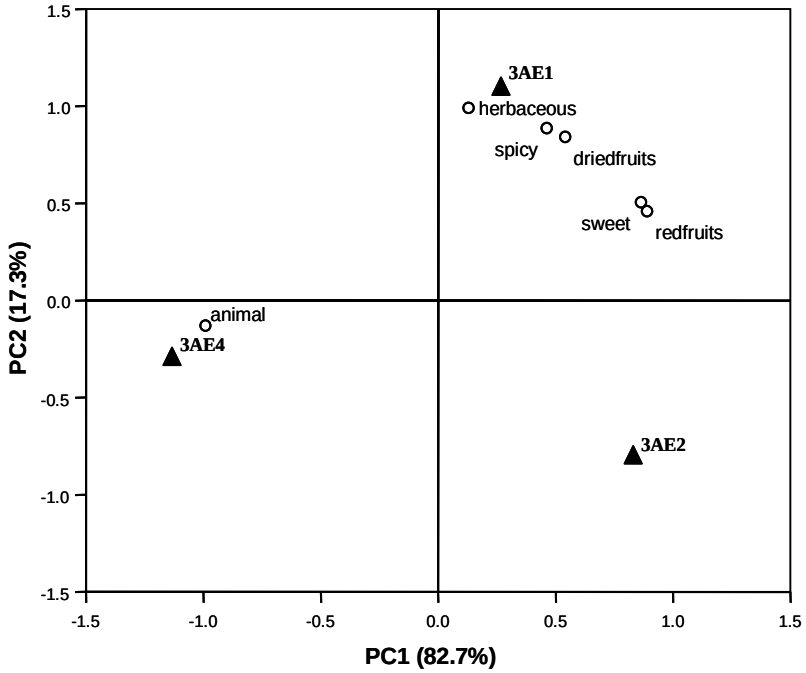


Fig. V.6 - Plot of the first and second principal components (PCs) of the aroma descriptors data and the three Aragonez clonal wines. The percentage of variation explained by each PC is indicated between brackets.

According to the distribution of clonal wines from the 2003 vintage, in the product space PC1 X PC2, the wines 3AE1, 3AE2 and 3AE4 are distant from each other. The wines 3AE1 and 3AE2 are located on the positive side of PC1. Wine 3AE1 is located on the positive side of PC2 while 3AE2 is located on the negative side of PC2. Wine 3AE4 is located on the negative side of both PC1 and PC2 and is positively highy correlated with the *animal* descriptor. This correlation is in accordance with the ANOVA and LSD test results previously shown in Table V.7. The 3AE1 and 3AE2 wines are located on the opposite sides of PC2 in spite of having similar intensity average attributes as shown in Table V.7. This separation on the PCA plot could probably be related to the fact that in wine 3AE1 all the average intensity attributes are relatively greater than those of 3AE2. However, these differences in average intensity attributes are not statistically significant (Table V.7).

5.3.8. Ribatejo Trincadeira clonal wines aroma evaluation

5.3.8.1. Aroma profiles of the five Trincadeira clonal wines from the Ribatejo DCO

Aroma profile characterisation of the five Trincadeira clonal wines from the Ribatejo DCO and from the 2001 and 2003 vintages was done using the seven aroma attributes described before (section 5.2.3.). Table V.10 shows the average intensities of these seven attributes and the results of its statistical analysis to find differences among clones and vintages. In the 2001 vintage, statistically significant differences in two aroma descriptors, *animal* and *dried fruits*, were found. The 1T6, 1T3 and 1T2 wines showed the highest average scores of *dried fruits* descriptor, with average scores of 1.1, 1.0 and 0.7 respectively.

Table V.10 – Average intensities of the aroma attributes and identification of statistically significant differences among clones by one-way ANOVA and LSD test analyses.

	Vintage 2001						Vintage 2003						
Descriptors	**1T2**	**1T3**	**1T4**	**1T5**	**1T6**	***Sig.***	**3T2**	**3T3**	**3T4**	**3T5**	**3T6**	***Sig.***	**Vintage effect**
sweet	0.5	0.5	0.7	0.3	0.6	*ns*	0.9	0.3	0.5	0.7	0.7	*ns*	*ns*
herbaceous	0.4	0.5	1.1	0.8	0.7	*ns*	1.0	0.7	1.0	0.8	0.7	*ns*	*ns*
animal	0.0a	0.1a	0.0a	0.4b	0.1a	**	0.0a	0.7b	0.8b	0.0a	0.0a	***	*
dried fruits	0.7ab	1.0ab	0.3a	0.3a	1.1b	**	0.3	0.4	0.2	0.4	0.3	*ns*	*ns*
red fruits	1.6	1.9	1.7	1.6	1.4	*ns*	2.1b	1.5b	0.9a	2.0b	2.1b	*	*ns*
spicy	0.2	0.6	0.0	0.2	0.5	*ns*	0.5	0.2	0.2	0.2	0.4	*ns*	*ns*
woody	0.1	0.1	0.0	0.2	0.2	*ns*	0.0	0.0	0.0	0.0	0.1	*ns*	*ns*

ns: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

The average scores of the *sweet*, *red fruits* and *woody* descriptors were similar for all the clonal wines. In relation to the *red fruits* descriptor, it should be emphasised that this descriptor presented the highest average score among overall descriptors. The clonal wine 1T4 presented the highest average score (1.1) for the *herbaceous* descriptor. On the other hand, the 1T2 wine showed the lowest average score (0.4). Regarding the *animal* descriptor, wine 1T5 had the highest score (0.4), which is statistically different from the other four wines. Table V.10 shows that in the 2003 vintage statistically significant differences were found in two aroma descriptors, *animal* and *red fruits*. In relation to the *animal* descriptor, it should be underlined that this descriptor presented the highest average scores in the 3T3 (0.7) and 3T4 (0.8) wines and, in opposition, it was not detected in wines 3T1, 3T2 and 3T5. Wine 3T4 showed the lowest average score (0.9) of *red fruits* descriptor, which was statistically different from the average scores of the other four wines. The *woody* descriptor was only detected in the 3T6 wine with an average score of 0.1. Finally, analysing the vintage effect on the descriptor average differences, statistically significant differences were found only in the *animal* descriptor averages. In order to better visualise the aroma profiles of the five Trincadeira clonal wines, from the 2001 and 2003 vintages, two spider web plots are presented in Figures V.7. and V.8.

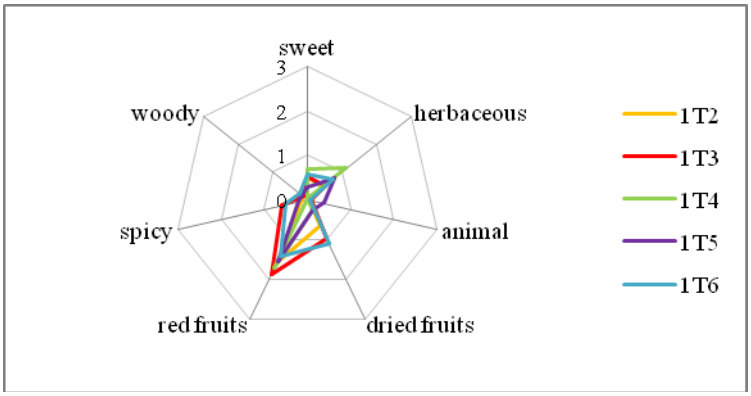


Fig. V.7 – Spider web plots showing the five Trincadeira clonal wines from the 2001 vintage. Average scores of attribute intensity are shown as the distance from the center.

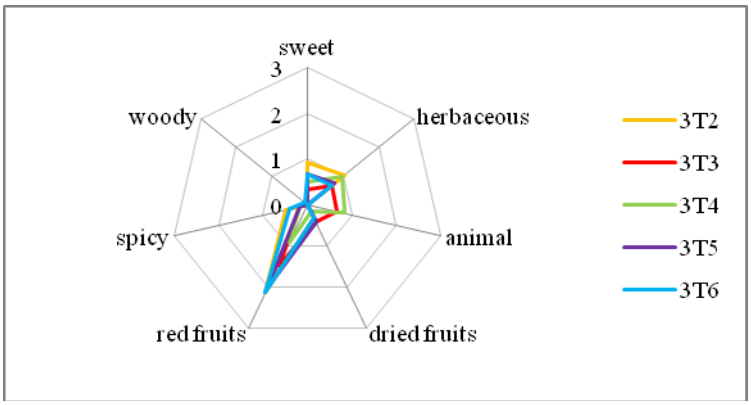


Fig. V.8 – Spider web plots showing the five Trincadeira clonal wines from the 2003 vintage. Average scores of attribute intensity are shown as the distance from the center.

5.3.8.2. Differentiation of Trincadeira clonal wines from the Ribatejo DCO

As summarised in Table V.11 two principal components were found accounting for 71.7% of the total variance.

Table V.11 - Principal component analysis (PCA) and eigenvalues associated with the factor analysis.

PC	Percentage of each PC (%)	Cumulative variance (%)	Eigenvalue
1	37.4	37.4	2.618
2	34.3	71.7	2.401

The Varimax rotated factor loadings are shown in Table V.12; these are the correlations between the PCs and the original data.

Table V.12 - Varimax rotated principal component factor loadings for aroma attributes of Trincadeira clonal wines.

Aroma attributes	PC1	PC2
sweet	**0.869**[a]	-0.335
herbaceous	0.180	**-0.731**
animal	**-0.861**	-0.236
dried fruits	0.178	**0.857**
red fruits	**0.851**	-0.097
spicy	0.547	0.460
woody	-0.027	**0.881**

[a]Loadings with an absolute value greater than **0.700** are shown in bold type.

Figure V.9 shows in the two dimensional plot of PC1 against PC2, the locations of the seven aroma attributes and the 10 clonal wine samples. The first principal component (PC1) was characterised by the contrast of *red fruits* and *sweet* attributes having a positive loading and the *animal* attribute displaying a negative loading. For the second PC, the attributes *woody* and *dried fruits* showed a positive loading and *herbaceous* showed a negative loading.

According to the distribution of clonal wines from the 2001 vintage, in the product space PC1 X PC2, all these wines are located on the positive side of PC1. The 1T2, 1T5 and 1T6 wines are on the positive side of PC2 and in opposition, the 1T3 and 1T4 wines are located on the negative side. The 1T3 and 1T4 wines are positively correlated with *red fruits*, *sweet* and *herbaceous* descriptors. The 1T2, 1T5 and 1T6 are positively correlated with *dried fruits*, *woody*

and *spicy*. Wine 1T6 is distant from the other two, mainly due to the positive correlation of this wine with the *dried fruits* attribute.

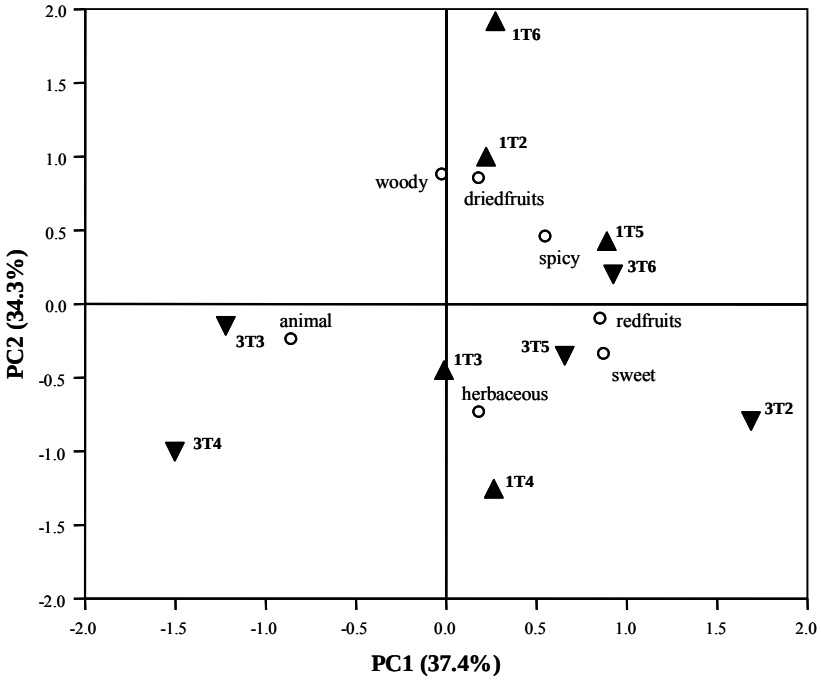


Fig. V.9 - Plot of the first and second principal components (PCs) of the aroma descriptors data and the five Trincadeira clonal wines from the 2001 and 2003 vintages. The percentage of variation explained by each PC is indicated between brackets.

In fact, this wine presents the highest average intensity of *dried fruits* attribute (1.1) as shown in Table V.10. With respect to the wines from the 2003 vintage, all the wines are located on the negative side of PC2 with the exception of 3T6. The 3T1, 3T2 and 3T5 wines are located on the positive side of PC1 while 3T3 and 3T4 wines are located on the negative side. The 3T3 and 3T4 wines are positively correlated with the *animal* descriptor and for this reason they are distant from the other wines. The 3T2, 3T5 and 3T6 wines are influenced by *herbaceous*, *red fruits* and *sweet* descriptors. However, wines 3T5 and 3T6 are distant from wine 3T2 because the last one has the lower average intensity of descriptor scores (Table V.10). Wine 3T5 is distant way from wine 3T6 mainly due to it highest average intensity score of *herbaceous* descriptor. Regarding the ten clonal wines on the PCA plot, it should be mentioned that only two wines, 3T3 and 3T4, are located on the negative side of PC1. Besides, both wines are positively correlated with *animal* descriptor which seems to show that these wines have a depreciative influence of *animal* descriptor in their aroma quality. The wines 3T6 and 3T5 were the best classified wines due to their high average aroma quality related descriptors. On the other hand, wine 3T4 was the one with the worst aroma quality. It must be highlighted that the clonal wine T6 showed the best aroma quality in the 2001 and 2003 vintages. In opposition, T4 clonal wine presented the lowest aroma quality.

With the objective of clarifying the similarities or dissimilarities among the five Trincadeira clonal wines in the two vintages, a hierarchical cluster analysis (HCA) was also done. Figure V.10 shows the dendogram obtained using the Ward method. The dendogram displays three clusters of wines in which the similar wines were classified. In the cluster with more elements, the 1T3, 3T5, 3T6, 3T2 and 1T4 wines were grouped together according to their similarities. In the cluster with the middle number of elements were wines 1T5, 3T3 and 3T4. Finally, wines 1T2 and 1T6 are grouped in another cluster.

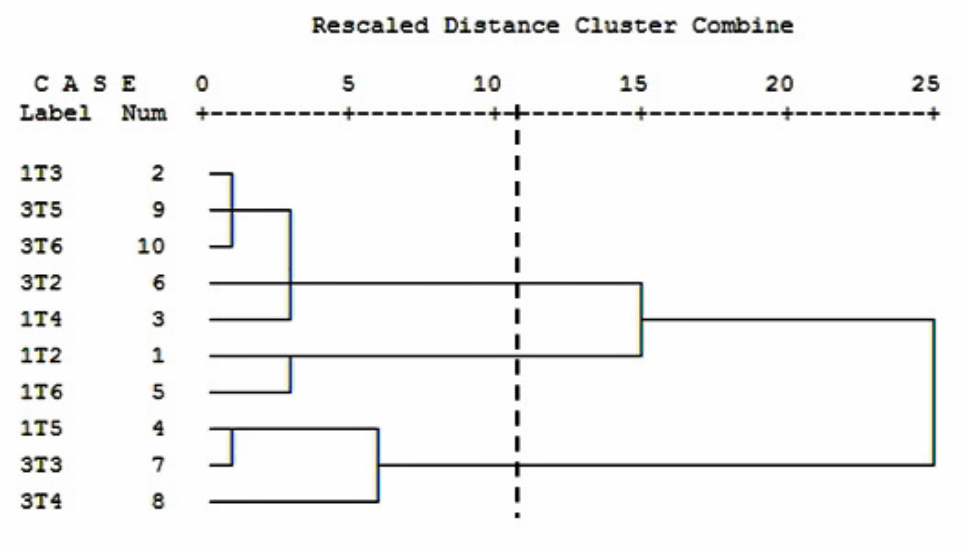


Fig. V.10 – Dendogram of Trincadeira clonal wines using the Ward method.

5.3.8.3. Discrimination of the five Trincadeira clonal wines between 2001 and 2003 vintages

After the PCA analysis of the Trincadeira clonal wines, a stepwise linear discriminant analysis (SLDA), using the aroma descriptors data, was performed in order to discriminate the five clonal wines under study. Table V.13 presents the number of steps, the selected variables, the value of *F*-to-remove of selected variable, the significance level (*Sig.*), and the standardised coefficients of discriminant functions (DFs). According to these results, three variables, *woody*, *spicy* and *sweet*, were found as discriminating variables.

Table V.13 – Stepwise linear discriminant analysis according to vintage (years, 2001 and 2003).

Step	Selected variable	F-to-remove of selected variable	Standardised coefficients of DF
1	*woody*	6.811	2.657
2	*spicy*	7.969	-2.616
3	*sweet*	5.545	1.383
Eigenvalues of DFs			6.618
p-values of DFs			0.004

Table V.14 presents the percentage of correctly classified clonal wines and shows that 100.0% of original grouped cases were correctly classified. In fact, considering the 2001 and 2003

vintages, the discriminant function obtained allowed the classification of all the wines in their correct groups.

Table V.14 - Percentage of correctly classified Trincadeira clonal wines.

			Predicted group membership		Total
	Wine year		2001	2003	
Original	Count	2001	5	0	5
		2003	0	5	5
	%	2001	100.0	0.0	100.0
		2003	0.0	100.0	100.0

In agreement with the results obtained in chapter 3, in which the application of GC-O analysis to the Trincadeira clonal wines allowed a good clonal wine separation from different vintages, also by sensory analysis through the SLDA, a classification of all the wines in their correct groups, regarding vintage year, was found. This result is particularly interesting as other studies (Falco, 2004) were unable to discriminate four Touriga Nacional clonal wines analysed by sensory analysis during three consecutive vintages.

The clear separation of the five Trincadeira clonal wines between the two vintages achieved by GC-O and descriptive sensory analyses revealed an important accordance between both analyses which highlighted their complementarity.

CHAPTER 6

Quantification of volatile compounds in clonal grapes and wines from Aragonez and Trincadeira

6. QUANTIFICATION OF VOLATILE COMPOUNDS IN CLONAL GRAPES AND WINES FROM ARAGONEZ AND TRINCADEIRA

6.1. INTRODUCTION

The aroma of wines and grapes is exceptionally complex, with contributions from many hundreds, possibly thousands of volatile compounds. After the recognition of volatile compounds that may contribute to the aroma of wines and grapes, it is important to improve our knowledge about their relative concentration in samples. Thus, the aim of this research was to determine volatile composition of clonal wines and the respective musts and grapes from Aragonez and Trincadeira cultivars.

6.2. MATERIALS AND METHODS

6.2.1. Samples

6.2.1.1. Aragonez and Trincadeira grapes

The grape samples of the certified clones from Aragonez and Trincadeira cultivars, used in this study, are codified in Table VI.1.

Table VI.1 – Codes of Aragonez and Trincadeira clonal grapes.

ARAGONEZ				TRINCADEIRA			
	Juices		Skins		Juices		Skins
Certified clone	Free fractions	Bound fractions	Free fractions	Certified clone	Free fractions	Bound fractions	Free fractions
T 54 EAN (PT)	3JAE1F	3JAE1B	3SAE1F	T 11 EAN (PT)	3JT2F	3JT2B	3ST2F
T 56 EAN (PT)	3JAE2F	3JAE2B	3SAE2F	T 12 EAN (PT)	3JT3F	3JT3B	3ST3F
T 58 EAN (PT)	3JAE4F	3JAE4B	3SAE4F	T 13 EAN (PT)	3JT4F	3JT4B	3ST4F
				T 14 EAN (PT)	3JT5F	3JT5B	3ST5F
				T 15 EAN (PT)	3JT6F	3JT6B	3ST6F

The extraction of the volatile compounds from the free fraction of juices and skins as well as from the bound fractions of juices was similar to the one used for musts (section 4.2.3., chapter 4). However, in the specific case of the preparation of juices and skin samples the procedure was performed as described below.

6.2.1.1.1. Juices

Juices were obtained through the use of a hand-crusher which presses the whole berries; the clarification of juices was done by centrifugation at 4 ºC (15000 rpm, Sorvall RC-5B, Newtown, USA) for 15 min. An amount of 100 mL of clarified juice sample was taken for free fractions analysis, while 200 mL of clarified juices were fractionated by solid-liquid chromatography using LiChroprep RP-18 non ionic resin in a glass column (30 x 3 cm i.d.). The glycosidically-bound compounds were then released as free aglycones by acid hydrolysis and extracted as previously described in section 4.2.3.3 (chapter 4).

6.2.1.1.2. Skins

The skins were obtained after peeling frozen whole berries of each clone. A total of 50 g of skins was put in contact with 100 mL of a hydroalcoholic solution (10% v/v) during 24 h in a dark place with a 20ºC controlled temperature. This mixture was stirred at 120 rpm in a stopped glass flask. Over the time, the liquid part was obtained by centrifugation at 5000 rpm during 4 min, at 4ºC (Sorvall RC-5B, Newtown, USA). Then, the extraction of free fraction of volatile compounds from the hydroalcoholic extract was done as was for the must or juice samples. An aliquot of 100 µL of 4-nonanol (82.7 $mg.L^{-1}$) was added before extraction as an internal standard.

6.2.1.2. Aragonez and Trincadeira clonal musts

Free and glycosidically-bound fractions of clonal musts were codified as Table VI.2 shows.

Table VI.2 – Codes of Aragonez and Trincadeira clonal musts.

ARAGONEZ			TRINCADEIRA		
2003 Vintage Estremadura			2003 Vintage Ribatejo		
Certified clone	**Free fractions**	**Bound fractions**	**Certified clone**	**Free fractions**	**Bound fractions**
T 54 EAN (PT)	3MAE1F	3MAE1B	T 11 EAN (PT)	3MT2F	3MT2B
T 56 EAN (PT)	3MAE2F	3MAE2B	T 12 EAN (PT)	3MT3F	3MT3B
T58 EAN (PT)	3MAE4F	3MAE4B	T 13 EAN (PT)	3MT4F	3MT4B
			T 14 EAN (PT)	3MT5F	3MT5B
			T 15 EAN (PT)	3MT6F	3MT6B

6.2.1.3. Aragonez and Trincadeira clonal wines

Aragonez and Trincadeira clonal wines were previously codified in chapter 3 (section 3.2.2.).

6.2.2. GC-FID analysis

The GC analysis was carried out in an Agilent Technologies 6890N series chromatograph equipped with a flame ionization detector (FID) and a fused silica capillary column of polyethylene glycol (INNOWAX, J&W Scientific, Agilent Technologies, USA) of 30 m, 0.32 mm i.d., and 0.25 µm film thickness. The injection volume was approximately 0.6 µL and 1.2 µL for wine and must or grape extracts, respectively. Operating conditions were as follows: injector and detector at 250 ºC; carrier gas hydrogen at a flow rate of 2.0 mL min^{-1} and split ratio 1:3; the temperature gradient used began at 45 ºC for 5 min, and was raised to 210 ºC at 3.5 ºC min^{-1} and was held at this temperature for 20 min. The compounds were quantified as 2-octanol (wines) or 4-nonanol (musts and grapes) equivalents. The extracts were obtained in duplicate and the GC-FID analysis was done twice for each sample extract.

6.3. RESULTS AND DISCUSSION

6.3.1. Characterisation of Aragonez grapes, musts and wines from the Estremadura DCO

6.3.1.1. Volatile quantification in Aragonez clonal grapes

6.3.1.1.1. Aragonez clonal juices

Aragonez clonal juices from the Estremadura DCO and from the 2003 vintage were analysed by FTIR analysis and are presented in Table VI.3. Statistically significant differences among Aragonez juices for potential alcohol, TA and pH were not found.

Table VI.3 – Analytical results of the three Aragonez clonal juices by FTIR analysis (n=4).

Clonal musts		Volumic mass ($g.mL^{-1}$)	Sugars ($g.L^{-1}$)	Potential alcohol (% vol.)	TA ($g.L^{-1}$ tartaric acid)	pH
3JAE1	*x*	1.09b	197.25c	11.6	4.75	3.33
	SD	*0.00*	*0.21*	*0.00*	*0.17*	*0.00*
3JAE2	*x*	1.08a	186.25b	10.9	4.85	3.21
	SD	*0.00*	*0.07*	*0.00*	*0.07*	*0.00*
3JAE3	*x*	1.08a	179.6a	10.5	5.05	3.26
	SD	*0.00*	*0.14*	*0.00*	*0.07*	*0.00*
Clonal effect		*	***	*ns*	*ns*	*ns*

x: average; *SD:* standard deviation*; ns*: not significant; * Significant ($p < 0.05$); ** Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same column, are not significantly different (LSD, 0.05).

The volatile compounds quantified by GC-FID analysis and the statistical significance of clone effect on average concentration values of volatile compounds in the three free fractions of Aragonez juices are presented in Table VI.4.

Table VI.4 – Average concentrations of volatile compounds (μg 4-nonanol.dm^{-3}) in the free fractions of clonal Aragonez juices (n=2).

Compounds	3JAE1F	3JAE2F	3JAE4F	Clonal effect
hexanal	178.06ab	150.34a	207.34b	*
SD	*0.46*	*0.14*	*0.34*	
(E)-2-hexenal	72.06a	85.52a	170.08b	***
SD	*0.17*	*0.06*	*0.19*	
1-hexanol	44.20a	71.17c	57.92b	***
SD	*0.09*	*0.05*	*0.07*	
(Z)-3-hexen-1-ol	35.58b	37.95b	26.45a	**
SD	*0.06*	*0.03*	*0.03*	
(E)-2-hexen-1-ol	15.94a	46.62c	26.40b	***
SD	*0.03*	*0.03*	*0.03*	
hexanoic acid	52.72a	51.23a	58.06b	*ns*
SD	*0.06*	*0.08*	*0.09*	
benzyl alcohol	61.66b	50.89a	50.46a	*
SD	*0.04*	*0.07*	*0.09*	
2-phenylethanol	23.15	21.02	19.37	*ns*
SD	*0.03*	*0.08*	*0.03*	
vanillin	47.71c	31.08b	9.67a	***
SD	*0.07*	*0.07*	*0.05*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

Only two of the nine quantified volatile compounds, hexanal and 2-phenylethanol, did not present statistically significant differences in their average concentrations among free fractions of juices. The 3JAE1F is the clone fraction with the highest concentration of benzyl alcohol and vanillin quantified in its juice free fraction. However, it also presents the lowest concentration in (*E*)-2-hexenal, 1-hexanol and (*E*)-2-hexen-1-ol. The 3JAE2F is the clone fraction with the highest average concentration of 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol. Finally, the 3JAE4F is the clone fraction with the highest average concentration of hexanal, (*E*)-2-hexenal, and hexanoic acid and it presents the lowest vanillin concentration.

The volatile compounds quantified by GC-FID analysis and the statistical significance of clone effect on average concentration values of volatile compounds in the three bound fractions of Aragonez juices are presented in Table VI.5.

Table VI.5 – Average concentrations of volatile compounds (μg 4-nonanol.dm^{-3}) in the bound fractions of clonal Aragonez juices (n=2).

Compounds	3JAE1B	3JAE2B	3JAE4B	Clonal effect
hexanal	1.09	2.00	1.01	*ns*
SD	*0.28*	*0.02*	*0.19*	
(*E*)-2-hexenal	24.96	47.84	39.80	*ns*
SD	*0.13*	*1.49*	*4.86*	
vitispirane	3.69	4.20	3.91	*ns*
SD	*0.02*	*0.14*	*0.17*	
β-damascenone	3.93b	6.82c	2.47a	***
SD	*0.09*	*0.06*	*0.28*	
hexanoic acid	nq	nq	nq	
SD				
benzyl alcohol	2.24a	2.20a	4.53b	*ns*
SD	*0.02*	*0.04*	*0.65*	
2-phenylethanol	nq	nq	nq	
SD				
vanillin	2.31a	3.73b	2.45a	***
SD	*0.05*	*0.03*	*0.13*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05); nq: not quantified.

The volatile compounds, (*Z*)-3-hexen-1-ol, hexanoic acid and 2-phenylethanol, previously detected in the free fractions were not quantified in bound fractions of Aragonez juices. On the other hand, vitispirane and β-damascenone were only quantified in bound fractions. Only two of the six quantified volatile compounds, β-damascenone and vanillin presented statistically significant differences in their average concentrations among bound fractions of juices ($p < 0.001$). The 3JAE2B is the clone fraction with the highest concentration of β-damascenone and vanillin. In opposition, the 3JAE4B and the 3JAE1B are the clone fractions with the lowest average concentrations of β-damascenone and vanillin, respectively.

6.3.1.1.2. Aragonez clonal skins

Table VI.6 presents the volatile compounds quantified by GC-FID analysis and the statistical significance of clone effect on average concentration values of volatile compounds in the three free fractions of Aragonez skins.

Table VI.6 – Average concentrations of volatile compounds (µg 4-nonanol.g^{-1} skin) in the free fractions of clonal Aragonez skins (n=2).

Compounds	3SAE1F	3SAE2F	3SAE4F	Clonal effect
hexanal	73.86b	99.08c	67.90a	***
SD	*0.06*	*0.19*	*0.25*	
(E)-2-hexenal	94.27a	148.07b	100.35a	***
SD	*0.06*	*0.24*	*0.25*	
1-hexanol	8.30a	14.69c	12.66b	***
SD	*0.01*	*0.06*	*0.00*	
(Z)-3-hexen-1-ol	4.25b	4.23b	3.43a	***
SD	*0.00*	*0.01*	*0.00*	
(E)-2-hexen-1-ol	5.07a	11.39c	8.34b	***
SD	*0.04*	*0.07*	*0.00*	
hexanoic acid	8.00	8.26	9.02	*ns*
SD	*0.03*	*0.03*	*0.02*	
benzyl alcohol	9.55	10.01	8.95	*ns*
SD	*0.00*	*0.06*	*0.01*	
2-phenylethanol	5.03b	3.48a	4.74ab	*
SD	*0.04*	*0.01*	*0.05*	
vanillin	3.80b	3.93b	2.09a	***
SD	*0.02*	*0.03*	*0.02*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

Only two of the nine quantified volatile compounds, hexanoic acid and benzyl alcohol, did not present statistically significant differences in their average concentrations among free fractions of skins. The 3SAE1F is the clone skin fraction with the lowest average concentrations of 1-hexanol and (*E*)-2-hexen-1-ol and with the highest average concentration of 2-phenylethanol. Regarding the 3SAE2F, this is the clone skin fraction with the highest concentration of (*E*)-2-hexenal, 1-hexanol and (*E*)-2-hexen-1-ol. Finally, the 3SAE4F is the clone skin fraction with the lowest concentrations of (*Z*)-3-hexen-1-ol and vanillin. Comparing all the free and bound fractions of Aragonez clonal juices and the free fractions of skins it should be emphasised that the Aragonez clone 3AE4 is the weakest when taking into account the vanillin concentration. This finding also occurs with free and bound fractions of musts as can be seen in section 6.3.1.2. However, this difference in average concentration of vanillin among clonal free and bound fractions of musts was not sufficient to generate differences in average intensity scores, among the three clonal musts, by GC-O analysis as showed in sections 4.3.1. and 4.3.2. of chapter 4.

6.3.1.2. Volatile quantification in Aragonez clonal musts

Aragonez clonal musts from the Estremadura DCO and from the 2003 vintage were analysed by FTIR analysis and the results are shown in Table VI.7. Only the potential alcohol presented an insignificant difference among the three Aragonez clonal musts.

Since the odourant compounds detected by GC-O analysis (section 4.3.1., chapter 4) and correctly identified by GC-MS analysis are few, it was decided to present the quantification results of all detected volatile compounds by GC-FID in the following two Tables (VI.8 and VI.9).

Table VI.7 – Analytical results of the three Aragonez clonal musts by FTIR analysis (n=4).

Clonal musts		Volumic mass ($g.mL^{-1}$)	Sugars ($g.L^{-1}$)	Potential alcohol (% vol.)	TA ($g.L^{-1}$ tartaric acid)	pH
3MAE1	*x*	1.07a	172.80a	10.20	6.05c	3.15b
	SD	*0.00*	*0.14*	*0.00*	*0.07*	*0.00*
3MAE2	*x*	1.08b	192.85c	11.30	5.35b	3.13a
	SD	*0.00*	*0.07*	*0.00*	*0.07*	*0.01*
3MAE3	*x*	1.08b	181.75b	10.70	5.05a	3.18c
	SD	*0.00*	*0.07*	*0.00*	*0.07*	*0.01*
Clonal effect		*	***	*ns*	**	**

x: average*; SD:* standard deviation*; ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); average values followed by the same letter, in the same column, are not significantly different (LSD, 0.05).

In Table VI.8 there are all the volatile compounds quantified by GC-FID analysis and the statistical significance of clonal effect on average concentration values of volatile compounds in the three free fractions of Aragonez musts.

Table VI.8 – Average concentrations of volatile compounds (μg 4-nonanol.dm^{-3}) in the free fractions of clonal Aragonez musts (n=2).

Compounds	3MAE1F	3MAE2F	3MAE4F	Clonal effect
hexanal	6.02	10.81	15.96	*ns*
SD	*0.03*	*0.12*	*0.02*	
(E)-2-hexenal	158.54a	194.95b	149.28a	*
SD	*0.09*	*0.31*	*0.26*	
1-hexanol	162.40a	125.25a	262.17b	***
SD	*0.08*	*0.17*	*0.46*	
(Z)-3-hexen-1-ol	97.32b	73.08a	80.90a	**
SD	*0.05*	*0.10*	*0.14*	
(E)-2-hexen-1-ol	167.05a	155.79a	270.56b	***
SD	*0.10*	*0.22*	*0.48*	
hexanoic acid	13.19a	15.83a	19.92b	*
SD	*0.02*	*0.04*	*0.02*	
benzyl alcohol	34.45b	35.46b	26.64a	**
SD	*0.03*	*0.05*	*0.04*	
2-phenylethanol	7.47a	6.30a	286.58b	***
SD	*0.01*	*0.03*	*0.03*	
vanillin	2.10	1.08	0.00	*ns*
SD	*0.02*	*0.02*	*0.00*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

Only three of the nine quantified volatile compounds were previously referred to as odourant compounds in section 4.3.1. of chapter 4: hexanal, 2-phenylethanol and vanillin. In two of these three odourant compounds, hexanal and vanillin, statistically significant differences in their average concentrations among clonal wines were not found. Comparing these results with those obtained by GC-O analysis regarding vanillin, described in section 4.3.1. (chapter 4), also no statistically significant differences were detected in average intensity scores within the three Aragonez clonal wines. Considering the other six volatile compounds, all show statistically significant differences in their average concentrations among clonal wines.

In Table VI.9 all the volatile compounds quantified by GC-FID analysis and the statistical significance of clonal effect on average concentration values of volatile compounds in the three bound fractions of Aragonez musts are presented. Only two of the six quantified volatile

compounds were previously referred to as odourant compounds in section 4.3.2. of chapter 4: hexanal, and β-damascenone. In these two odourant compounds, statistically significant differences were found in their average concentrations among clonal wines.

Table VI.9 – Average concentrations of volatile compounds (μg 4-nonanol.dm^{-3}) in the bound fractions of clonal Aragonez musts (n=2).

Compounds	3MAE1B	3MAE2B	3MAE4B	Clonal effect
hexanal	20.49a	22.87a	36.58b	**
SD	*0.69*	*0.56*	*1.92*	
(*E*)-2-hexenal	0.54a	nq	0.92b	***
SD	*0.10*		*0.00*	
vitispirane	2.30a	4.04b	4.32b	***
SD	*0.09*	*0.05*	*0.15*	
β-damascenone	2.10a	2.45a	3.00b	**
SD	*0.04*	*0.05*	*0.11*	
hexanoic acid	1.12	1.06	1.07	*ns*
SD	*0.03*	*0.02*	*0.06*	
benzyl alcohol	2.59b	1.89a	1.66a	*ns*
SD	*0.06*	*0.16*	*0.10*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05); nq: not quantified compound.

Comparing these results with those obtained by GC-O analysis described in section 4.3.2. (chapter 4), it can be verified that the differences detected in GC-FID analysis were not sufficient to show differences in the average intensity scores attributed by the panel of sniffers to those odourant compounds.

Considering the other four volatile compounds, two of them, (*E*)-2-hexenal and vitispirane show statistically significant differences in their average concentrations among clonal wines. On the other hand, hexanoic acid and benzyl alcohol, did not present statistically significant differences.

6.3.1.3. Volatile quantification in Aragonez clonal wines

Table VI.10 shows the results of the volatile compounds analysed for clonal Aragonez wines from the 2003 vintage which were identified as odourant compounds by GC-O analysis. Average values, standard deviations, ANOVA and LSD test results are reported in this Table.

In the twenty odourant compounds quantified by GC-FID analysis, statistically significant differences ($p < 0.05$) among clones in the ten odourant compounds: 2-methyl-1-propanol (Q6), isoamyl acetate (Q7), ethyl hexanoate (Q9), (Z)-3-hexen-1-ol (Q10), ethyl octanoate (Q11), 3-(methylthio)-1-propanol (Q18), hexanoic acid (Q22), 4-ethylguaiacol (Q25), ethyl vanillate and acetovanillone (Q36), were not found. These results are identical to those found for the same odourant compounds in the GC-O posterior intensity analysis of the three Aragonez clonal wines referred to in section 3.3.2.2. (chapter 3). This relationship between both quantitative data by GC-FID and by GC-O analysis seems to indicate that there were very small differences in the amounts of those odourant compounds and that these differences were not perceived by the eight sniffers.

Table VI.10 – Average concentrations of volatile compounds (mg 2-octanol.dm^{-3}) identified as odourant compounds by GC-O analysis and quantified by GC-FID analysis (n=2).

No.	Compounds	3AE1	3AE2	3AE4	Clonal effect
Q6	2-methyl-1-propanol	25.16	34.02	29.15	*ns*
	SD	*7.75*	*1.09*	*1.39*	
Q7	isoamyl acetate	0.55	0.57	0.56	*ns*
	SD	*0.04*	*0.04*	*0.05*	
Q8	2+3-methyl-1-butanol	159.37a	237.74b	227.50b	*
	SD	*37.31*	*3.51*	*16.01*	
Q9	ethyl hexanoate	0.19	0.20	0.17	*ns*
	SD	*0.01*	*0.01*	*0.02*	
Q10	(*Z*)-3-hexen-1-ol	0.03	0.04	0.03	*ns*
	SD	*0.00*	*0.00*	*0.00*	
Q11	ethyl octanoate	0.28	0.30	0.25	*ns*
	SD	*0.01*	*0.03*	*0.03*	
Q13	2-methylpropanoic acid	0.67b	1.02a	0.73a	*
	SD	*0.20*	*0.01*	*0.03*	
Q14	γ-butirolactone	8.29a	13.29b	16.03c	**
	SD	*1.78*	*0.01*	*1.00*	
Q15	butanoic acid	0.28a	0.44b	0.27a	**
	SD	*0.06*	*0.00*	*0.00*	
Q16	3-methylbutanoic acid	0.47a	0.92c	0.64b	**
	SD	*0.12*	*0.02*	*0.05*	
Q18	3-(methylthio)-1-propanol	1.40	1.56	1.82	*ns*
	SD	*0.43*	*0.01*	*0.10*	
Q22	hexanoic acid	0.99	0.98	0.93	*ns*
	SD	*0.10*	*0.00*	*0.06*	
Q23	guaiacol	0.04ab	0.03a	0.08b	*
	SD	*0.01*	*0.03*	*0.02*	
Q24	2-phenylethanol	47.74a	75.10b	69.79b	**
	SD	*2.89*	*0.58*	*4.37*	
Q25	4-ethyl-guaiacol	0.12	0.15	0.13	*ns*
	SD	*0.02*	*0.00*	*0.01*	
Q30	4-ethylphenol	0.37b	0.07a	0.56c	***
	SD	*0.00*	*0.00*	*0.04*	
Q31	4-vinylguaiacol	0.30a	0.73c	0.62b	***
	SD	*0.04*	*0.02*	*0.03*	
Q36	ethyl vanillate+acetovanillone	0.04	nq	0.03	*ns*
	SD	*0.05*		*0.00*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05); nq: not quantified compound.

On the other hand, statistically significant differences among the average concentrations of the three Aragonez clonal wines in ten odourant compounds: 2+3-methyl-1-butanol (Q8), 2-methylpropanoic acid (Q13), γ-butirolactone (Q14), butanoic acid (Q15), 3-methylbutanoic acid (Q16), guaiacol (Q23), 2-phenylethanol (Q24), 4-ethylphenol (Q30) and 4-vinylguaiacol (Q31), were found. Comparing these results with those described in section 3.3.2.2. (chapter 3), it can be concluded that the results are divergent. In fact, all the ten odourant compounds did not show any statistically significant differences in the GC-O analysis, in opposition to the GC-FID analysis. In other words, the differences detected in the GC-FID analysis were not sufficient to be detected by the panel of the eight sniffers during GC-O experiments.

6.3.2. Characterisation of Aragonez clonal wines from the Alentejo DCO

The results of the volatile compounds analysed in the five clonal Aragonez wines from the 2001 vintage, as well the average values, standard deviations, ANOVA and LSD test results are reported in Table VI.11.

Table VI.11 – Average concentrations of volatile compounds (mg 2-octanol.dm^{-3}) identified as odourant compounds by GC-O analysis and quantified by GC-FID analysis (n=2).

No.	Volatile compound	1AA1	1AA2	1AA3	1AA4	1AA5	Clonal effect
Q6	isoamyl acetate	0.57	0.62	0.64	0.66	0.77	*ns*
	SD	*0.16*	*0.10*	*0.12*	*0.06*	*0.11*	
Q7	2+3-methyl-1-butanol	270.53	250.92	232.84	278.54	241.04	*ns*
	SD	*67.17*	*39.55*	*45.90*	*7.52*	*13.74*	
Q8	ethyl hexanoate	0.29	0.26	0.24	0.22	0.24	*ns*
	SD	*0.08*	*0.04*	*0.05*	*0.02*	*0.03*	
Q9	ethyl octanoate	0.38	0.36	0.32	0.29	0.34	*ns*
	SD	*0.10*	*0.05*	*0.07*	*0.02*	*0.05*	
Q11	2-methylpropanoic acid	1.12b	0.97b	0.71a	0.96b	0.95b	*
	SD	*0.22*	*0.16*	*0.15*	*0.02*	*0.01*	
Q12	γ-butirolactone	7.93a	7.54a	7.72a	10.20b	6.34a	*
	SD	*1.52*	*1.08*	*1.83*	*0.24*	*0.21*	
Q13	butanoic acid	0.23	0.21	0.14	0.14	0.20	*ns*
	SD	*0.00*	*0.04*	*0.13*	*0.11*	*0.03*	
Q14	3-methylbutanoic acid	0.99bc	0.76a	0.78a	1.18b	0.91ca	*
	SD	*0.24*	*0.09*	*0.15*	*0.00*	*0.03*	
Q15	3-(methylthio)-1-propanol	1.99b	1.28a	1.35a	1.53a	1.53a	*
	SD	*0.37*	*0.19*	*0.31*	*0.04*	*0.03*	
Q20	2-phenylethanol	86.27	77.48	84.83	94.79	84.82	*ns*
	SD	*18.77*	*9.27*	*13.47*	*3.82*	*10.02*	
Q21	4-ethylguaiacol	0.14b	0.15b	0.09a	0.12b	0.12ab	*
	SD	*0.02*	*0.01*	*0.02*	*0.03*	*0.01*	
Q26	4-ethylphenol	0.26bc	0.69d	0.31c	0.19ab	0.20b	***
	SD	*0.05*	*0.06*	*0.04*	*0.00*	*0.03*	
Q27	4-vinylguaiacol	0.32ab	0.38b	0.49c	0.30ab	0.27a	**
	SD	*0.06*	*0.05*	*0.04*	*0.01*	*0.05*	
Q30	vanillin	0.10	0.12	0.12	0.09	0.11	*ns*
	SD	*0.02*	*0.01*	*0.01*	*0.00*	*0.02*	
Q31	ethyl vanillate+acetovanillone	0.05	0.04	0.02	0.06	0.05	*ns*
	SD	*0.01*	*0.01*	*0.04*	*0.00*	*0.01*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

In the seventeen odourant compounds quantified by GC-FID analysis, statistically significant differences ($p < 0.05$) among clones in the nine odourant compounds: isoamyl acetate (Q6), 2+3-methyl-1-butanol (Q7), ethyl hexanoate (Q8), ethyl octanoate (Q9), butanoic acid (Q13), 2-phenylethanol (Q20), vanillin (Q30), ethyl vanillate and acetovanillone (Q31), were not found. These results are identical to those found for the same odourant compounds in the GC-O posterior intensity analysis of the five Aragonez clonal wines referred to in section 3.3.1.2. (chapter 3). This relationship between quantitative data by GC-FID and by GC-O analyses seems to indicate that there were very small differences in the amounts of those odourant compounds and that these differences were not perceived by the panel of sniffers. On the other

hand, statistically significant differences among the average concentrations of five Aragonez clonal wines in seven odourant compounds: 2-methylpropanoic acid (Q11), γ-butyrolactone (Q12), 3-methylbutanoic acid (Q14), 3-(methylthio)-1-propanol (Q15), 4-ethylguaiacol (Q21), 4-ethylphenol (Q26) and 4-vinylguaiacol (Q27), were found. Comparing these results with those described on the section 3.3.1.2. (chapter 3), it can be concluded that only in two compounds are the results similar. In fact, γ-butyrolactone and 4-ethylguaiacol showed statistically significant differences among the clonal wines in both GC-FID and GC-O analyses. The other five odourant compounds did not show any statistically significant differences in GC-O analysis in opposition to the GC-FID analysis.

6.3.3. Characterisation of Trincadeira grapes, musts and wines from the Ribatejo DCO

6.3.3.1. Volatile quantification in Trincadeira clonal grapes

6.3.3.1.1. Trincadeira clonal juices

Trincadeira clonal juices from the 2003 vintage were analysed by FTIR analysis and are expressed in Table VI.12. Only the potential alcohol and pH showed no statistically significant differences among the Trincadeira clonal juices.

Table VI.12 – Analytical results of the five Trincadeira clonal juices (n=4) by FTIR analysis.

Clonal juices		Volumic mass ($g.mL^{-1}$)	Sugars ($g.L^{-1}$)	Potential alcohol (% vol.)	TA ($g.L^{-1}$ tartaric acid)	pH
3JT2	*x*	1.09b	206.35b	12.10	3.50b	3.58
	SD	*0.00*	*0.07*	*0.00*	*0.00*	*0.00*
3JT3	*x*	1.08a	201.85a	11.90	3.05a	3.54
	SD	*0.00*	*0.07*	*0.00*	*0.07*	*0.00*
3JT4	*x*	1.09b	201.85a	11.90	2.95a	3.44
	SD	*0.00*	*0.07*	*0.00*	*0.07*	*0.00*
3JT5	*x*	1.09b	208.60c	12.30	3.65c	3.42
	SD	*0.00*	*0.00*	*0.00*	*0.07*	*0.00*
3JT6	*x*	1.09b	208.60c	12.30	3.90d	3.48
	SD	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
Clonal effect		**	***	*ns*	***	*ns*

x: average*; SD:* standard deviation*; ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same column, are not significantly different (LSD, 0.05).

In order to know the volatile composition of the five Trincadeira clones, the free and bound fractions of grape juices and free fractions of skins, were studied. The volatile compounds quantified by GC-FID analysis and the statistical significance of clone effect on average concentration values of volatile compounds in the three bound fractions of Trincadeira juices are presented in Table VI.13.

Table VI.13 – Average concentrations of volatile compounds (µg 4-nonanol.dm^{-3}) in the free fractions of clonal Trincadeira juices (n=2).

Compounds	3JT2F	3JT3F	3JT4F	3JT5F	3JT6F	Clonal effect
hexanal	105.98b	72.78a	115.07b	157.20c	79.41a	***
SD	*0.10*	*0.03*	*0.09*	*0.14*	*0.10*	
(E)-2-hexenal	175.98c	102.37a	226.11d	250.65e	140.90b	***
SD	*0.17*	*0.04*	*0.17*	*0.27*	*0.16*	
1-hexanol	35.36a	46.52b	45.55b	64.11c	41.11b	***
SD	*0.04*	*0.02*	*0.01*	*0.08*	*0.05*	
(Z)-3-hexen-1-ol	3.89a	8.65d	7.01c	6.36c	4.93b	***
SD	*0.01*	*0.00*	*0.00*	*0.01*	*0.01*	
(E)-2-hexen-1-ol	31.79a	48.38c	48.72c	51.07c	42.75b	***
SD	*0.04*	*0.02*	*0.01*	*0.07*	*0.05*	
hexanoic acid	53.62a	68.86b	55.88a	56.89a	67.46b	***
SD	*0.07*	*0.05*	*0.01*	*0.03*	*0.08*	
benzyl alcohol	78.10b	89.47c	90.07c	114.41d	60.90a	***
SD	*0.13*	*0.06*	*0.04*	*0.08*	*0.09*	
2-phenylethanol	30.00c	29.28c	20.22b	31.64c	14.77a	***
SD	*0.08*	*0.02*	*0.00*	*0.01*	*0.03*	
vanillin	7.65a	10.86b	8.74ab	9.06ab	11.95b	**
SD	*0.01*	*0.02*	*0.01*	*0.02*	*0.03*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

All the nine quantified volatile compounds showed statistically significant differences in their average concentrations among free fractions of juices. The 3JT5F is the clone juice fraction with the highest concentration of hexanal, (*E*)-2-hexanal, 1-hexanol, (*E*)-2-hexen-1-ol, benzyl alcohol and 2-phenylethanol. The 3JT3F is the clone juice fraction with the highest average concentration of (*Z*)-3-hexen-1-ol and hexanoic acid. Finally, 3JAE4F is the clone juice fraction with the highest average concentration of hexanal, (*E*)-2-hexenal, and hexanoic acid. The 3JT6F juice fraction presents the highest average concentration of vanillin. On the other hand, the 3JT2F juice fraction shows the lowest average concentrations of five compounds, 1-hexanol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexan-1-ol, hexanoic acid and vanillin.

The volatile compounds quantified by GC-FID analysis and the statistical significance of clone effect on average concentration values of volatile compounds in the five bound fractions of Trincadeira juices are presented in Table VI.14.

Statistically significant differences in average concentrations of two volatile compounds, vitispirane and 2-phenylethanol were not found. On the other hand, statistically significant differences were found for the other six compounds. The 3JT4B is the clone juice fraction with the highest concentration of hexanal, (*E*)-2-hexanal, hexanoic acid and 2-phenylethanol. The 3JT2B is the clone juice fraction with the highest average concentration of β-damascenone, benzyl alcohol and vanillin.

Table VI.14 – Average concentrations of volatile compounds (µg 4-nonanol.dm^{-3}) in the bound fractions of clonal Trincadeira juices (n=2).

Compounds	3JT2B	3JT3B	3JT4B	3JT5B	3JT6B	Clonal effect
hexanal	0.67ab	0.19a	1.01b	0.92b	0.49ab	*
SD	*0.11*	*0.09*	*0.01*	*0.02*	*0.14*	
(*E*)-2-hexenal	25.12b	18.31a	35.81c	33.38c	23.44ab	***
SD	*1.95*	*0.06*	*0.11*	*0.33*	*0.22*	
vitispirane	1.47	3.21	2.57	2.40	2.94	*ns*
SD	*0.41*	*0.01*	*0.01*	*0.05*	*0.04*	
β-damascenone	1.34b	0.36a	0.00a	0.00a	0.00a	***
SD	*0.02*	*0.18*	*0.00*	*0.00*	*0.00*	
hexanoic acid	0.43a	1.09b	1.38b	1.29b	1.22b	**
SD	*0.12*	*0.07*	*0.07*	*0.08*	*0.01*	
benzyl alcohol	3.47b	3.06bc	2.92c	2.95c	2.37a	**
SD	*0.08*	*0.11*	*0.04*	*0.06*	*0.03*	
2-phenylethanol	1.28	1.47	1.69	1.77	1.27	*ns*
SD	*0.11*	*0.10*	*0.08*	*0.11*	*0.05*	
vanillin	1.98ab	1.49a	1.72a	1.66a	2.63b	*
SD	*0.05*	*0.25*	*0.02*	*0.06*	*0.03*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

6.3.3.1.2. Trincadeira clonal skins

Table VI.15 presents the volatile compounds quantified by GC-FID analysis and the statistical significance of clone effect on average concentration values of volatile compounds in the five free fractions of Trincadeira skins.

Table VI.15 – Average concentrations of volatile compounds (µg 4-nonanol.g^{-1} skin) in the free fractions of clonal Trincadeira skins (n=2).

Compounds	3ST2F	3ST3F	3ST4F	3ST5F	3ST6F	Clonal effect
hexanal	50.94bd	32.04a	77.42c	52.20b	47.11b	***
SD	*0.09*	*0.09*	*0.23*	*0.17*	*0.05*	
(E)-2-hexenal	89.56d	60.71a	151.86b	91.61d	104.12c	***
SD	*0.07*	*0.20*	*0.39*	*0.36*	*0.18*	
1-hexanol	11.00a	11.16a	23.39d	14.94b	18.72c	***
SD	*0.13*	*0.05*	*0.20*	*0.08*	*0.09*	
(Z)-3-hexen-1-ol	0.40a	1.10bc	1.22c	1.07bc	0.75ac	*
SD	*0.02*	*0.00*	*0.00*	*0.00*	*0.02*	
(E)-2-hexen-1-ol	10.08a	9.75a	28.75c	13.12a	19.79b	***
SD	*0.14*	*0.05*	*0.25*	*0.11*	*0.12*	
hexanoic acid	9.49a	10.96b	10.53b	9.21a	10.88b	***
SD	*0.01*	*0.04*	*0.01*	*0.03*	*0.01*	
benzyl alcohol	18.05a	17.28a	20.39b	23.47c	17.72a	***
SD	*0.03*	*0.05*	*0.00*	*0.04*	*0.05*	
2-phenylethanol	6.32b	5.96a	9.51e	8.83d	7.60c	***
SD	*0.01*	*0.02*	*0.01*	*0.00*	*0.01*	
vanillin	3.83bc	3.98c	2.91ab	2.59a	3.27ac	*
SD	*0.04*	*0.05*	*0.02*	*0.02*	*0.01*	

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

All the nine quantified volatile compounds showed statistically significant differences in their average concentrations among free fractions of skins. The 3ST4F is the clone skin fraction with the highest concentration of hexanal, (*E*)-2-hexanal, 1-hexanol, (*Z*)-3-hexan-1-ol and 2-phenylethanol. The 3ST3F is the clone skin fraction with the highest average concentration of hexanoic acid and vanillin. Finally, the 3ST5F is the clone skin fraction with the highest average concentration of benzyl alcohol. On the other hand, the 3ST3F clone skin fraction shows the

lowest average concentrations of five compounds: hexanal, (*E*)-2-hexanal, (*E*)-2-hexan-1-ol, benzyl alcohol and 2-phenylethanol.

6.3.3.2. Volatile quantification in Trincadeira clonal musts

Trincadeira clonal musts from the 2003 vintage were analysed by FTIR analysis and the results are expressed in Table VI.16.

Table VI.16 – Analytical results of the five Trincadeira clonal musts (n=4) by FTIR analysis.

Clonal musts		Volumic mass ($g.mL^{-1}$)	Sugars ($g.L^{-1}$)	Potential alcohol (% vol.)	TA ($g.L^{-1}$ tartaric acid)	pH
3MT2	*x*	1.09a	210.85b	12.40	7.70d	3.23b
	SD	*0.00*	*0.07*	*0.00*	*0.00*	*0.00*
3MT3	*x*	1.09a	208.65a	12.30	6.15b	3.28d
	SD	*0.00*	*0.07*	*0.00*	*0.07*	*0.00*
3MT4	*x*	1.09a	208.65a	12.30	5.95a	3.19a
	SD	*0.00*	*0.07*	*0.00*	*0.07*	*0.00*
3MT5	*x*	1.09a	208.65a	12.30	5.95a	3.24b
	SD	*0.00*	*0.07*	*0.00*	*0.07*	*0.01*
3MT6	*x*	1.10b	219.85c	12.90	6.50c	3.25c
	SD	*0.00*	*0.07*	*0.00*	*0.00*	*0.00*
Clonal effect		**	***	*ns*	***	***

x: average*; SD:* standard deviation*; ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same column, are not significantly different (LSD, 0.05).

As can be verified in section 6.3.1.2. with the musts from the Aragonez variety, the potential alcohol of Trincadeira musts also presented an insignificant difference among them. In fact, there was a clonal effect on the average values of volumic mass, sugars, TA and pH. In respect to the vintage effect on the average concentration differences of volatile compounds among the five free fractions of Trincadeira clonal musts, the one-way ANOVA allowed to find statistically significant differences in all compounds as shown in Table VI.17.

Table VI.17 – Average concentrations of volatile compounds (µg 4-nonanol.dm^{-3}) in the free fractions of clonal Trincadeira musts (n=2).

Compounds	3MT2F	3MT3F	3MT4F	3MT5F	3MT6F	Clonal effect
hexanal	63.60b	12.66a	13.92a	13.11a	12.61a	***
SD	*0.04*	*0.01*	*0.04*	*0.00*	*0.01*	
(E)-2-hexenal	29.90a	112.85c	66.36b	58.67b	38.47a	***
SD	*0.00*	*0.01*	*0.19*	*0.01*	*0.02*	
1-hexanol	137.66c	54.67a	47.51a	64.74b	64.26b	***
SD	*0.02*	*0.01*	*0.14*	*0.00*	*0.02*	
(Z)-3-hexen-1-ol	7.62c	4.26b	3.06a	2.48a	3.74b	***
SD	*0.01*	*0.00*	*0.01*	*0.00*	*0.00*	
(E)-2-hexen-1-ol	278.65d	85.38c	62.20a	73.95b	71.99ab	***
SD	*0.03*	*0.02*	*0.19*	*0.00*	*0.02*	
hexanoic acid	15.51b	11.19a	9.96a	10.72a	11.07a	***
SD	*0.00*	*0.01*	*0.04*	*0.00*	*0.01*	
benzyl alcohol	56.24c	35.93ab	39.00b	30.28a	28.92a	***
SD	*0.01*	*0.06*	*0.13*	*0.02*	*0.00*	
2-phenylethanol	151.14c	27.06a	32.92a	32.11a	42.99b	***
SD	*0.02*	*0.01*	*0.10*	*0.00*	*0.02*	
vanillin	6.50d	0.00ab	0.60abc	1.91c	0.00ab	***
SD	*0.01*		*0.01*	*0.03*		

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

The 3MT2F is the free fraction with the highest average concentration of the majority of the compounds: hexanal, 1-hexanol, *(Z)*-3-hexen-ol and *(E)*-2-hexen-1-ol, hexanoic acid, benzyl alcohol, 2-phenylethanol and vanillin. Table VI.18 shows the average concentrations of the compounds quantified in the five bound fractions of Trincadeira musts.

Table VI.18 – Average concentrations of volatile compounds (µg 4-nonanol.dm^{-3}) in the bound fractions of clonal Trincadeira musts (n=2).

Compounds	3MT2B	3MT3B	3MT4B	3MT5B	3MT6B	Clonal effect
hexanal	4.45	0.22	9.72	9.44	7.16	*ns*
SD	*0.83*	*0.11*	*1.64*	*1.86*	*0.81*	
(*E*)-2-hexenal	1.58c	0.00a	0.00a	0.67ab	0.82b	**
SD	*0.10*			*0.21*	*0.14*	
β-damascenone	1.13b	1.29b	1.31b	1.20b	0.43a	**
SD	*0.03*	*0.05*	*0.05*	*0.04*	*0.12*	
hexanoic acid	0.00a	0.00a	0.00a	0.49ab	1.10b	**
SD				*0.14*	*0.19*	
benzyl alcohol	5.06bc	3.60a	3.93a	4.35ab	5.73c	*
SD	*0.12*	*0.11*	*0.16*	*0.10*	*0.16*	
vanillin	0.00a	0.27b	0.00a	0.51c	0.00a	*
SD		*0.13*		*0.14*		

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05).

Statistically significant differences in two compounds, hexanal and vanillin among the bound fractions were not found. The 3MT4B is the bound fraction with the highest average concentration of β-damascenone, while the 3MT6B is the bound fraction with the highest average concentration of 2-phenylethanol.

Concerning the results of the free fractions of Aragonez and Trincadeira grapes and musts, it is possible to differentiate these varieties, through the concentration average ratio of isomers (*Z*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol. Oliveira (2000) has previously mentioned a differentiation between Loureiro and Alvarinho white wines based on the concentration ratio of the isomers (*E*) and (*Z*)-3-hexen-1-ol. Later, Câmara (2004) found a differentiation between Malvazia and Verdelho musts from Boal and Sercial musts based on the relative abundance of the isomers (*E*) and (*Z*)-3-hexen-1-ol. This author also found that (*E*) and (*Z*) isomers ratio was constant for each wine from Boal, Malvazia, Sercial and Verdelho cultivars which permitted their differentiation.

The results obtained in the current work indicate that the concentration average ratio of the two isomers (*E*)-2-hexen-1-ol and (*Z*)-3-hexen-1-ol is constant for the free fractions of skins, juices and musts, within the clones of each variety, in the following proportion between Aragonez and Trincadeira, respectively: (i) for musts, 2.4 and 25.2 (ii) for juices, 0.9 and 7.5; (iii) for skins, 2.1 and 19.3. Besides, the proportion of this ratio among musts, juices and skins is constant for all clones of each variety: musts > skins > juices.

6.3.3.3. Characterisation of volatile compounds in Trincadeira clonal wines

6.3.3.3.1. Volatile compounds quantification in Trincadeira clonal wines

Table VI.19 gives the results of the volatile compounds analysed in the clonal Trincadeira wines from the 2001 and 2003 vintages. Average values, standard deviations, ANOVA and LSD test results are reported in this Table.

In respect to the vintage effect on the average concentration differences of volatile compounds among the five clonal wines, the one-way ANOVA allowed to find statistically significant differences in fourteen compounds as is shown in Table VI.19. Two of these compounds ethyl 3-methylbutanoate and vanillin, were only quantified in one of the vintages, in the 2001 and 2003, respectively. The compounds in which statistically significant differences were not found are 2-methyl-1-butanol, 3-methyl-1-butanol, hexanoic acid, 2-phenylethanol, ethyl vanillate and acetovanillone.

Analysing the clonal wines from the 2001 vintage, no statistically significant differences were found among wines in seven compounds, ethyl 3-methylbutanoate (Q5), ethyl hexanoate (Q9), ethyl octanoate (Q10), hexanoic acid (Q19), 4-vinylguaiacol (Q35), ethyl vanillate and acetovanillone (Q40).

Comparing the results of Table VI.19 with the results described in section 3.3.3.2. (chapter 3), we can verify that the statistically significant differences found in the concentrations of twelve compounds were not sufficient to provoke significant differences in GC-O analysis of the same compounds. The exception was the odourant compound 4-ethylphenol. In fact, statistically significant differences in both quantitative and GC-O analysis of this compound were found. Moreover, a positive correlation (r = 0.685, Pearson correlation) between the quantitative data by GC-FID analysis and GC-O data for 4-ethylphenol was found.

In respect to the clonal wines from the 2003 vintage, statistically significant differences were found among wines in five compounds, γ-butyrolactone (Q13), 3-methylbutanoic acid (Q15), 2-phenylethanol (Q22), 4-ethylphenol (Q34) and 4-vinylguaiacol (Q35). Similarly to the discussion above regarding the clonal wines from the 2001 vintage, the 4-ethylphenol also showed statistically significant differences between quantitative and GC-O data. Furthermore, a positive correlation (r = 0.732, Pearson correlation) between the quantitative data by GC-FID analysis and GC-O data for this compound was found.

Table VI.19 - Odourant compounds quantified by GC-FID analysis (mg 2-octanol.dm^{-3}) in Trincadeira 2001 and 2003 clonal wines (n=2).

		2001 Vintage						2003 Vintage						Vintage
No.	**Volatile compound**	**1T2**	**1T3**	**1T4**	**1T5**	**1T6**	***Sig.***	**3T2**	**3T3**	**3T4**	**3T5**	**3T6**	***Sig.***	**efect**
Q5	ethyl 3-methylbutanoate	0.04	0.04	0.04	0.05	0.03	*ns*	nq	nq	nq	nq	nq		***
	SD	*0.00*	*0.00*	*0.00*	*0.00*	*0.04*								
Q6	2-methyl-1-propanol	45.84d	30.86a	34.06b	35.09b	38.46c	***	27.72	27.42	31.07	29.26	33.57	*ns*	**
	SD	*0.02*	*1.63*	*0.27*	*0.17*	*1.61*		*3.97*	*2.00*	*9.75*	*2.80*	*1.91*		
Q7	isoamyl acetate	0.27a	0.36c	0.32b	0.32b	0.35c	**	0.42	0.45	0.41	0.44	0.44	*ns*	***
	SD	*0.01*	*0.03*	*0.02*	*0.00*	*0.01*		*0.00*	*0.11*	*0.02*	*0.11*	*0.04*		
Q8	2+3-methyl-1-butanol	223.47b	194.01a	186.28a	211.11bc	197.44ac	**	145.73	184.82	199.70	182.45	202.80	*ns*	*ns*
	SD	*7.07*	*18.14*	*4.51*	*0.82*	*2.87*		*19.41*	*0.37*	*50.30*	*34.95*	*15.93*		
Q9	ethyl hexanoate	0.11	0.11	0.11	0.11	0.11	*ns*	0.09	0.09	0.09	0.09	0.10	*ns*	***
	SD	*0.01*	*0.01*	*0.01*	*0.00*	*0.00*		*0.00*	*0.02*	*0.01*	*0.02*	*0.02*		
Q10	ethyl octanoate	0.15	0.15	0.14	0.16	0.15	*ns*	0.18	0.16	0.19	0.18	0.21	*ns*	***
	SD	*0.00*	*0.02*	*0.01*	*0.00*	*0.01*		*0.00*	*0.03*	*0.01*	*0.05*	*0.02*		
Q12	2-methylpropanoic acid	1.45b	1.51b	1.27a	1.64c	1.66c	**	0.49	0.63	0.80	0.73	0.72	*ns*	***
	SD	*0.05*	*0.16*	*0.02*	*0.01*	*0.01*		*0.11*	*0.06*	*0.24*	*0.09*	*0.02*		
Q13	γ-butyrolactone	13.35b	11.84a	11.84a	14.29b	14.23b	**	14.10a	17.70a	31.11c	15.53a	22.24b	**	**
	SD	*0.42*	*1.56*	*0.06*	*0.04*	*0.01*		*2.23*	*1.08*	*9.61*	*2.53*	*1.07*		
Q14	butanoic acid	0.17b	0.15a	0.14a	0.17b	0.16b	**	0.35	0.56	0.56	0.53	0.55	*ns*	***
	SD	*0.01*	*0.01*	*0.00*	*0.00*	*0.00*		*0.06*	*0.11*	*0.15*	*0.02*	*0.05*		
Q15	3-methylbutanoic acid	1.06b	1.14bc	0.51a	1.40d	1.18c	***	0.20a	0.39b	0.53c	0.54c	0.57c	**	***
	SD	*0.04*	*0.13*	*0.06*	*0.01*	*0.01*		*0.02*	*0.06*	*0.17*	*0.03*	*0.02*		
Q16	3-(methylthio)-1-propanol	1.21b	0.54a	0.51a	1.26b	0.78a	**	1.06	1.32	1.35	1.12	1.24	*ns*	**
	SD	*0.05*	*0.39*	*0.04*	*0.00*	*0.00*		*0.19*	*0.21*	*0.37*	*0.21*	*0.07*		
Q19	hexanoic acid	0.48	0.46	0.43	0.49	0.43	*ns*	0.43	0.42	0.50	0.42	0.46	*ns*	*ns*
	SD	*0.02*	*0.05*	*0.01*	*0.01*	*0.01*		*0.04*	*0.03*	*0.10*	*0.09*	*0.01*		
Q22	2-phenylethanol	75.64b	67.52a	61.13a	66.72a	61.48a	*	46.50a	69.78b	85.42b	70.46b	72.46b	*	*ns*
	SD	*2.81*	*9.30*	*1.45*	*1.78*	*1.96*		*5.22*	*2.69*	*17.57*	*14.78*	*1.60*		
Q26	4-ethylguaiacol	0.23d	0.14a	0.17bc	0.19c	0.17b	**	0.13	0. 16	0.14	0.13	0.14	*ns*	**
	SD	*0.01*	*0.02*	*0.00*	*0.00*	*0.00*		*0.02*	*0.01*	*0.04*	*0.01*	*0.02*		
Q34	4-ethylphenol	0.48c	0.37a	0.70d	0.42b	0.33a	***	0.05a	0.15b	0.64c	0.14b	0.13b	**	***
	SD	*0.04*	*0.03*	*0.04*	*0.02*	*0.01*		*0.00*	*0.04*	*0.06*	*0.02*	*0.03*		
Q35	4-vinilguaiacol	0.56	0.46	0.52	0.50	0.39	*ns*	0.48b	0.43bc	0.33a	0.37ac	0.42bc	**	*
	SD	*0.04*	*0.17*	*0.08*	*0.03*	*0.01*		*0.01*	*0.02*	*0.03*	*0.07*	*0.01*		
Q39	vanillin	nq	nq	nq	nq	nq		0.05	0.08	0.07	0.06	0.08	*ns*	***
	SD							*0.00*	*0.01*	*0.00*	*0.05*	*0.03*		
Q40	ethyl vanillate + acetovanillone	0.18	0.16	0.19	0.16	0.16	*ns*	0.16	0.10	0.26	0.06	0.06	*ns*	*ns*
	SD	*0.03*	*0.00*	*0.00*	*0.01*	*0.01*		*0.26*	*0.02*	*0.44*	*0.02*	*0.01*		

SD: standard deviation; *ns*: not significant; *Significant ($p < 0.05$); **Highly significant ($p < 0.01$); ***Very highly significant ($p < 0.001$); Average values followed by the same letter, in the same line, are not significantly different (LSD, 0.05); nq: not quantified compound.

Comparing the average concentration of vanillin in free and bound fractions of the five clonal grapes and musts, with its average concentration in corresponding wines, it is interesting to verify that statistically significant differences were not found among the clonal wines. In fact, the musts and grapes showed statistically significant differences of average concentration of vanillin among clones. Hence, a linear relationship between vanillin concentration of grapes, musts and wines cannot be established. Furthermore, linear relationships of the other quantified compounds could not be demonstrated by the previous results.

6.3.3.3.2. Discrimination of the five Trincadeira clonal wines between 2001 and 2003 vintages

A stepwise linear discriminant analysis (SLDA), using the quantitative data, was performed in order to discriminate the five clonal wines under study. Table VI.20 presents the number of steps, the selected variables, the value of *F*-to-remove of selected variable, the significance level (*Sig.*), and the standardised coefficients of discriminant functions (DFs). According to these results four variables, vanillin, ethyl hexanoate, ethyl octanoate and 2-methylpropanoic acid, were found as discriminating variables.

Table VI.20 – Stepwise linear discriminant analysis according to vintage (years, 2001 and 2003).

Step	Selected variable	F-to-remove of selected variable	Standardised coefficients of DF
1	vanillin	109.475	1.040
2	ethyl hexanoate	14.234	-1.340
3	ethyl octanoate	5.843	1.339
4	2-methylpropanoic acid	4.650	-0.882
Eigenvalues of DFs			168.700
p-values of DFs			0.000

Table VI.21 presents the percentage of correctly classified clonal wines and shows that 100.0% of original grouped cases were correctly classified. Thus, considering the 2001 and 2003 vintages, the discriminant function obtained allowed the classification of all the wines in their correct groups.

Table VI.21 - Percentage of correctly classified Trincadeira clonal wines.

	Wine year		Predicted group membership		Total
			2001	2003	
Original	Count	2001	5	0	5
		2003	0	5	5
	%	2001	100.0	0.0	100.0
		2003	0.0	100.0	100.0

Comparing the present results with those obtained in chapter 3 with GC-O data and with those obtained in chapter 5 with descriptive sensory data, a good accordance was found among the LSDA results, each showing a 100% correct classification for the five Trincadeira clonal wines regarding the two vintages. These results underlined that, despite the approach used, sensory or analytical approach, a clear differentiation between Trincadeira clonal wines from the two vintages was observed.

CHAPTER 7

Conclusions and future outlook

7. CONCLUSIONS AND FUTURE OUTLOOK

7.1. CONCLUSIONS

The characterisation of the aroma components of clonal red grapes and wines from Aragonez and Trincadeira *Vitis vinifera* L. cultivars undertaken in the current work allowed verify that there were no important qualitative differences among clones of each cultivar regarding to the volatile compounds considered as odour-active compounds by GC-O analysis.

It can be emphasised that the sequential application of GC-O posterior intensity method, descriptive sensory and quantitative analyses demonstrated to be an interesting tool for the aroma characterisation. It was clearly demonstrated here that differences among aroma of Aragonez or Trincadeira clonal wines were largely due to the amount of odourant compounds, or more specifically due to the relative proportion of compounds found in each sample, rather than due to the presence or absence of a specific compound.

Several odourant compounds were detected, having the highest average intensities in all clonal wines: 3-methylbutanoic acid, 2-phenylethanol, Furaneol™, and 4-vinylguaiacol. The quantification by gas chromatography-flame ionization detector (GC-FID) of some of the odourant compounds found in the clonal wines as well as those found in musts and grapes showed several statistical differences among clones.

Furaneol™ and homofuraneol, described with a *burnt sugar* (*caramel-like*) and *candy-cotton* odour descriptors, were identified in Aragonez and Trincadeira clonal wines as well as in both free and bound fractions of Aragonez musts, indicating their grape-derived origin.

The odourant profiles of the free aroma fractions of the three Aragonez clonal musts revealed double the number of odourant compounds than those found in the bound aroma fractions.

The GC-O and GC-MS analyses revealed that the compounds that mainly contribute to the aroma profile of the different clonal wines were mainly fermentative-derivates rather than grape-derivates. The inexistence of quantified monoterpenic compounds and the poorness in C_{13}-norisoprenoid compounds in clonal musts and grapes, lead us to conclude that Aragonez and Trincadeira can clearly be classified as neutral cultivars.

The GC-O results demonstrated that if only GC-MS analysis is used to study the aroma of a wine extract the odourant importance of many volatile compounds will be over emphasised and many of the most important odourant compounds will be under emphasised or not detected. As a result, the complementary application of both techniques should be a routine procedure in wine aroma analysis.

The stepwise linear discriminant analysis (SLDA) when applied to the GC-O data, descriptive sensory analysis data or quantitative (by GC-FID) data, revealed in all cases, a good differentiation and, consequently, a correct classification (100%) of the five Trincadeira clonal wines regarding the vintage factor.

In fact, six odourant compounds: isoamyl acetate, 2-methyl-1-propanol, unknown, 2-methylpropanoic acid, hexanoic acid and ethyl hexanoate obtained with GC-O data analysed by SLDA were found to be discriminating variables. With respect to the descriptive sensory data, three aroma descriptors, *woody*, *spicy* and *sweet* were found to be discriminating variables. By other hand, it has been shown that the variables resulting from the SLDA application to the quantitative data with greater discriminant power were the four odourant compounds: vanillin, ethyl hexanoate, ethyl octanoate and 2-methylpropanoic acid.

These results were the first that highlighted the accordance of the results obtained with the application of SLDA to three distinct sets of data achieved by the GC-O, descriptive sensory and GC-FID analyses of the five Trincadeira clonal wines from two distinct vintages.

The aroma attributes *sweet*, *herbaceous*, *animal*, *dried fruits*, *red fruits*, *spicy* and *woody*, used by the trained sensory panel, were useful in obtaining the aroma profile of the Aragonez and Trincadeira clonal wines. Furthermore, the statistical analyses applied to the sensory data enabled us to detect differences in the aroma of the clonal wines of each variety.

According to the descriptive sensory analysis, the Trincadeira clonal wine T 15 EAN (PT) coded as T6 was the wine among the Trincadeira ones and in both vintages with the highest aroma quality appreciation. Regarding the Aragonez clonal wines, the clonal wine with the highest aroma quality appreciation from the Alentejo DCO was the wine T 58 EAN (PT) coded as 1AA4, while from the Ribatejo DCO were the wine T 54 EAN (PT) coded as 3AE1 and the wine T 56 EAN (PT) coded as 3AE2.

The knowledge about the global aroma of cultivars through the study of their clones certified or under certification process reveals their great usefulness, since it allows the viticultural agents of productive sector to do a critical and well scientific supported choice of cultivars in order to obtain higher quality wines.

7.2. FUTURE OUTLOOK

The work presented here points to a number of studies that still need to be considered in more detail. Since there are several odourant compounds that remain unknown, it is our future goal to pursue their identification in order to get a better understanding of their specific role in the aroma of the Aragonez and Trincadeira clonal wines. GC-O analysis cannot determine synergistic or antagonist effects but can be particularly useful in identifying components responsible for wine aroma. Sample matrix interactions studies such as sensory reconstitution studies or omission tests should be done in future for a better understanding of mutual interaction of odourant compounds of these wines.

Since the choice was made to study clonal wines resulting from spontaneous alcoholic and malolactic fermentations in order to preserve the *terroir* characteristics of each wine, it will be of a great importance in future to conduct ecological studies of the *"microbiota"* of grape-berry and wine to better understand the role of indigenous yeast and bacteria on wine aroma quality.

This research work should be considered as a contribution to the characterisation of aroma of wine and grapes from the Portuguese Aragonez and Trincadeira *Vitis vinifera* L. red cultivars which can be very useful for clonal certification. The approach followed during this work can also be very useful for similar studies with other cultivars to be done in the future. The knowledge about the aroma compounds and the aroma quality of different cultivars is very important for researchers, oenologists, winemakers, vineyardists, and obviously, for maximisation of wine quality which is the main objective of the clonal selection.

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