UNIVERSIDADE da MADEIRA

EFFECT OF THE *ESTUFAGEM* PROCESS ON THE CHEMICAL CONSTITUENTS OF MADEIRA WINES

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Dedicated to my Parents, my Sisters and Bruno

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RESUMO

O vinho Madeira é um produto de reputação bem estabelecida, cujo aroma e sabor característico resulta de combinações únicas. Particularmente, a sua maturação pode incluir estufagem, processo no qual o vinho é normalmente aquecido a 45 °C durante três meses. Durante este período, várias alterações químicas podem acontecer, por isso torna-se essencial avaliar o seu impacto sobre o vinho. Neste sentido, o principal objectivo da tese foi avaliar o efeito da estufagem sobre os principais constituintes químicos do vinho Madeira, especificamente sobre as moléculas potencialmente importantes no desenvolvimento das suas características típicas.

Primeiramente, desenvolveram-se metodologias analíticas capazes de determinar os compostos-alvo, combinando precisão e reprodutibilidade à eficácia de execução. Depois, vários vinhos Madeira monovarietais foram analisados durante a estufagem em condições padrão e de sobreaquecimento de modo a avaliar o seu efeito. Os seguintes compostos foram avaliados: furanos, aminoácidos, aminas biogénicas, polifenóis, ácidos orgânicos e compostos voláteis. Além disso, também foi avaliada a composição polifenólica total e o potencial antioxidante destes vinhos, assim como a cor.

Os resultados mostram que a maior parte dos constituintes sofrem alterações devido ao processo de aquecimento. Particularmente, o aquecimento promove o desenvolvimento de 5-hidroximetilfurfural (HMF) em vinhos doces submetidos a temperaturas de estufagem mais elevadas. Por outro lado, a estufagem propicia o decréscimo da maior parte dos aminoácidos, sugerindo o seu envolvimento na formação do *bouquet* destes vinhos. No que diz respeito ao teor total de polifenóis e ao potencial antioxidante destes vinhos a estufagem não parece afectar grandemente, no entanto a maioria dos polifenóis monoméricos decrescem durante este processo. O processamento térmico dos vinhos proporciona o desenvolvimento da composição volátil, especialmente de voláteis considerados típicos do aroma do vinho Madeira. Finalmente, foi demonstrado que a degradação térmica dos açúcares, nomeadamente da fructose, promove o aparecimento de compostos voláteis previamente identificados em vinhos estufados.

Palavras-chave: Vinho; Aquecimento; Aminoácidos; Ácidos orgânicos; Polifenóis; Voláteis.

SUMMARY

Madeira wine is a product of well-established reputation, whose aroma and flavour is the result of unique combinations. Particularly, its maturation may include *estufagem*, wherein wine is usually heated at 45 °C for three months. During this period, several chemical changes may occur, so it is essential to assess its impact on the wine. In this sense, the main objective of the thesis was to evaluate the effect *estufagem* on the chemical constituents of Madeira wine, specifically on those molecules potentially important in the development of its typical features.

Firstly, analytical methodologies capable of determining the target compounds, combining precision and reproducibility to execution effectiveness, were developed. Then various monovarietal Madeira wines were analysed during *estufagem* under standard and overheating conditions in order to assess its effect. The following compounds were evaluated: furans, amino acids, biogenic amines, polyphenols, organic acids and volatile compounds. In addition, the total polyphenolic composition, the antioxidant potential and the colour of these wines were also evaluated.

The results show that most constituents change due to the heating process. Particularly, the heating promotes the development of 5-hydroxymethylfurfural (HMF) in sweet wines submitted to *estufagem* at higher temperatures. Moreover, *estufagem* provides the decrease of most amino acids, suggesting their involvement in the formation of the bouquet of these wines. Regarding the total polyphenol content and antioxidant potential of these wines they do not seem to be greatly affected by the heating step, however most monomeric polyphenols decrease during this process. The thermal processing of Madeira wines favours the development of the volatile composition, especially of volatiles usually reported as typical aromas of Madeira wines. Finally, it was demonstrated that the thermal degradation of sugars, especially of fructose, promotes the emergence of volatile compounds identified in baked wines.

Keywords: Wine; Heating; Amino acids; Organic acids; Polyphenols; Volatiles.

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LIST OF ABBREVIATIONS

ABTS – 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid.

AEDA – Aroma extract dilution analysis.

ANOVA – Analysis of variance.

AP – Antioxidant potential.

ATP – Adenosine-5'-triphosphate.

AU – Absorbance units.

%Bl – Blue percentage.

°C - Degree Celsius.

°C/min – Degree Celsius per minute.

$CaCO_3$ – Calcium carbonate.

CE – Capillary electroforesis.

CI – Colour intensy.

cL – Centilitre.

CO_2 – Carbon dioxide.

CQM – *Centro de Química da Madeira*.

CRA-Cl – Carbazole-9-yl-acetyl chloride.

CV – Coefficient of variation.

Cyd-3-glu – Cyanidin-3-glycoside.

D65 – Daylight source.

Da – Dalton.

DDMP – 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one.

DMP – 3,5-Dihydroxy-2-methyl-4H-pyran-4-one.

DPPH – 2,2-Diphenyl-1-picrylhydrazyl.

EMF – 5-Ethoxymethylfurfural.

ET - Electronic tongue.

eV – Electron volt.

EVN – *Estação Vitivinícola Nacional*.

F – Furfural.

FCT – *Fundação para a Ciência e Tecnologia.*

Fe^{3+} – Ferric iron.

FMOC-Cl – 9-fluorenylmethyl chloroformate.

FMOC-OSu – N-(9-fluorenylmethoxycarbonyloxy)succinimide.

FRAP – Ferric reducing/antioxidant power.

GAE – Gallic acid equivalent.

g/cm^3 – Gram per cubic centimetre.

g/L – Gram per litre.

GC×GC-ToFMS – Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry.

GC-MS – Gas chromatography – mass spectrometry.

GC-O – Gas chromatography – olfactometry.

GHP – GH Polypro.

ha – Hectare.

hL – Hectolitre.

IARC – International Agency for Research on Cancer.

INRB – *Instituto Nacional de Recursos Biológicos*.

IVBAM – *Instituto do Vinho, do Bordado e do Artesanato da Madeira*.

HMF – 5-Hydroxymethylfurfural.

HS-SPME – Headspace solid-phase micro-extraction.

H_2S – Hydrogen sulphide.

kg – Kilogram.

$KHCO_3$ – Potassium bicarbonate.

KI – Kovats índex.

km – Kilometre.

km^2 – Square kilometre.

kNN - k-Nearest neighbour.

IDA – Iodoacetic acid.

L – Litres.

LAB – Lactic acid bacteria.

LDA – Linear discriminant analysis.

LC–MS/MS – Liquid chromatography coupled with tandem mass spectrometry.

LOD – Limit of detection.

LOQ – Limit of quantification.

m – Metre.

MCE – 2-Mercaptoethanol.

MECC – Micellar electrokinetic capillary chromatography.

MIBP – 2-Methoxy-3-isobutyl pyrazine.

mg/L – Milligram per litre.

min – Minutes.

MLC – Micellar liquid chromatography.

MLF – Malolactic fermentation.

4-MMP – 4-Mercapto-4-methyl-pentan-2-one.

MR – Maillard reaction.

MS – Model systems.

m/z – Mass-to-charge ratio.

ng/L – Nanogram per litre.

NIST – National Institute of Standards and Technology.

NH_3 – Ammonia.

nm – Nanometres.

NP – Portuguese Official Standards.

OAV – Odour activity values.

OIV – International Office of Vine and Wine or *Organisation Internationale de la Vigne et du Vin* in French.

OPA – *ortho*-phthaldialdeyde.

PBS – Phosphate buffered saline.

PCA – Principal component analysis.

PITC - Phenyl isothiocyanate.

PhD – Doctor of Philosophy or *philosophiae doctor* in Latin.

PLS-DA – Partial least squares-discriminant analysis.

PTFE – Polytetrafluoroethylene.

QE – Quercetin equivalents.

%Re – Red percentage.

RP-HLPC-DAD – Reversed phase – high performance liquid chromatography – diode array detection.

RSD – Residual standard deviation.

SBSE – Stir bar sorptive extraction.

SIM – Single ion monitoring.

SD – Standard deviation.

SPE – Solid phase extraction.

SO_2 – Sulphur dioxide.

SRARN – *Secretaria Regional do Ambiente e Recursos Naturais*.

TDN – 1,1,6-Trimethyl-1,2-dihydronaphthalene.

TF – Total flavonoids.

TMA – Total monomeric anthocyanins.

TNM – *Tinta Negra Mole.*

To – Tonality.

TP – Total polyphenols.

TPB – 1-(2,3,6-Trimethylphenyl)buta-1,3-diene.

t_R – Retention times.

Unk – Unknown.

UV – Ultraviolet.

Vis – Visible.

VLQPRD – *Vinho Licoroso de Qualidade Produzido em Região Determinada*.

VTP – Vitispirane.

v/v - Volume to volume.

%Ye – Yellow percentage.

µg/L – Microgram per litre.

SHORT CURRICULUM VITAE

Vanda Nulita Gomes Pereira was born in 1981, in Ribeira Brava, Portugal. She is graduated in Chemistry (2005) by the University of Madeira. In 2006, she was awarded with a PhD grant from the *Fundação para a Ciência e Tecnologia* (*FCT*) and, since then, she is a PhD student at the University of Madeira, developing her experimental work integrated in the Natural Products Research Group at Madeira Chemistry Research Centre (*CQM - Centro de Química da Madeira*), Center of Exact Sciences and Engineering, University of Madeira. Her PhD work was done under the supervision of Prof. José Carlos Marques (University of Madeira) and co-supervision of Prof. Juan Cacho Palomar (University of Zaragoza).

During the PhD-time, she participated in 12 Advanced Courses/Conferences covering diverse topics related with the theme of her PhD thesis. As a result of her research efforts, she is author or co-author of 9 publications in international peer-reviewed journals (5 published and 4 submitted for publication). Her work also resulted in 3 oral presentations and 25 poster presentations mostly in international scientific meetings. She has also contributed for several research projects and activities developed at *CQM* that are described in more detail below.

PROFESSIONAL FORMATION

2010 – EFFoST Annual Meeting - Food and Health (Dublin, Irland).

2009 – VI Encontro Nacional de Cromatografia (Funchal, Portugal).

2009 – Pedagogical Training Course for Trainers (96 hours, Funchal, Portugal).

2009 – VI In Vino Analytica Scientia (Angers, France).

2008 – 12as Jornadas de Análisis Instrumental – JAI (Barcelona, Espanha).

2007 – V Encontro Nacional de Cromatografia (Aveiro, Portugal).

2007 – TrainMIC Course (Monte da Caparica, Lisbon).

2007 – II Encontro Nacional de Metrologia (Funchal, Portugal).

2007 – ExTech®2007 Ninth International Symposium on Advances in Extraction Technologies (Ålesund, Norway).

2007 – Jornadas do Projecto ANTIVINMAC (Funchal, Portugal).

2006 – XXIX World Congress of Vine and Wine (Logroño, Spain).

2006 – 3rd Central European Congress on Food – CEFood (Sofia, Bulgaria).

COLLABORATION IN PROJECTS

VALIMED – *Estudo e validação do cálculo de incertezas de medição associadas a calibrações e ensaios*. Funding through FEDER - Intervir+, QREN (Project MDFDR-01-0224-FEDER-000006). Collaboration since 2010.

Chemical Characterization and Hierarchization (by Chemometry) of the Ageing Aromas of Fortified Wines (PhD project of Ana Cristina Pereira). Collaboration since 2007.

ANTIVINMAC – Determination of Antioxidant Potential, Amino Acids and Biogenic Amines in Wines from Canárias, Azores and Madeira. Funding through Interreg IIIB (Project 05/MAC/2.3/M28). Collaboration since 2006.

IMPACT – Impact of Processing Technologies in the Quality of Madeira Wine. Funding through ADI, POCTI, action 2.3. Collaboration since 2005.

CARVINMAC – Characterization of wines produced from traditional grapes in Madeira, Açores and Canárias. Funding through FEDER - INTERREG III (Project MAC/2.3/M51). Collaboration since 2005.

OTHER ACTIVITIES

Collaboration in the 6th Conference on Inorganic Chemistry, Funchal, Portugal, March 31 – April 2, 2005. Event organized by the Portuguese Society of Chemistry (*SPQ – Sociedade Portuguesa de Química*).

Collaboration in *A Química é Divertida* (Chemistry is Fun): one week of experimental demonstrations to promote science among young people – A project regularly organized by Madeira Chemistry Research Centre (*CQM*).

Collaboration in practical lessons (laboratory works) of the disciplines Analytical Chemistry and Experimental Chemistry (Degree in Chemistry/Biochemistry, University of Madeira).

Collaboration in the supervision of graduate personal which were training their laboratory skills.

LIST OF PUBLICATIONS

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PART 1

General introduction

CHAPTER 1
Madeira wine

CHAPTER 1A

A brief introduction

1A.1 Introduction

For centuries, wine produced in Madeira Island (Portugal) has been internationally recognized. Certainly that the discovery of America by Christopher Columbus, at the end of the 15th century, encouraged the Madeira wine exportation and consequently marked the History of these wines (Liddell, 1998a). Nowadays, is well-known, along with others, such as Porto in Portugal mainland, Sherry in Spain and Marsala in Sicily. The island is located in the Atlantic Ocean at about 970 km southwest of the Portuguese capital and at 600 km from the North African coast and is part, together with the islands of Porto Santo, Desertas and Selvagens, of the Madeira archipelago. The wine, considered one of the hallmark images of Madeira Island, is traditionally named as Madeira wine and classified as fortified. This classification is usually attributed to wines with high alcohol content, since at some production stage natural grape spirit is added. In the case of Madeira wine, it holds an alcoholic content between 17 and 22% v/v (*Decreto Regulamentar Regional n. º 20/85/M, de 30 de Agosto*).

The history of the island and its wine has been minutely studied by several authors, namely Vieira (Vieira, 1993, 2003b) and Liddell (Liddell, 1998b). Briefly, it seems that the fortification step was only introduced by the middle of the 18th century (Liddell, 1998a) to prevent the wine from spoiling, given that the earliest unfortified examples usually decayed at sea, when ships were heading to the New World or East Indies, during the Age of Exploration. Soon, the wine producers have discovered that the long sea expeditions improved the wine quality, due to its exposition to excessive heat, typical of the tropical climates, transforming their flavour. Currently, Madeira winemaking process can involve the wine baking up to temperatures of 50 °C for 3 months, the so-called *estufagem* procedure, which meant to reproduce the wine ageing during those long sea voyages. The first known *estufa* was introduced by Pantaleão Fernandes in 1794 (Vieira, 2003a).

Madeira wine is characterized by marked and intense flavour, and therefore is rarely consumed with meals, but usually served as an aperitif or dessert wine. This wine is so robust that even after opening its quality is still maintained. Nevertheless, Madeira wine bottles need to be conveniently stored for being enjoyed at its best. Therefore, they are usually stored upright in temperatures between 18 to 20 °C (Elliott, 2010b).

Nowadays, the Madeira wine commercialization is not the main source of receipt of this Autonomous Region, but it is still considerable. Sales have been significant over the last years, namely from 2005 to 2009, about 3.4 million litters/year were sold (Elliott, 2010a). The Madeira wine production verified in 2008 was of about 4.3 million litres, while, in 2009, the marketing of Madeira wine reached the 3.2 million litres (IVBAM, 2009). In 2009, the European Union represented the biggest market, with France (35.4%), Germany (9.5%) and United

Kingdom (9.3%) constituting the greatest consumers (Elliott, 2010a). The domestic market, representing 13.6%, is also an important consumer of Madeira wine, mainly the tourists visiting the island. Outside Europe, the main markets are the United States of America and Japan.

1A.2 Wine-growing region

Madeira wine distinctive features are influenced by the characteristics of the island as well, namely by the soil (mainly basalt because of its volcanic origin), the proximity to the sea and also by the moderated climatic conditions: hot humid summers and mild winters, with annual temperatures of about 17.5 °C along the coast (Elliott, 2010d). The rainfall is usually abundant in the autumn and winter, about 75%, while in spring are attained the 20%. The soils are in general clayey, acids and abundant in organic matter, magnesium and iron, poor in potassium and sufficient in phosphorous (Liddell, 1998d).

In 2010, the wine-growing region of Madeira wine is about 5 km^2 whilst the total area of the island is approximately 735 km^2 (Elliott, 2010d). Despite of the vine cultivation being spread all over the archipelago, in south coast the viticulture is very common in Câmara de Lobos (about 188 ha), while on the north coast, São Vicente (about 142 ha) and Santana (around 70 ha) are the main producing areas (see Figure 1A.1).

FIGURE 1A.1 – Main viticultural regions of Madeira Island (adapted from Elliott (2010d)).

The agricultural land is characterized by sharply elevated terrains, usually made into terraces known as *poios* sustained by walls of basaltic stone (IVBAM, 2009). Here, the vines are cultivated in low trellises, where the vines are horizontally settled along wires and suspended between 1 to 2 m off the ground with stakes. This traditional system of conducting the vine is commonly known as *latada* (Figure 1A.2) and similar to that used in Minho for the *vinho verde*

cultivation. The densities of the plantations vary between 2500 and 4000 plants/ha and their irrigation is done through canals that bring water from the upper points of the island, the so-called *levadas*. The explorations have in average 0.3 ha, divided into more than one plot where the production cannot exceed the 80 hL/ha (Elliott, 2010d; IVBAM, 2009). The mechanisation is almost impossible because the vines are planted in these small terraces, thus the whole cultivation cycle is usually made by man hand, increasing the costs of the entire process.

FIGURE 1A.2 – *Latada* system [1].

Indeed, the island orography has imposed many difficulties in Madeira wine production, even nowadays the harvesting is still manually made, which constitutes an arduous assignment. Alternatively, in lands with milder slopes the vines cultivation through *Espaldeira* or *espalier* method (Figure 1A.3) has been recurrent in the latest years. Here the vines grow vertically in rows on wires.

FIGURE 1A.3 – *Espaldeira* system [1].

1A.3 Grape varieties

All varieties used in Madeira wine production are *Vitis Vinifera L.* species (IVBAM, 2009). Actually, European Community imposed the use of *V. Vinifera* species rather than others, in defence of European varieties. Therefore, *Vitis Labrusca* species commonly produced in Madeira Island for internal consumption are also interdicted to the winemaking of these fortified wines. These species were introduced in Madeira Island in the second half of the 19th century due to the *phylloxera vastatrix* attack to the *V. Vinifera* roots (Liddell, 1998d). Moreover, *V. Labrusca* grapes are usually considered inappropriate to making quality wines due to the unpleasant aroma and low alcohol content achieved during fermentation. *V. Vinifera* mostly contains anthocyanin monoglucosides while others, particularly *V. Labrusca*, have mainly anthocyanin diglucosides, especially malvidin-3,5-diglucoside (Wang, Race, & Shrikhande, 2003), thus this anthocyanin has been used to discriminate the occurrence of this grape species in the production of European wines.

There are recommended and authorized *V. Vinifera* varieties for the Madeira wine production (Table 1A.1), but the white varieties *Sercial*, *Verdelho*, *Boal* and *Malvasia* and the red *Tinta Negra Mole* are the most common (also known as the traditional varieties), with the latest representing at least 80% of the production (IVBAM, 2009). *Tinta Negra Mole* can also be designated as *Tinta Negra*, namely to prevent confusion with the variant produced in Algarve.

[1] Picture kindly supplied by *Secretaria Regional do Ambiente e Recursos Naturais* (SRARN).

TABLE 1A.1 – Recommended and authorized grape varieties for production of Madeira wines (adapted from D*ecreto Regulamentar Regional n. º 20/85/M, de 30 de Agosto*).

	Recommended	**Authorized**
Red varieties	*Bastardo*	*Tinto Negro*
	Tinta da Madeira	*Complexa*
	Malvasia Cândida Roxa	*Deliciosa*
	Verdelho Tinto	*Triunfo*
	Tinta Negra Mole	
White varieties	*Boal (Malvasia Fina)*	*Carão de Moça*
	Malvasia Cândida	*Moscatel de Málaga*
	Sercial	*Malvasia Babosa*
	Verdelho	*Malvasia de S. Jorge*
	Terrantez	*Rio Grande*
		Valveirinho
		Listrão
		Caracol

Another important variety is *Terrantez* but its cultivation is extremely rare nowadays. The finest wines are produced almost exclusively from the previously described white grapes while the inexpensive ones are commonly prepared from *Tinta Negra Mole*, although recently it has been attained excellent wines from this variety.

Tinta Negra Mole (*TNM*) was firstly introduced in Madeira viticulture in the 18th century, and since then it has been essentially cultivated in Estreito de Câmara de Lobos, Câmara de Lobos and São Vicente (Elliott, 2010d; IVBAM, 2009). *TNM* variety corresponds to the *Molar* variety produced in Portugal mainland (Eiras-Dias, Paulos, Mestre, Martins, & Goulart, 2006) and is known to be more robust than white ones, whereat adapted easily to the specific conditions of the island. It is indeed quite resistant to some pests. Dry, medium-dry, medium-sweet and sweet wines can be produced from this variety. In addition to its versatility, grapes are elliptic-globose, black with light pulp, vary from small to medium-size and are characterized for having fine and soft skins (Figure 1A.4). Its musts can achieve an alcoholic potential of 9 – 12%. This variety accounts for 80 to 85% of the total production.

FIGURE 1A.4 – *TNM* grape variety [2].

FIGURE 1A.5 – *Malvasia* grape variety [2].

The most appreciated Madeira wines are from *Malvasia Cândida* grape variety. Indeed, some of them have reached astronomical prices (IVBAM, 2009). This grape variety traditionally produces sweet rich wines (residual sugars can range from 3.5 to 6.5° Baumé) and is considered the richest and smoothest variety of Madeira wines. Its wines usually detain high acidity well balanced with sweetness (Elliott, 2010c). *Malvasia* wines are also characterized by

[2] Picture kindly supplied by Doctor Jorge Cunha (EVN).

their dark colour with caramel, honey and raisin aromas, and usually accompany desserts (Elliott, 2010b). *Malvasia*, also known as Malmsey or *Malvazia*, was the first to be planted in the island and is usually planted at 150 to 200 m height above sea level. It is a fastidious fruit requiring sheltered, sunny locations and perfect conditions for ripening (IVBAM, 2009). Therefore, grows well in Câmara de Lobos, Estreito de Câmara de Lobos, Campanário, São Jorge, Arco de São Jorge and Santana (Elliott, 2010d). *Malvasia* bunches are medium-large and conical (Figure 1A.5), while berries are big, elliptic or globe-elliptic and greenish-yellowish to golden in colour (IVBAM, 2009). Their musts commonly attained up to 13% of alcoholic potential (Elliott, 2010d).

Boal (or *Bual*) is also known as *Malvasia Fina* (Lopes et al., 1999). From this white variety are usually produced rich and bodied medium-sweet wines (2.5 to 3.5° Baumé), fruity with a splendid bouquet, in which acidity and sweetness are well balanced. These wines are also characterized by their dark colour and raisin aromas. It is said that this white variety came from Portugal mainland and its cultivation is frequently made at altitudes of 100 to 300 m, yielding medium-large and dense bunches with berries of resistant skin, usually elliptic, greenish-yellowish or golden when riped (Figure 1A.6) (IVBAM, 2009). It is frequently found in the south, namely in Estreito da Calheta, Calheta, Arco da Calheta, Campanário, Estreito de Câmara de Lobos and Câmara de Lobos (Elliott, 2010d). Their musts normally hold up a potential alcohol between 11 to 13%.

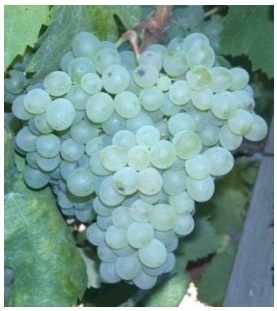

FIGURE 1A.6 – *Boal* grape variety [3].

From *Verdelho* grapes are prepared medium-dry wines with residual sugars between 1.5 to 2.5° Baumé. These aromatic wines are less acidic and more bodied than wines made from *Sercial* grapes and are frequently served as aperitif (Elliott, 2010b; IVBAM, 2009). Nutty and caramel nuances are evident. Even nowadays there are some doubts about its origin, but seems to be similar to the variety found in Azores (Lopes et al, 1999). This variety is usually cultivated at latitudes of about 400 m and are characterised by small to medium-sized bunches and golden oblong grapes with resistant skin (Figure 1A.7). Furthermore, *Verdelho* is very difficult to reproduce. Verdelho produces musts with a potential alcohol of 10 – 12%.

FIGURE 1A.7 – *Verdelho* grape variety [3].

Finally, *Sercial* is known to produce excellent dry wines with very little residual sugar, varying from 0.5 to 1.5° Baumé. The resulting wines are characterized with golden to very pale colours, almond aromas as well as a notable astringency and acidity. Generally, require large periods of ageing to be conveniently appreciated. *Sercial* is the same variety encountered in the Portugal mainland, known as *Esgana Cão* (Lopes et al, 1999). *Sercial* cultivation is often made on the north of the island at altitudes up to 200 m and in the south up to 600 m (IVBAM, 2009).

FIGURE 1A.8 – *Sercial* grape variety [3].

[3] Picture kindly supplied by Doctor Jorge Cunha (EVN).

Therefore, is abundantly found in Seixal, Porto Moniz, Ponta Delgada, São Vicente, Arco de São Jorge and Jardim da Serra (Elliott, 2010d). The vines are very resistant (namely to mildew and *oidium*) and produce medium-small, compact and pinecone shape bunches, with very acidic greenish-yellow grapes (Figure 1A.8) (IVBAM, 2009; Liddell, 1998d). *Sercial* is frequently the last variety to be harvested, producing musts with alcoholic potentials up to 11% (Elliott, 2010d).

Occasionally are produced wines from *Terrantez* and *Bastardo* varieties, although these are now increasingly rare especially due to *phylloxera* epidemic that hit the island in the past (Liddell, 1998d).

1A.4 Winemaking [4]

Madeira wine is characterized by a unique winemaking process, mainly by using a baking step so-called *estufagem*. Briefly, the fermentation extension is defined according to the sugar content of the grape variety involved (dry, medium-dry, medium-sweet and sweet wines may be produced) and is blocked by the addition of natural grape spirit (containing 95% v/v of ethanol) when the desired sweetness is attained, typically from 0 to 130 g/L. Then, a baking step can be followed, where the fortified wine is usually heated at about 45 °C for 3 months. After this, the wine is allowed to undergo a normal maturation process in oak casks (usually used casks) for a minimum period of 3 years. With the heating step introduction a premature ageing takes place, being originated the typical colour and bouquet of these wines, besides contributing to their exceptional longevity. This baking step also contributes for the distinction among others fortified wines. The main steps for the Madeira wine production are briefly discussed below.

Harvesting – Usually begins in mid-August and ends in October and at this period, grapes are at their maturity peak, with the desirable sugar content and acidity. At this point, the grapes must have a minimum potential alcohol of 9%. Once grapes are picked, after the official order, they are taken in containers with capacity of about 25 kg to the wine cellars, to be processed as soon as possible. At wine cellars, grapes are triaged in order to assess their fitosanitary condition. Then, they are weighed and the probable degree of alcohol is verified using a refractometer. Finally, the grape selection is made in accordance with the type of wine to obtain.

Subsequently, the stems are removed and the grapes are crushed to squeeze out the so-called free-run juice with the help of a mechanical crusher/de-stemmer. The resultant is guided to pressing/fermentation through a mass pump. At this stage, sulphite is usually added to inhibit the growth of the natural microbial flora (bacteria), through the addition of an aqueous solution with 5% of potassium metabisulphite ($K_2S_2O_5$). Some wine producers also add

[4] This caption is based on IVBAM web pages (IVBAM, 2009) and on Elliot's book (Elliott, 2010e).

pectolytic enzymes for helping extract more aroma and colour to musts. This addition can also increase the rate of must clarification.

Pressing – This step is the act of applying pressure to grapes in order to separate juice from the skins, seeds and pulp, usually named as the pomace, and in Madeira wine winemaking can be performed through two fermentative processes: *bica aberta* or *curtimenta*. In the first one pressing occur as soon as grapes are crushed and before fermentation, while in the second the must is only pressed after alcoholic fermentation being conducted with the grape solids.

Fermentation – This process starts when yeasts begin to digest the sugars present in the grape juice, producing ethanol and carbon dioxide as by-products. The amount of sugar corresponding to each Baumé degree (about 17 g/L) is transformed into about 1% of alcohol. The volume of carbon dioxide gas released during fermentation is equivalent to the amount of ethanol produced.

Despite of be common in wine industry the inoculation of a selected pure yeast culture for the fermentation process accomplishment, in Madeira winemaking, fermentation starts spontaneously, due to the naturally present yeasts derived from grapes surface or picked up from cellar equipment and tanks. Generally, as the fermentation progresses the activity of metabolizing yeasts increases the temperature through heat production. The raise of the fermentation temperature affects not only the speed of the fermentation but also the quality of the final wine. It is recognized that low temperatures and consequently slower fermentations avoid the loss of volatile aromas, thus these conditions are the required to improve the wine quality. The Madeira wine fermentation is industrially elaborated usually in stainless steel tanks of the local wine-producing cellars, with temperature normally controlled bellow 25 °C. The fermentation is carried out according to the sugar content of the grape variety involved and the type of wine being produced (extra-dry, dry, medium-dry, medium-sweet and sweet). Sweet wines are submitted to a soft fermentation with the purpose of maintaining the high content of residual sugars. Usually this process takes no longer than 5 days. In contrast, dry wines are thoroughly fermented obtaining low sugar levels. This can take up to eight days.

Fortification - Once the required level of sweetness is attained, usually below 130 g/L, the fermentation is stopped by the addition of natural grape spirit (containing 95% (v/v) of ethanol) raising the alcohol content up to 17 to 22% (v/v). This ethanol concentration usually promote multiple effects on taste and mouth-feel: can enhance sweetness (through ethanol sweet taste) and influence the perception of acidity, making these wines (usually acidic) appear less sour and more balanced (Jackson, 2000). Besides halting the alcoholic fermentation, fortification can also prevent the metabolism of lactic acid bacteria, which could produce too much acetic acid.

The fortification is regulated and should never be done before being attained 4% of alcohol exclusively derived from alcoholic fermentation, to guarantee sufficient quantities of fermentative aromas, ensuring the quality of wine.

Clarification and stabilization – After fortification, yeast cells (lees) and other materials gradually settle to the bottom of the storage vessel by the force of gravity and so, the wine can be decanted into a new tank (racking process). Eventually, racking can be repeated until most of the lees, bacteria, tartrates, proteins and other insoluble matters have been separated from the wine, but the use of fining agents can speed up the process at lower the cost. Therefore, clarification is generally achieved with bentonite clays and/or gelatines. These ingredients are added to wine to combine with the suspended particles, making them larger molecules that can gradually precipitate. Unlike filtration, fining not only removes dead yeast cells and grape fragments, but also removes soluble substances such as tannins, phenols and proteins (gelatines). Finally, the wines are racked/filtered into new tanks or casks, free of any sludge. At this stage some corrections can be done, especially the alcohol addition.

Ageing – This step can be achieved following one of the two ageing processes: *estufagem* or *canteiro*. In *canteiro* system, wines are aged in casks for at least 2 years, in the lofts of the wine cellars where the temperature is higher. Indeed wines are warmed exclusively from the natural temperatures of Madeira sub-tropical climate. Generally, the casks are made of oak, but eventually, chestnut, satinwood and mahogany are also used. Moreover, wines are deliberately exposed to air with a certain amount at casks top. This oxidative ageing makes wine develop intense and complex aromas. This kind of maturation promotes wine losses, often 4 to 5% per year, due to wine evaporation. Passed few years, wines are usually placed in cooler lodges, on the ground floor, where they continue their slow maturation before being bottled. Usually, these wines are commercialised only after 3 years of ageing.

Most wines are aged through the artificial heating, *estufagem*. Presently, the *estufagem* system consists in putting the wine in stainless steel vats usually fitted with stainless steel serpentines. These coils allow hot water circulate inside the container gradually releasing heat throughout the wine, up to 55 °C at least during 3 months. *Estufas* are sealed and never filled to full capacity, ensuring that there is room for wine expansion and promoting oxidation. This kind of ageing accelerates the wine maturation and tends to suppress the secondary fermentation given that, in fact, consists in a soft pasteurization. Once this process is completed, wine is cooled, frequently for 3 or 4 weeks, before being clarified again. Then, rests, usually in oak casks, for at least 90 days, as regulated by the Madeira wine, Embroidery and Handicraft Institute, locally designated by IVBAM. At this moment, the oenologist can evaluate the wine characteristics and decide if the wine is ready to be bottled or continues to age until the required features are achieved. This decision is also dependent on the market law. The resulting wines can only be sold later than October 31 of the 2nd year following the harvest date, to ensure the quality guarantee.

Another accelerated ageing method exposes wine to heat, usually up to 55 °C for 6 months, in large wooden casks stored in wharehouses outfitted with steam producing tanks that heat the room, resembling a sauna. This ageing process is named as *Armazém de Calor* (Warehouse of Heat) but currently is rarely used.

Bottling – Before bottling, the wines blending is frequently performed and several procedures may be done. These procedures may include racking and/or cold stabilization,

which consists in submitting wine (especially 3 and 5 years old wines) at low temperatures, to remove some remaining materials, preventing wine to become cloudy and gassy; fining, to eliminate the remaining brown sediments produced during the baking process and/or wood-ageing; decolourization, if necessary, using charcoal – the opposite is achieved by using concentrated must; and finally the addition of natural grape spirit up to 17-22%, frequently performed to compensate the alcohol lost during the ageing process. Additionally, small amounts of sulphite may be added. Young wines may also be filtered prior to bottling. Then, wines can be bottled, generally in 75, 50 or 37.5 cL dark green, brown or black bottles to decrease the detrimental effects on quality commonly triggered by light. At this time, wine must have the adequate qualities and fulfil the analytical characteristics applied to liqueur wines and the legally established for wines in general. These requirements are controlled by the IVBAM, issuing the wines certificates after performing the analytic and organoleptic tests.

1A.5 Styles

Madeira wine presentation can vary from extra-dry to sweet (or rich) wines according to their proportion of residual sugars (see Table 1A.2) and range between very pale (typical of extra-dry wines) to dark brown (sweet wines) colours, passing through golden tones (see Figure 1A.9).

TABLE 1A.2 – Madeira wine designations in terms of richness degree (adapted from *Portaria Regional n. º 40/82, de 2 de Fevereiro*).

Wine type	Baumé scale
Extra-dry	< 0.5°
Dry	< 1.5°
Medium-dry	1.0° - 2.5°
Medium-sweet	2.5°- 3.5°
Sweet	> 3.5°

FIGURE 1A.9 – Colours of Madeira wines (Elliott, 2010c).

Moreover, the following designations can be used to describe Madeira wine structure:

Light – Wine slightly full-bodied but with well balanced consistency.

Full-bodied – Well balanced wine with complex flavours that fulfil the mouth.

Fine – Elegant wine, having a perfect balance between the acid freshness, the body maturity and the set of aromas developed during its ageing in cask.

Soft - Fine wine with an evolved bouquet.

Velvety – Soft wine, usually viscous, resultant from the ageing in cask.

Mellow – Soft wine with evident ageing characteristics, with a perfect balance in its organoleptic features.

These traditional designations are regulated in *Portaria n.º 40/82 de 2 de Fevereiro* and *Portaria n.º 125/98 de 24 de Julho.* Consequently, Madeira wines can be commercialized according to the following styles:

Styles regarding the age:

Selected or Finest (*Seleccionado*) **–** Wine that fulfils the standard features and ages for at least 3 years. This is the youngest level of Madeira wine and is usually prepared from *TNM* wines blended with others or not. Generally, bottles do not mention the varieties that gave rise.

Rainwater – This wine has a maximum age of 5 years and its organoleptic characteristics are in conformity with the typical quality standards of the corresponding age. Presents a golden colour, with residual sugars ranging from 1.0 to 2.5° Baumé and density equal or below 1.0150 g/cm^3 (medium-dry wine). Usually this kind of wine has 18% of alcohol.

Reserve or Old (*Reserva*) – Quality wines with an ageing period equivalent to 5 years. These wines contain at least 80 % of *Sercial*, *Verdelho*, *Boal* or *Malvasia*. Currently, these wines can also be produced from *TNM* grapes. Reserve wines are often aged naturally without any artificial heat source. The bottle label indicates the name of the major vine variety.

Old Reserve or Very Old (*Reserva Velha*) – Wine complying characteristics and qualities of a 10-year-old style.

Special Reserve (*Reserva Especial*) – With the same features of 10-year-old Madeiras but is of outstanding quality.

Extra Reserve (*Reserva Extra*) - This style is rarely encountered, but is usually richer and more complex than 10-year-old Madeiras, since can aged at least 15 years. Moreover, other styles can also be produced such as 20, 30 and over 40-year-old Madeiras.

Styles regarding the year of harvest:

Solera – These wines are aged trough a dynamic method of blending and ageing, in such a way that the bottled wine is a mixture of ages. This kind of ageing is known as solera system, which consists in a succession of casks, usually lined up in a cascade structure. In this

system, a base wine fills all the containers and age through the *canteiro* procedure for at least 5 years, from which, every year, a small portion (up to 10%) is removed from the bottom cask for bottling, being replaced by an equal amount of the wine from the casks above, while a new wine is introduced into the top cask, and so on up to a maximum of nine cycles. The resulting soleras indicate the harvest year of the base wine.

Harvest (*Colheita*) – Quality wine obtained from a single harvest, with the indication of the harvest year. This style must be aged in casks at least 6 years.

Vintage (*Frasqueira/Garrafeira*) – These wines are obtained from one of the traditional varieties of a particular fine year. This style must be aged in casks at least 20 years and 2 years in bottle prior to being sold. These wines are the best example of Madeira wine remarkable longevity.

The wines produced by the Autonomous Region of Madeira have been categorized as *VLQPRD* (*Vinho Licoroso de Qualidade Produzido em Região Determinada*) and are authorized to bear the Protected Designation of Origin (PDO) locally designated as *Denominação de Origem Protegida* (*DOP*), the highest category obtained for wines. The *DOP* designation is given to wines traditionally produced in a defined geographic area, holding high quality patterns or characteristics inherent to the geographical location (natural and human factors). In addition, they are produced according to restricted rules, established by law, to make sure that all winemaking stages, from vineyard to final consumer, are strictly controlled. Therefore, several parameters are monitored by IVBAM, the regional wine committee, to ensure the genuineness and quality of the wines produced in the Madeira demarcated region.

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CHAPTER 1B

Scientific overview

1B.1 Introduction

Madeira wine is one of the most important products of the Autonomous Region of Madeira economy, essentially due to its use as an aperitif or digestive, as well as to make sauces all over the world. Until nowadays, Madeira wine production and ageing process has been accomplished mainly according to the empirical understanding acquired over years. However, Madeira wine scientific knowledge may play an important role in its competitiveness in the current phase of the global economy. In fact, in the last decade, Madeira wine has been the target issue of some researchers. The scientific studies can actually extend the producers knowledge about important phenomena taking place during the winemaking process or even regarding wine quality evaluation. So far, at least 18 papers deal directly with Madeira wine scientific investigation, especially studying its volatile profile. Nogueira and Nascimento (Nogueira & Nascimento, 1999) performed one of the first studies, but was Câmara who developed the Madeira wine scientific research with its work for his doctoral thesis, regarding essentially the volatiles of these fortified wines (Câmara, 2004), from which resulted several scientific papers (Câmara, Alves, & Marques, 2006a, 2006b, 2006c, 2007; Câmara, Herbert, Marques, & Alves, 2004; Câmara, Marques, Alves, & Silva Ferreira, 2003; Câmara, Marques, Alves, & Silva Ferreira, 2004) .

Nogueira and Nascimento (1999), firstly performed the analytical characterization of Madeira wines, analysing the physicochemical and sensorial parameters of 52 samples from different types and ages. They concluded that all parameters are generally below of national and/or international regulations. They also applied statistical concepts to data, namely principal component, discriminant and cluster analysis, achieving the wines differentiation regarding the type and age. After that, the following studies have placed great emphasis on the aromatic characterization of Madeira wine, from grape musts to aged wines. These studies not only characterize these wines in chemical terms but also by sensory analysis. Particularly, it was performed the first study on the process of *estufagem*. Later, some researchers sought to take advantage of the experimental data by applying advanced multivariate statistical techniques in order to obtain adequate tools to predict the age of Madeira wines. Finally, it was started the study of other compounds with interest on the safety of Madeira wines, for example ethyl carbamate and metallic ions.

1B.2 Studies regarding Madeira wine volatile composition

Firstly, Câmara et al. (2003) studied the evolution of heterocyclic acetals (1,3-dioxanes and 1,3-dioxolanes) as possible indicators of Madeira wines age. They found a linear

correlation between these acetals and wine ageing. They also suggested that oxidative conditions observed during maturation did not appear to cause any influence into acetalization reaction.

In 2004, the same authors determined the sotolon levels, a very powerful odorant, and its association with sugar content. To accomplish this purpose, they analysed 86 Madeira wines produced from the most common white varieties, with different sugar concentrations and aged from 1 to 25 years (Câmara, Herbert, et al., 2004). This study revealed that the highest sotolon levels reached 2 mg/L, especially in the sweetest Madeira wines. They found that sotolon concentration linearly increased with age. They also suggested that sotolon formation may derive from sugars as well as observed for furanic derivatives.

In the same year, Câmara and co-workers (2004) also characterized the varietal volatiles of four musts of Madeira wine grape varieties: *Boal*, *Malvasia*, *Sercial* and *Verdelho*. They validated a dynamic headspace solid-phase micro-extraction (HS-SPME) method, coupled with gas chromatography–mass spectrometry (GC-MS), to evaluate the free terpenoids profile of 39 musts (3 replicates of the same variety of the harvests: 1998, 1999 and 2000). The validation procedure was later published (Câmara, et al., 2006b). *ϐ*-Damascenone and *ϐ*-ionone were the predominant terpenoids in these musts, both present at concentrations above their perception threshold (45 ng/L), hence responsible of musts fruity and exotic notes. They concluded that *Malvasia* presented the highest terpenoid levels while *Verdelho* showed the lowest ones. Moreover, the four musts were well distinguished regarding the varietal origin, when were applied common statistical approaches to data, especially when linear discriminant analysis (LDA) was used.

Then, the volatile profile of 33 monovarietal Madeira wines of the 5 most used varieties, with different ages and degree of sweetness, was characterized by means of SPME and stir bar sorptive extraction (SBSE) followed GC–MS, by Alves et al. (2005). These researchers highlight SBSE technique for its capability of being used for major and trace compounds, with potential impact on the aroma complexity of Madeira wines. They found esters (>80.7%), alcohols (<8.2%), C_{13}-norisoprenoids (<6.5%), carboxylic acids (<4.2%), aldehydes (<3.7%), lactones (<3%), pyrans (< 1.7%) and terpenes (<1.4%) as constituents of the aroma profile of Madeira wine. Using the SBSE methodology, these researchers identified two important aroma compounds (woody, coconut, vanilla and chocolate notes) in Madeira wine reserves derived from oak wood, *trans*-oak lactone and *cis*-oak lactones, showing an increase with ageing. Moreover, the C_{13}-norisoprenoids, *ϐ*-damascenone, *ϐ*-ionone and dihydroactinidiolideome were detected in these wines. Other compounds, usually associated with the thermal degradation of sugars and carotenoids, were also found, namely 5-hydroxymethylfurfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), vitispirane (VTP), 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and the TDN derivative 1,1,6-trimethyl-1,2,5,6-tetrahydro-6-ethoxynaphthalene. Once again, authors applied multivariate techniques to evaluate the statistical analysis of data. According to them, the differentiation between the young wines dry/medium-dry and sweet/medium-sweet was remarkable, as well as for young and old wines (reserves). Diethyl succinate, *cis*-oak lactone and ethyl octanoate reveal to be the discriminating variables.

Câmara et al. (2006a) also evaluated the volatiles changes of the four most used white varieties during 1, 11 and 25 years of wood-ageing, by means of dichloromethane liquid-liquid extracts. At least 120 volatile compounds were encountered in these wines, mainly isoamyl alcohols, 2-phenylethanol, hexanoic and octanoic acids and also 20 different furanic derivatives. Additionally, *β*-damascenone, TDN, the isomeric vitispiranes, the *cis*- and *trans*-furan linalool oxide, the *cis*- and *trans*-whisky lactone, the dioxanes and dioxolanes isomers and many other constituents were also identified. Besides the furanic derivatives, other compounds usually associated with sugar thermal degradation were also found, such as cyclotene, maltol, DDMP and 3,5-dihydroxy-2-methyl-4H-pyran-4-one (DMP). Their results revealed a great increase with age of ethyl esters of diprotic acids and furanic derivatives whilst the concentrations of fatty acids ethyl esters, acetates and fatty acids declined. Methanol, acethaldehyde and ethyl acetate concentration enlarged with ageing as well. Indeed, they suggest that sotolon, 5-methylfurfural, furfural, HMF, 5-ethoxymethylfurfural (EMF), may be considered ageing markers of these wines.

Similar to Alves et al. (2005), Câmara and co-workers (2006c) also applied HS-SPME followed by GC-MS to differentiate 36 young monovarietal Madeira wines (8 months in oak casks) according to the grape variety, using multivariate statistical analysis. In this study, the identified compounds (42) were grouped into four different sets (higher alcohols, fatty acids, ethyl esters and carbonyl compounds) to compute the principal component analysis (PCA) model. Actually, this procedure may seem contradictory with PCA goal. PCA is proposed to lead with a high number of collinear variables. Therefore, grouping variables in order to perform analysis based on a lower number of variables may, in some cases, limit the exploratory analysis that PCA seeks to carry out. LDA was applied to classification purposes, highlighting the variables with more contribute to the differentiation of these young Madeira wines (non-commercial wines), which were *(E)*-hex-3-en-1-ol, diethyl succinate, EMF, ethyl octanoate and hexanoic acid. Later, these authors applied the same analytical and statistical procedures to classify these wines in terms of terpenoid patterns, concluding that this class of compounds also allows the distinction of Madeira wines according to variety, especially vitispirane, *α*-terpineol, farnesol and linalool (discriminating variables) (Câmara, et al., 2007). They also verified that young *Malvasia* wines presented the highest levels of total free monoterpenols while *Verdelho* exhibits the lowest contents, like previously observed for monovarietal musts of the same varieties (Câmara, Herbert, et al., 2004).

Different styles (dry, medium-dry, medium-sweet and sweet) of *Tinta Negra Mole* (*TNM*, red variety) young wines were also characterized regarding their volatile profile (Perestrelo, Fernandes, Albuquerque, Marques, & Câmara, 2006). Wine volatiles were extracted with dichloromethane through a validated liquid–liquid extraction, allowing their quantification. More than 90 compounds were found in the volatile fraction of these *TNM* red wines, mainly belonging to the following chemical families: higher alcohols, ethyl esters of medium-chain fatty acids, fatty acids, carbonyl compounds and higher alcohols acetates. Others were also detected but in minor quantities: furans, lactones, dioxanes, dioxolanes, volatile phenols and sulphur-containing compounds. The dry style presented the highest amount in volatiles (about 570 mg/L). Indeed, this result was expected since in a young wine the abundant volatiles are essentially derived from the fermentative step, which is more

extensive in dry wines. *TNM* sweet wines presented the highest concentration of carbonyl compounds, indicating that sugars can play an important role for the formation of these volatiles. They also tentatively established the most powerful odorants of *TNM* wines, concluding that ethyl octanoate, phenylacetaldehyde, ethyl hexanoate, isoamyl acetate, octanoic acid and 2-phenylethyl acetate were the most prominent.

The aroma profile of Madeira wines was also studied through sensorial, GC-O (gas chromatography–olfactometry) and GC-MS analysis by Campo et al. (2006). They characterized 4 emblematic 10-year-old monovarietal wines (*Malvasia*, *Boal*, *Verdelho* and *Sercial*) as candy, nutty, maderized, toasty, lacquer and dried fruit. The GC-O results let them conclude that Madeira wines aroma is particularly complex (41 odorants). This study also presented rankings of Madeira wine odorants, obtained by quantitative GC-O analysis and maximum odour activity values (OAVs are obtained through GC-MS quantitative analysis), among which, isoamyl alcohol (fusel), 2,3-butanedione (butter), 2-methoxy-4-vinylphenol (bitumen), phenylacetaldehyde (green, honey), sotolon (spicy), 2-methoxyphenol (smoky), *(Z)*-whiskylactone (coconut) and some volatile phenols from wood, stand out. The powerful odorant 2-furfurylthiol (toasty) was only perceived in *Sercial* wine. Moreover, they also detected through the GC-O analysis several important unknown odorants specific from Madeira wines. They also highlighted the absence of varietal aromas such as terpenoids, leading to infer that these volatiles are not important odorants in aged Madeira wines.

Oliveira e Silva and colleagues (2008) also performed sensorial and GC-O analysis but with the purpose of determining the optimal temperature and baking time to achieve Madeira wines with typical features. To accomplish this aim, young *Malvasia* and *Sercial* wines (two of each, produced under different fermentation periods) were submitted to baking at three temperatures: 30, 45, and 55 °C during 4 months. Firstly, an expert panel carried out sensorial analyses to find out the best descriptors of reference Madeira wines, which were: dried fruit, nutty, musty, baked, oak, mushroom, and brown sugar. The same panel gave the highest scores to both wines baked at 45 °C during 4 months, but considered the wines baked at 45 °C during 3 months more similar with the reference Madeira wine (selected wines). Indeed, *Malvasia* was considered (the sweetest) the wine which most closely matches with a typical Madeira wine, suggesting that sugar can play an important role in the aroma of Madeira wines. Then, the ranking of descriptors found in the selected wines was performed by GC-O analysis according to aroma extract dilution analysis (AEDA). Some odorants were common to both *Malvasia* and *Sercia*l wines, with the following descriptors: toasty, dried fruits (nutty) and burnt sugar. Others were specific of *Malvasia* and *Sercial* wines, for example, an odorant described as baked vegetable was only found in *Sercial* wines and was identified as methional. Finally, these researchers realized that sotolon, identified as the odorant described as nutty (dried fruits), is responsible for a huge impact on the flavour of both *Malvasia* and *Sercial* wines, especially because this odorant presented the highest scores in GC-O/AEDA ranking.

1B.3 Chemometric studies

There are other studies that deal with the development and application of advanced multivariate statistical methodologies, entitled chemometric analysis, aiming monitoring the quality of Madeira wines over ageing period, centring on their aromas (Pereira, Reis, Saraiva, & Marques, 2010) and on their polyphenolic and organic acid profile (Rudnitskaya, Rocha, Legin, Pereira, & Marques, 2010). Particularly, classification methodologies for evaluating ageing period have been developed using LDA and/or k-nearest neighbor (kNN) classifiers methods on features extracted from partial least squares discriminant analysis (PLS-DA) (Pereira, et al., 2010). Moreover, these kind of studies are important tools to find out potential adulterations and falsifications.

In a first approach, 8 Madeira wines, produced from the four most common white varieties and aged for 5 and 10 years, were studied by Pereira and co-workers (2010) in order to assess whether the information of the major volatile compounds, along with the proper techniques of advanced multivariate statistical, allowed proceed with the appropriate procedures to identify the different types of Madeira wines in terms of ageing time. The results reached in that paper give a solid base in this sense: the evolution trends were identified based on wines aroma profiles (GC-MS experimental data). PCA results showed that dry and medium-dry wines (*Sercial* and *Verdelho*) have similar behaviours with ageing, whilst the medium-sweet and sweet wines together follow the opposite direction. Based on the same results, they also verified that 5-year-old wines showed more similarity amongst themselves than 10-year-old ones. Furthermore, they also demonstrated that *Malvasia* wines showed faster maturation kinetics, perhaps by having higher sugar content. Moreover, a new approach based on the contribution plot concept was developed and tested to evaluate the importance of volatiles in the explanation of such trends. Hence, this study identified the volatiles which had contribution for the notorious evolution trends in *Malvasia* and *Boal*, which indeed were quite similar. Several carbonyl compounds seem to play an important role in the ageing trend of medium-sweet and sweet wines, such as vanillin, butyrolactone, furfural, furfuryl alcohol, diethyl malate, HMF and especially acetoin. In the case of medium-dry and dry wines the compounds with great contribution in their ageing trend were alcohols, quite abundant in these wines, and especially the esters diethyl succinate, ethyl citrate, diethyl malate and the corresponding malic acid as well.

According to the previous results, the same authors decided to analyse the aroma composition and its evolution through ageing process for wines produced from a single grape variety, since different ageing trends for different Madeira wines were identified. In this regard, 26 aged *Malvasia* wines were characterized in terms of volatiles in order to assess the aroma composition and its evolution through an extended ageing period (20 years), and then use such information in order to develop a classification framework, to allow the proper estimation of the wine age (Pereira, et al., 2010). The proposed classification methodology, based on the kNN algorithm using eight latent variables extracted from the PLS-DA method, achieved a performance of 4%. This error rate could be improved to lower than 1% if the classification resolution was reduced (considering 9 classes instead of the original 10).

On the other hand, Rudnitskaya et al. (2010) purposed a prediction methodology to estimate Madeira wine age based on electronic tongue (ET) response to organic acids, phenolics and furanic compounds (24 variables). Furthermore, they also evaluated the use of ET for quantifying organic acids and phenolic compounds. First of all, these researchers determined the amounts of these compounds in 14 Madeira wines (3, 6, 10 and 17 years old *Boal*, *Malvasia*, *Verdelho* and 3 and 6 years old *TNM*) by high performance liquid chromatography coupled with diode array detection (HLPC-DAD), for calibrating the ET multisensor system (26 potentiometric chemical sensors). Then, they built calibration models for age prediction and for the quantification of compounds using PLS1 regression, validating through cross-validation. According to these authors, the HPLC and ET classification models could predict the wine age with the accuracy of 2.6 and 1.8 years, respectively. Additionally, they found out that ET was capable of detecting 3 organic acids: tartaric, citric, formic and 5 polyphenols: protocatehuic acid, vanillic acid, sinapic acid, catechin, vanillin and *trans*-resveratrol, with less than 18% of error.

1B.4 Other studies

Further studies have been published about Madeira wines, but this time regarding other compounds besides wine volatiles. Perestrelo and colleagues (2010) proposed a methodology to determine ethyl carbamate, a potential carcinogenic, in fortified wines, based on HS-SPME combined with comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC-ToFMS). The developed analytical methodology presented low detection limits (at least 2.75 μg/L) but accuracy levels of about 16%. These researchers applied this method to 20 monovarietal Madeira wines produced from *TNM*, *Sercial*, *Verdelho*, *Malvasia* and *Boal* varieties from different harvests. They concluded that the winemaking procedure plays an important role in the ethyl carbamate levels found, from 54.1 (medium dry) to 162.5 μg/L (medium sweet), in which 50% of the analysed Madeira wines exceeded the limit imposed by Canada (100 μg/L), but unfortunately, they did not studied the origin of its formation in these wines.

Finally, Pérez Trujillo et al. (2011) determined the contents of 11 metallic ions in wines from the archipelagos of Madeira and Azores. Among the 64 wines analysed, 36 were from Madeira Island, 15 of which were table wines and 21 were fortified wines. They found out that Madeira and Azores wines presented high contents of sodium than wines from continental regions. However, these contents were similar to those found in wines made of vines surrounded by the sea, such as those from Canary Islands. The levels found of iron were less than 5.7 mg/L, so they concluded there was no ferric cloudiness. Conversely, they observed that the copper levels of some Madeira wines exceeded the limit imposed by the International Office of Vine and Wine (OIV) of 1 mg/L, perhaps by the cellar's winemaking technology or even by some excessive phytosanitary treatments against *oidium*. Additionally, they verified that these contents were in average higher in the wines produced in Madeira (0.63 mg/L) comparatively with those produced in Azores Islands (0.22 mg/L). Regarding zinc levels, they were always lower than 3 mg/L, well below the limit established by OIV, of 5 mg/L. Little

differences were observed in the levels of Na, Ca, Mg, Sr, Li and K between the wines of these two islands, but with respect to Fe, Cu, Zn and Mn their values were higher in the wines from Madeira than those from Azores, which presented the highest contents in Rb. These authors also observed that Madeira liquor wines have greater amounts of Fe, Cu and Mn and lower amounts of Ca, Mg and Rb than table wines, maybe due to the influence of the alcohol degree in the solubility of the metallic salts.

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CHAPTER 2

Brief introduction to some wine chemical constituents

CHAPTER 2A

Nitrogen-containing compounds

2A.1 Introduction

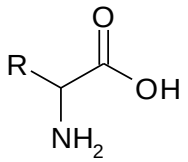
Grapes hold several forms of nitrogen-containing compounds, inorganic and organic sources (Hutkins, 2007), which all together contribute to the total nitrogen of grapes. The inorganic forms include ammonia (in the form of ammonia salts) and nitrates whereas the organic forms encompass several chemical substances. The main organic nitrogen-containing compounds are amino acids, proteins and peptides. Actually, the organic nitrogen at grape harvest is usually constituted by 70% of amino acids, while proteins and peptides do not represent more than 5% (Moreno-Arribas & Polo, 2009).

Total nitrogen can be measured in wineries usually through the Kjeldahl method (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006d). Generally, the total nitrogen of grape must is quite low (between 150 to 650 mg/L) but enough for the initial growth of yeasts and varies each year, certainly due to variations in grape ripeness (Jacobson, 2006; Ribéreau-Gayon, et al., 2006d). Furthermore, nitrogen also varies greatly between varietals and vineyards. Generally, red table wines at their final stage keep higher total nitrogen contents (143 – 666 mg/L) than white wines (77 – 377 mg/L), since their vinification usually includes maceration at greater temperatures, which facilitates the dissolution of nitrogenated substances from grape seeds, skins and also from dead yeasts cells (Ribéreau-Gayon, et al., 2006d).

Undeniably, nitrogenous compounds are essential nutrients to yeast during the entire fermentative process. Yeasts nitrogen needs are influenced by temperature, sugar and oxygen concentration (Moreno-Arribas & Polo, 2009). Occasionally, the nitrogen content may become insufficient, sluggish or stuck fermentations can occur and hydrogen sulfide (off-odour compound) may be produced, compromising the wine quality (Hutkins, 2007 74). To prevent this problem, several winemakers usually add ammonium salts to the must, especially in the case of grapes deficient in nitrogenous compounds, however this practice can reduce the concentration of some higher alcohols, which contribute to the aromatic complexity of wines (Purificación Hernández-Orte, Cacho, & Ferreira, 2002). On the other hand, if this procedure is carefully handled can actually decrease the volatile acidity up to 70%, depending on the type of yeast involved (Hernandez-Orte, Bely, Cacho, & Ferreira, 2006). Indeed, the quantity and quality of available nitrogen affects the fermentation kinetics, the production of ethanol, glycerol, aromatic and spoilage compounds, and therefore the wine quality (P. Hernández-Orte, Ibarz, Cacho, & Ferreira, 2005). Thus, it is important to study the nitrogenous components.

2A.2 Amino Acids

Amino acids are organic compounds whose general structure usually encloses at least one amino group (-NH_2) attached to a carbon containing a carboxyl group (-COOH) (Jackson, 2000a):



Actually, this structure corresponds to the *α*-amino acids. Amino acids have molecular weights below 200 Da (Ribéreau-Gayon, et al., 2006d). They play a central role as building blocks of peptides and proteins, namely as components of cell walls, promoting their growth, repair and maintenance (Eggeling & Sahm, 2009).

Amino acids are already present in wine grapes, constituting up to 80% of berry nitrogen content essentially as non-soluble components of the fruit (Jackson, 2000b). Free amino acids are then transferred to wine musts through crushing and pressing practices. At this stage, they serve as nutrients for yeasts conducting the alcoholic fermentation, but can also be metabolized if malolactic fermentation (secondary fermentation) progresses (Moreno-Arribas & Polo, 2009). Besides their importance during the fermentative process, amino acids concentration and composition can also indirectly contribute to the aromatic complexity of wines, since they are involved in the synthesis of flavour substances. Amino acid profile and concentration in wines are dependent from grape variety (including rootstock), climatic and growing conditions of grapes (mainly nitrogen fertilization), winemaking procedures and vintage (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006a).

In winemaking, amino acids are an important fraction (25–30%) of the nitrogenous source released in the crushing and pressing practices and constitute a relevant source (30–40%) to yeast growing and vitality during the alcoholic fermentation, namely because their digestion produces energy in the ATP form (Jackson, 2000a; Ribéreau-Gayon, et al., 2006d; Zamora, 2009). Other nitrogen sources are also available in grape musts such as ammonia (3–10% of total nitrogen), peptides (25–40%) and proteins (5–10%), but only some of them can be assimilated by the wine yeasts (Moreno-Arribas & Polo, 2009; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006b). Yeast nitrogen needs can be firstly ensured by ammoniacal nitrogen. Indeed, some yeast can synthesize amino acids only from ammoniacal nitrogen assimilation, but amino acids earlier presence stimulates yeasts more than ammonium. During the fermentative process, yeasts also enzymatically degrade the proteins into peptides and peptides into amino acids, easily assimilated. These occurrences together with yeasts self-digestion through the action of their own enzymes (also known as autolysis) usually increase the concentration of free amino acids at the end of fermentation.

Among the 32 free amino acids found in musts and wines, proline, arginine and alanine are usually the most abundant (Ribéreau-Gayon, et al., 2006d). The most important amino acids are presented in Figure 2A.1 and all have *L* configurations. Some are acidic compounds (aspartic acid and glutamic acid), other are basic (lysine, histidine, ornithine, citrulline and

arginine) and others have no marked acidic or basic character (serine, threonine, tyrosine cysteine and methionine). Amino acids assimilation by yeasts is quite different, proline can only be assimilated under aerobic conditions while the others are readily assimilated after the disappearance of the ammonium cation from the medium (Boulton, Singleton, Bisson, & Kunkee, 1996; Ribéreau-Gayon, et al., 2006a). Arginine, alanine, glutamic acid (precursor of proline and arginine), glutamine, aspartic acid, asparagine, threonine and serine, are favourably assimilated (Moreno-Arribas & Polo, 2009).

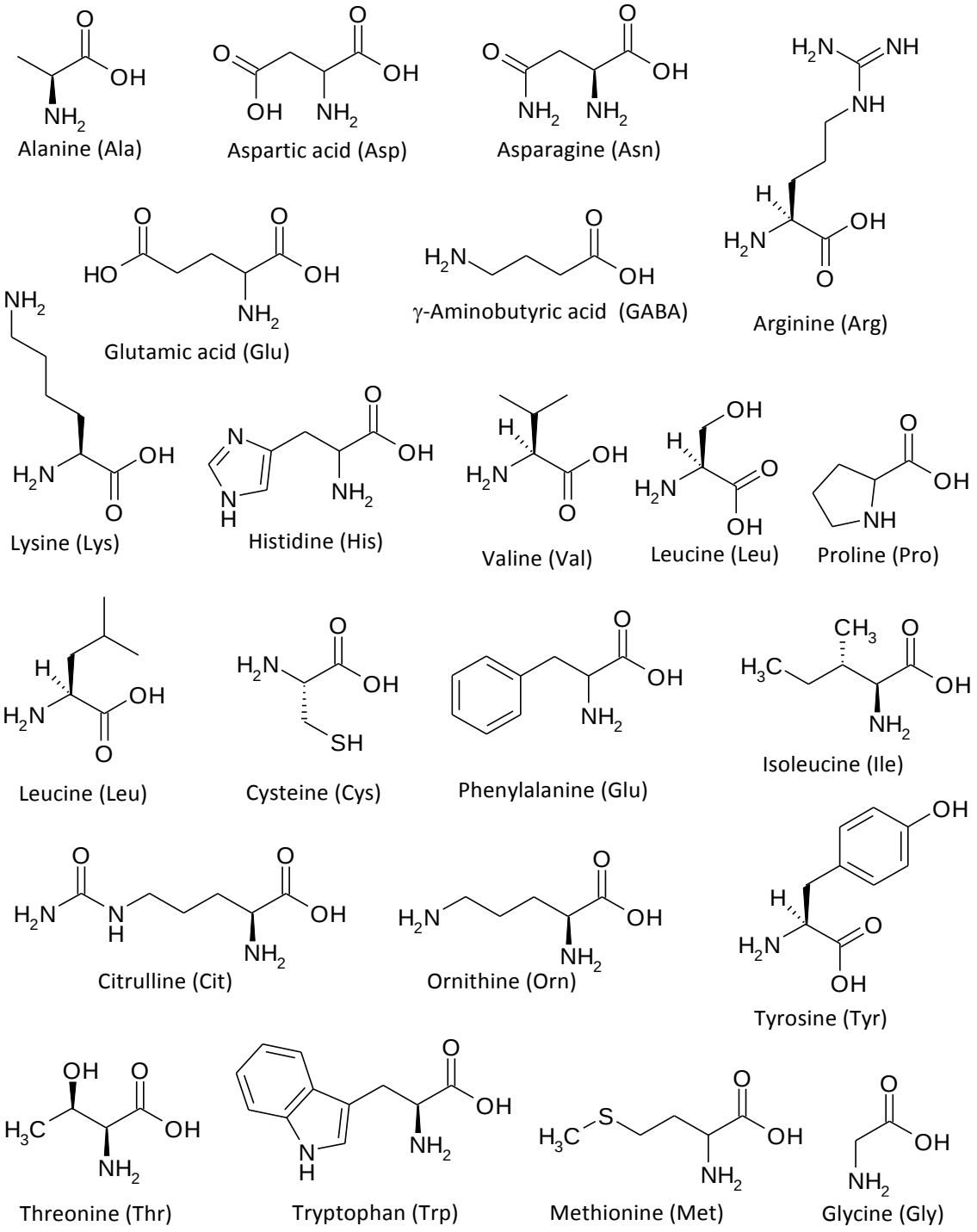


FIGURE 2A.1 – Main amino acids found in wines.

Amino acids have motivated researchers to study their profile and influence in wine production (Valero, Millán, Ortega, & Mauricio, 2003), not only for their importance in the fermentation step but essentially for their important effect on the wine flavour development (Garde-Cerdán & Ancín-Azpilicueta, 2008; Hernandez-Orte, et al., 2006; Purificación Hernández-Orte, et al., 2002; Hernández-Orte, et al., 2005; Pozo-Bayon et al., 2005). They are metabolic precursors of higher alcohols (also called as fusel alcohols), esters, aldehydes, and ketonic acids (Soufleros, Bouloumpasi, Tsarchopoulos, & Biliaderis, 2003). Amino acids are converted into keto-acids and therefore mostly into their corresponding alcohols through yeast's Ehrlich pathway (Garde-Cerdán & Ancín-Azpilicueta, 2008). For example, valine, leucine and isoleucine catabolism yields 2-methylpropanol, 2-methylbutanol and 3-methylbutanol, respectively, whereas phenylalanine, tyrosine, tryptophan and methionine originate 2-phenylethanol, tyrosol, tryptophol, methionol (Ugliano & Henschke, 2009). Subsequently, higher alcohols become precursors of esters, especially acetate esters (Sumby, Grbin, & Jiranek, 2010).

Amino acids participate in several enzymatic and chemical reactions in wines. They can react with α-dicarbonyl compounds, during fermentative process or even during maturation (via Strecker degradation according to Maillard reaction), to form several compounds, namely volatiles with different aromas: sulphury, floral, toasted and roasted notes (Costantini, García-Moruno, & Moreno-Arribas, 2009; Marchand, de Revel, & Bertrand, 2000; Pripis-Nicolau, de Revel, Bertrand, & Maujean, 2000). For example, cysteine can play an important role in wine aroma since it produces sulphur volatiles with low olfactory threshold. Their metabolism not only favours the wine aroma complexity but can also be responsible for the arising of undesirable compounds, namely off-odour compounds, biogenic amines and precursors of ethyl carbamate.

In the case of off-odour formation, cysteine and methionine metabolism are the best example. They are metabolised by lactic acid bacteria (LAB) into several sulphur-containing compounds, namely into hydrogen sulphide and methanethiol (Costantini, et al., 2009). Moreover, the chemical metabolism of methionine during wine oxidative ageing yields methional, an unpleasant aroma compound (Escudero, Hernández-Orte, Cacho, & Ferreira, 2000).

On the other hand, amino acid enzymatic decarboxylation results in the formation of biogenic amines, undesirable compounds at high levels, due to the physiological effects in the human organism consequently of their toxicology (Santos, 1996).

Arena and co-workers have studied the arginine metabolism by wine LAB on the formation of ethyl carbamate precursors (Arena & Manca de Nadra, 2005; Arena, Saguir, & Manca de Nadra, 1999). Arginine, usually abundant in grape juice, is mostly metabolized by yeasts during the alcoholic fermentation, namely into proline and urea (through the action of arginase-urease) but still remains present in wine after this fermentative step (Arena, et al., 1999; Moreno-Arribas & Polo, 2009). Thus, arginine is usually accessible for the LAB metabolism during the malolactic fermentation (MLF), conducted after alcoholic fermentation depletion, especially in red wines. During MLF, arginine can be catabolised by LAB into

citrulline and ammonia (NH_3) via arginine deiminase. In turn, citrulline is converted into ATP, CO_2, NH_3 and ornithine in two enzymatic reactions (Arena, et al., 1999).

At last, the knowledge of this nitrogenous compounds in wines has been important since amino acid profile has been successfully used for authenticity studies and criteria for differentiation (Etiévant, Schlich, Bouvier, Symonds, & Bertrand, 1988; Heberger, Csomos, & Simon-Sarkadi, 2003; Kim, Kim, Cheong, & Jeong, 1996; Pet'ka, Mocák, Farkaš, Balla, & Kováč, 2001; Soufleros, et al., 2003).

2A.3 Biogenic amines

Biogenic amines are low-molecular weight nitrogenous bases (containing an amino group), whose chemical structure may be aliphatic (putrescine and cadaverine), aromatic (tyramine) and heterocyclic (histamine) (Santos, 1996). They are naturally present in grapes but in wines derive mostly from the fermentative processes and can also be developed during ageing and storage (Moreno-Arribas & Polo, 2009). Biogenic amines can also be originated from microbial contaminations due to poor sanitary conditions of grapes and processing equipment. Certainly, contaminations during winery operations and raw material quality together with winemaking processes, ageing time and storage conditions can explain the variability of the amine contents in wines.

Occurrence of amines in different types of foods is usually associated to proteins degradation through microbial activity which cannot be easily extended to wines, as the protein content is low. However, amines occurrence in wines may be related with: their naturally presence in grapes; amino acid decarboxylation through the substrate-specific enzymes of yeasts (during alcoholic fermentation) or spoilage bacteria (during malolactic fermentation); aldehydes and ketones amination and/or transamination (Anli, Vural, Yilmaz, & Vural, 2004; Santos, 1996). Nevertheless, biogenic amines are mainly produced by decarboxylation of amino acids by microorganisms and inadequate sanitary conditions, that at some stage of winemaking, can contribute for their increase in wines because of the presence of spoilage bacteria (Leitão, Marques, & San Romão, 2005). Most contaminations happen during the spontaneous (non-controlled) malolactic fermentation (Moreno-Arribas & Polo, 2009). Moreover, the practice of amino acid addition can intensify their occurrence in wines (Soufleros, Bouloumpasi, Zotou, & Loukou, 2007). Histidine can be enzymatically decarboxylated into histamine, tyrosine into tyramine, tryptophan into tryptamine, lysine into cadaverine, ornithine into putrescine and arginine into spermine and spermidine (Santos, 1996). Figure 2A.2 presents the most common biogenic amines found in wines.

It seems that cadaverine, spermine and spermidine are essentially formed by yeasts during alcoholic fermentation, while putrescine, already present in grapes, histamine and tyramine greatly enhanced during malolactic fermentation and ageing (Landete, Ferrer, & Pardo, 2007; Moreno-Arribas & Polo, 2009; Soufleros, et al., 2007).

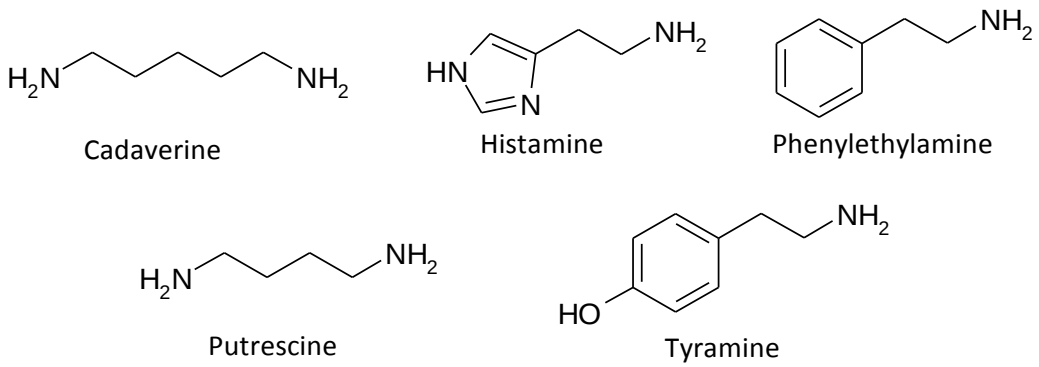


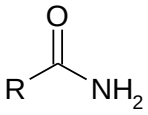
FIGURE 2A.2 – Most common biogenic amines present in wines.

Biogenic amines are usually found in wines at low concentrations when comparing with other fermented foods, such as beers and some sausages (Moreno-Arribas & Polo, 2009; Soufleros, et al., 2007). However, the ingestion of some biogenic amines, namely histamine, tyramine and phenylethylamine, especially at higher concentrations, can induce adverse effects, like headache, nausea, rushes, hypo- and hypertension, digestive problems and allergic reactions (Jackson, 2000a; Santos, 1996). In general, these biogenic amines are considered toxic in alcoholic beverages if they are in the following ranges: 8 – 20 mg/L for histamine, 25 – 40 mg/L for tyramine (Moreno-Arribas & Polo, 2009). In the case of phenylethylamine little as 3 mg/L can cause negative physiological effects. Thus, and from a toxicological point of view, histamine content have been recommended to be regulated in wines by several countries: Germany (2 mg/L), Belgium (5-6 mg/L), France (8 mg/L), Holland (3 mg/L), Switzerland and Austria (10 mg/L) (Landete, Ferrer, Polo, & Pardo, 2005; Moreno-Arribas & Polo, 2009). Putrescine and cadaverine are not themselves toxic but are usually associated with wine deficient sanitary conditions, improving negative flavours of putrefaction or rotting flesh, respectively. These amines can increase the toxicity of histamine, tyramine and phenylethylamine, since they obstruct the removal of these toxic substances from the body. However, putrescine can be dangerous if react with nitrites to form a carcinogenic nitrosamine (Costantini, et al., 2009).

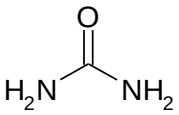
Frequently, the levels of biogenic amines are higher in red wines than in white wines given that they usually undergo maceration during the alcoholic fermentation (increasing the amino acid content) and suffer malolactic fermentation (Moreno-Arribas & Polo, 2009). Naturally, there are other wine types which are susceptible to the biogenic amines problematic, since they include peculiar practices which favour the occurrence of these compounds, among which are example the sparkling wines, biologically aged wines and wines made of botrytized grapes such as Tokaji Aszú wines (Kiss, Korbász, & Sass-Kiss, 2006; Moreno-Arribas & Polo, 2009).

2A.4 Amides

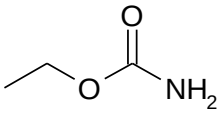
Amides are substances which contain an amino group (-NH_2) directly linked to a carbonyl function (-CO) (Jackson, 2000a):



The most common examples found in wines are urea and ethyl carbamate:



urea



ethyl carbamate

Urea formation in wines is related to arginine metabolism carried out by yeasts during the alcoholic fermentation. Wines usually enclose less than 1 mg/L in urea (Ribéreau-Gayon, et al., 2006d). Urea importance in wine is related to the development of ethyl carbamate.

Apprehensive with the ethyl carbamate genotoxic characteristics, Canada has introduced the following acceptable limits in alcoholic beverages: 30 µg/L in table wines, 100 µg/L in fortified wines, 150 µg/L in distilled spirits, 200 µg/L in sake and 400 µg/L in fruit brandies and liqueurs (Hasnip et al., 2007). Nonetheless, at present time there are no maximum levels for ethyl carbamate established by any reference entity as the Codex Alimentarius, the European Union or others, and only few countries follow the example of Canada of drawing up their own recommendations. In fact, the biggest implication for the economy is the paralysation of wines exportation to these countries if these guidelines are not accomplished.

Ethyl carbamate or urethane was recently re-classified as potentially carcinogenic (Group 2A) by the International Agency for Research on Cancer (IARC) (Lachenmeier, Kanteres, Kuballa, López, & Rehm, 2009). For this reason, great efforts have been done by several researchers to examine ethyl carbamate occurrence in several foods (Hasnip, et al., 2007; Nóbrega, Pereira, Paiva, & Lachenmeier, 2009; Park et al., 2007), namely in wines (Uthurry et al., 2004), as well as its origin and factors affecting its formation (Arena & Manca de Nadra, 2005; Arena, et al., 1999; Bruno, Vaitsman, Kunigami, & Brasil, 2007; Mira de Orduña, Liu, Patchett, & Pilone, 2000; Uthurry, Lepe, Lombardero, & García Del Hierro, 2006). The low concentrations of ethyl carbamate and the occurrence of interferences demand precise and sensitive methods. Therefore, in recent years, great importance has been given to the development of new methodologies to determine this amide, especially in decreasing the time-consuming of the oldest ones (Herbert, Santos, Bastos, Barros, & Alves, 2002; Lachenmeier, Nerlich, & Kuballa, 2006; Park, et al., 2007; Perestrelo, Petronilho, Câmara, & Rocha, 2010; Zhang & Zhang, 2008).

In wines, ethyl carbamate is mainly formed from the ethanolysis of precursors, mostly urea and citrulline, derived from the arginine metabolism during the fermentative processes (Weber & Sharypov, 2009). Additionally, it has also been reported that hydrogen cyanide derived from cyanogenic glycosides, present in raw materials, can also potentiate the ethyl carbamate development (Weber & Sharypov, 2009). Besides being developed during the fermentative steps, ethyl carbamate formation is also related with storage and ageing periods.

Indeed, its development is accelerated by high temperature at any winemaking stage or storage, combined with high concentrations of its precursors, ethanol, urea and citrulline. Actually, high concentrations of arginine can potentiate the formation of ethyl carbamate, given that urea and citrulline are formed by its metabolism through yeast and lactic acid bacteria during the alcoholic and malolactic fermentations, respectively (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006c). Furthermore, grape variety and excessive nitrogen fertilization in the vineyards can also contribute to the ethyl carbamate content in wines (Ribéreau-Gayon, et al., 2006d).

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CHAPTER 2B

Polyphenols

2B.1 Introduction

The chemical family of polyphenols encompass numerous compounds that share a common structure, based on cyclic derivatives of benzene with one or more hydroxyl groups directly connected with the ring (Jackson, 2000a). Polyphenols are plant secondary metabolites widespread in nature, constituting one of the most abundant substances in plants, derived from the acetate and the shikimate pathways (Lattanzio, Kroon, Quideau, & Treutter, 2009; Pandey & Rizvi, 2009). They are ubiquitous in fruits and vegetables but also in others foodstuffs, such as beverages, cocoa and wine (L. Bravo, 1998; Manach, Williamson, Morand, Scalbert, & Remesy, 2004).

It is recognized that phenolics have several significant functions in wine: are responsible for the bitterness and astringency in wine taste; contribute for the wine colour, namely for the red wine colour; participate in the wine aroma, although to a lesser extend; and are the key compounds in wine preservation being considered one of the basis of long ageing (Monagas, Bartolomé, & Gómez-Cordovés, 2005). Because phenolics are readily oxidizable, due to their reactivity centred in the acidic character of the phenolic function and in the nucleophilic character of the benzene ring, they are usually associated to the browning phenomenon occurred in wines exposed to air, especially white wines. In addition to oenological properties, polyphenols are known to potentiate some health benefits effects often associated with the well-known "French paradox". They prevent the oxidation of low-density lipoprotein cholesterol, and promote the inhibition of platelet aggregation, in that way reducing heart disease risks (Woraratphoka, Intarapichet, & Indrapichate, 2007). These effects are related with their pharmacological activities, such as antioxidant (free radical scavenging and metal chelating activities), anti-inflammatory, anti-allergic, antiviral, anti-carcinogenic, anti-microbial and vasodilatory actions (Clifford et al., 1996; Frankel, German, Kinsella, Parks, & Kanner, 1993; Fremont, 2000; Teissedre, Frankel, Waterhouse, Peleg, & German, 1996; Vaquero, Alberto, & de Nadra, 2007).

Others classifications can be made, but usually, grape and wine polyphenols are classified as flavonoids and non-flavonoids. Flavonoids comprise flavonols like quercetin, flavanonols as astilbin, flavones as apigenin, flavan-3-ols as catechins, and also anthocyanins like malvin (Monagas, et al., 2005). The non-flavonoid constituents are structurally simpler, from which are example the phenolic acids: hydroxybenzoic (e.g. gallic acid) and hydroxycinnamic (e.g. caffeic acid) acids. Others minor non-flavonoids are the volatile phenols (e.g. guaiacol) usually considered as off-flavours, the stilbenes, namely *trans*-resveratrol, and miscellaneous compounds like lignans and coumarins (Jackson, 2000a; Moreno-Arribas & Polo, 2009; Rentzsch, Wilkens, & Winterhalter, 2009).

In grapes, polyphenols arise conjugated with sugars (mono- or polysaccharides) through hydroxyl groups, or even through aromatic carbons (Pandey & Rizvi, 2009).

Frequently, polyphenols can also appear linked to organic acids, amines, lipids, or even associated with other phenols. Grape phenolics are usually found in juice (hydroxycinnamoyl tartaric acid esters), pulp (proanthocyanidins, hydroxybenzoic acids), seeds (flavan-3-ols, proanthocyanidins, gallic acid) and skins (anthocyanins, flavan-3-ols, proanthocyanidins, flavonols, dihydroflavonols, hydroxycinnamoyl tartaric acid esters, hydroxybenzoic acids, hydroxystilbenes) and accumulate when grapes are ripening (Lattanzio, et al., 2009). Most of wine phenolics are originated from grape berry, therefore, the amount and composition of wine's polyphenols is strongly affected by the viticultural factors such as grape variety, growing region, environmental conditions (especially sun exposure), cultural techniques and grape ripening state. Moreover, the winemaking practices have additional effect, essentially maceration and fermentation in contact with the grape skins and seeds but also pressing, fining and ageing.

2B.2 Non-flavonoid polyphenols

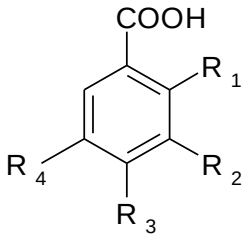
Non-flavonoid polyphenols are non-coloured compounds, structurally simpler than flavonoids (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). Non-flavonoids have being essentially subdivided into phenolic acids and their derivatives, and stilbenes. In grapes, non-flavonoids are essentially present in the pulp and are easily extracted on crushing, especially hydroxycinnamic acid derivatives since they are very abundant, namely those esterified with tartaric acid (hydroxycinnamoyl tartaric acid esters), caftaric, coutaric, and fertaric acids (Jackson, 2000a; Monagas, et al., 2005). In wines, the most common non-flavonoids are hydroxybenzoates and hydroxycinnamates.

Non-flavonoids are known to increase the stabilization of red wines due to intra- and intermolecular reactions, and also for being associated to white wine browning (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). Additionally, some of them exhibit strong antioxidant effects, especially resveratrol.

2B.2.1 *Phenolic acids and their derivatives*

Phenolic acids constitute a great fraction of wine phenolics. Actually, their levels in red wines range from 100 to 200 mg/L while in white wines usually does not exceed 20 mg/L (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). These compounds are colourless when present in hydroalcoholic solutions, however, they can be oxidised into yellow compounds. On the other hand, phenolic acids are not odorants but can be precursors of volatile phenolic acids, by the action of some yeasts and bacteria. Frequently, the volatile phenols are found in wines at low concentrations, namely vinyl and ethyl phenols, but due to their high odorant activity they can influence the sensory characteristics of wine and be responsible for unpleasant aromas (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). Indeed, the most common phenolic acids in wines are the derivatives of benzoic and cinnamic acids.

Hydroxybenzoic acids are characterized for having a C_6-C_1 skeleton, indeed an aromatic six-carbon ring with one carbon attached to a carboxylic group (Figure 2B.1).

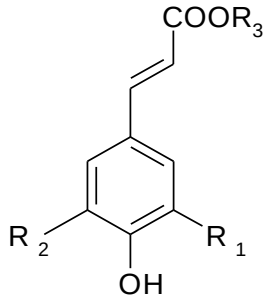


Hydroxybenzoic acids	R_1	R_2	R_3	R_4
Gallic acid	H	OH	OH	OH
Gentisic acid	OH	H	H	OH
p-Hydroxybenzoic acid	H	H	OH	H
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Vanillic acid	H	OCH_3	OH	H

FIGURE 2B.1 – Basic structure of hydroxybenzoic acids and the most common examples in wines.

In wines, hydroxybenzoic acids are predominantly found in their free form but in grapes, they are mainly present as hydrolysable glycosides and tannins (ellagitannins and gallotannins) (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009; Ribéreau-Gayon, et al., 2006). Several hydroxybenzoates can be found in wines with different substitutions at position R_2, R_3, R_4 and R_5 of the benzene ring. The most common are gallic, gentisic, *p*-hydroxybenzoic, protocatechuic, salicylic, syringic, and vanillic acids (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). Gallic acid is frequently the most abundant, while gentisic and salicylic acids are usually found in trace amounts (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009; Ribéreau-Gayon, et al., 2006). Furthermore is also frequent to found ellagic acid in wines, especially in oak-aged wines (Jackson, 2000a). This phenolic acid derives from the breakdown of ellagitannins, polymers of ellagic acid, or gallic and ellagic acids with glucose. By the way, ellagic acid is indeed formed by the interaction of two molecules of gallic acid. Moreover, it has been reported the occurrence of some derivatives of hydroxybenzoic acids in wines, namely the ethyl esters of vanillic, p-hydroxybenzoic, and protocatechuic acids and the methyl esters of vanillic and protocatechuic acids (Baderschneider & Winterhalter, 2001; Güntert, Rapp, Takeoka, & Jennings, 1986).

Regarding to hydroxycinnamic acids, these phenolic acids encompass a C_6-C_3 skeleton as depicted in Figure 2B.2. In grapes and, thereby, in wines, they are usually found in the free form, but especially as esters of tartaric acid (Ribéreau-Gayon, et al., 2006).



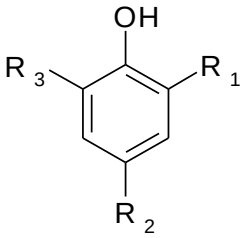
Hydroxycinnamic acids	R_1	R_2	R_3
Caffeic acid	OH	H	H
Caftaric acid	OH	H	Tartaric acid
p-Coumaric acid	H	H	H
p-Coutaric acid	H	H	Tartaric acid
Ferulic acid	OCH_3	H	H
Fertaric acid	OCH_3	H	Tartaric acid
Sinapic acid	OCH_3	OCH_3	H

FIGURE 2B.2 – Chemical structures of hydroxycinnamic acids most common in wines.

The most common esterified hydroxycinnamates (hydroxycinnamoyltartaric acids) are caffeoyltartaric acid (caftaric acid), *p*-coumaryltartaric acid (coutaric acid) and feruloyltartaric acid (fertaric acid). These compounds are highly oxidizable and the enzymically generated derivatives (*o*-quinones), especially those from caftaric and coutaric acids, developed much of the appreciated straw yellow–gold coloration of some white wines but may also contribute for the undesired browning of other white wines. Actually, *o*-quinones by themselves are slightly

coloured as well as their condensation products, but, for being powerful oxidants, oxidize other compounds, in particular flavan-3-ols, to form brown polymers (Cheynier, Fulcrand, Guyot, Oszmianski, & Moutounet, 1995; Li, Guo, & Wang, 2008). On the other hand, the hydrolysis of the above mentioned hydroxycinnamoyltartaric acids originates their correspondent free form, which is, respectively, caffeic acid, *p*-coumaric acid and ferulic acid (Jackson, 2000a). Another hydroxycinnamate regularly found in wines is sinapic acid, but often in small amounts as well as all hydroxycinnamates in the free form (Ribéreau-Gayon, et al., 2006). Cinnamic acids derivatives can be found in the isomeric forms *cis* and *trans*, but as the *trans* form is more stable is also the most abundant (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). The most predominant hydroxycinnamate is *trans*-caftaric acid, representing up to 50% of the hydroxycinnamic acids. In comparison with hydroxybenzoic acids, concentration of hydroxycinnamic acids in wines is higher and values about 100 mg/L are frequently described.

From the esterification of caffeic acid and *p*-coumaric acid with the glucose of anthocyanin monoglucosides are formed acylated anthocyanins (flavonoids). Furthermore, the breakdown of *p*-coumaric and ferulic acids generates the formation of volatile phenols, essentially ethyl and vinyl phenols (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). Particularly, the enzymatic decarboxylation of *p*-coumaric acid and ferulic acid, through the action of some yeast during alcoholic fermentation, originates vinyl phenols and vinyl guaiacols, respectively, with odours resembling gouache paint (Clarke & Bakker, 2007; Ribéreau-Gayon, et al., 2006).



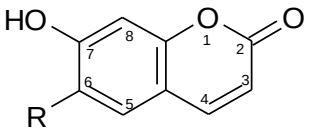
Volatile Phenols	R_1	R_2	R_3
Ethyl phenol	H	$CH_2–CH_3$	H
Vinyl phenol	H	$CH=CH_2$	H
Guaiacol	OCH_3	H	H
Methyl guaiacol	OCH_3	CH_3	H
Ethyl guaiacol	OCH_3	$CH_2–CH_3$	H
Vinyl guaiacol	OCH_3	$CH=CH_2$	H
Propyl guaiacol	OCH_3	$CH_2–CH_2–CH_3$	H
Allyl guaiacol	OCH_3	$CH=CH–CH_3$	H
Syringol	OCH_3	H	OCH_3
Methyl syringol	OCH_3	CH_3	OCH_3

FIGURE 2B.3 – Most common volatile phenols in wines.

Volatile phenols are indeed a small fraction of the polyphenols present in wines but due to their high odour activity they play an important role in wine's aroma, being responsible for off-flavours (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). Besides being formed from precursors present in wines through enzymatic route, they can also migrate from wood during maturation, due to the lignin breakdown promoted by the toasting of the casks (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009; Ribéreau-Gayon, et al., 2006). Figure 2B.3 depicts some examples. In this case, these phenols are characterized by smoky, toasty and burnt smells.

Besides volatile phenols, yeast metabolism may also provide other non-flavonoid compounds as tyrosol (Jackson, 2000a). Tyrosol, frequently found in wines between 20 to 30 mg/L, is formed during alcoholic fermentation from tyrosine (Ribéreau-Gayon, et al., 2006).

Coumarins can be considered as cinnamic acid derivatives (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009; Ribéreau-Gayon, et al., 2006). Coumarins encompass in their structure fused benzenes and *α*-pyrone rings (Figure 2B.4) (Hoult & Payá, 1996).



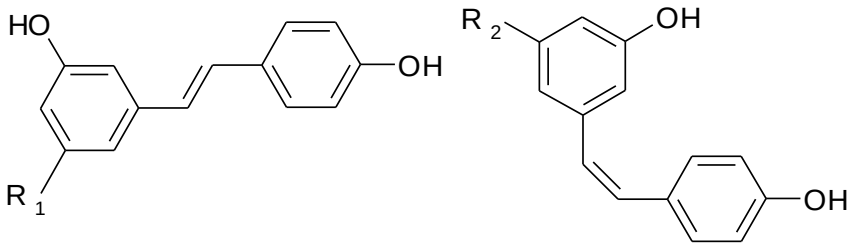
Coumarins		R
Esculetin	aglycone	OH
Esculin	glycoside	Glc 6
Scopoletin	aglycone	OCH_3
Scopolin	glycoside	Glc 7

FIGURE 2B.4 – Structure of coumarins in wines.

The occurrence of these non-flavonoids in wines usually appears in small quantities and is frequently associated with oak-wood ageing (Goode, 2005; Ribéreau-Gayon, et al., 2006). The most common examples are the aglycones (free forms) esculetin and scopoletin, and the glycosides (glycosylated forms) esculin and scopoline (Ribéreau-Gayon, et al., 2006). Even at low concentrations, they influence the sensory attributes of wines: the glycosides, present in green wood, are bitter while the aglycones, present in naturally seasoned wood, are acidic (Goode, 2005; Ribéreau-Gayon, et al., 2006).

2B.2.2 *Stilbenes*

Stilbenes are an important class of polyphenols in wines since they exhibit potent antioxidant, anticarcinogenic and antimutagenic properties (Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). Indeed, grapes and wines are pointed out as the most significant dietary sources of stilbenes. Their occurrence in grapes is associated to the response of grapevines against fungal infection, particularly *Botrytis cinerea*, or to UV irradiation. Stilbenes are essentially located in grape skins, mainly in the glycosylated form, and are transferred to wine musts during the initial winemaking steps, especially when fermentation is conducted in the presence of grape solid parts (skins, seed and stem) (Sun, Ribes, Leandro, Belchior, & Spranger, 2006). Therefore, red wines generally have higher stilbene contents than rosé or white wines (Rentzsch, et al., 2009). Additionally, red grapes frequently present greater amounts of stilbenes. The stilbene structures contain two benzene rings bonded by a carbon chain, normally ethane or ethylene (Ribéreau-Gayon, et al., 2006). The most relevant example is resveratrol (3,5,4'-trihydroxystilbene) not only for being the most abundant stilbene in wines but also for being extensively studied (M. N. Bravo et al., 2008; Moreno-Arribas & Polo, 2009; Rentzsch, et al., 2009). The two isomeric free forms, *cis*- and *trans*-, can be found in wines as well as their *β*-glucoconjugated forms, the *cis*- and *trans*-piceid. Nevertheless, the most abundant is *trans*-resveratrol (Sun, et al., 2006). The levels of *trans*-resveratrol in wines varies greatly, even between the red and white grape varieties, ranging from 0.2 to 14 mg/L in red wines and from 0.1 to 0.8 mg/L in white wines (Rentzsch, et al., 2009; Stervbo, Vang, & Bonnesen, 2007). It is said that *cis*-resveratrol, absent in grapes, appears in wines from the photochemical isomerization of *trans*-resveratrol or by the breakdown of resveratrol polymers, the so-called viniferins, during skin fermentation (Sun, et al., 2006). Figure 2B.5 presents the several forms of resveratrol and its glycosides.



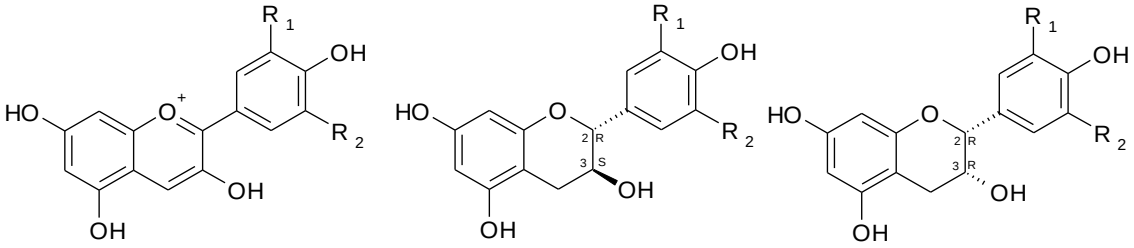
Stilbenes	R_1	R_2
trans-Resveratrol	OH	–
trans-Piceid	OGlc	–
cis-Resveratrol	–	OH
cis-Piceid	–	OGlc

FIGURE 2B.5 – Chemical structures of resveratrol and its glycosides present in wines.

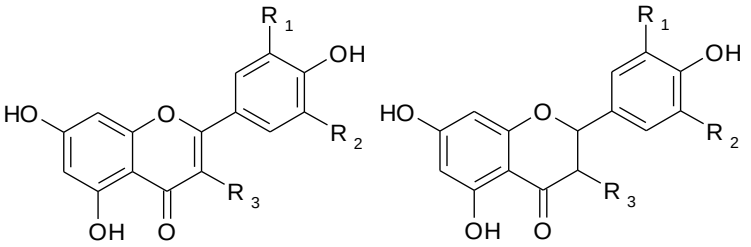
2B.3 Flavonoid polyphenols

Flavonoids are large-polymer molecules characterized by having two phenolic structures connected by an oxygen-containing carbon-ring structure (a pyran), sharing the common skeleton C_6-C_3-C_6 (Jackson, 2000a; Terrier, Poncet-Legrand, & Cheynier, 2009). Figure 2B.6 shows their general structure.

Flavonoids are the main source of phenolic compounds in grapes, especially in skins and seeds, but rarely are completely extracted for most wines (Jackson, 2000b). Their occurrence in wines is clearly influenced by the applied vinification techniques. Flavonoids usually are the major polyphenols in red wine, constituting more than 85% of the phenolic content and are usually found in concentrations ranging from 1000 to 1800 mg/L (Jackson, 2000a; López-Vélez, Martínez-Martínez, & Valle-Ribes, 2003). White wines flavonoids normally account less than 20%, namely fewer than 50 mg/L (Jackson, 2000a). In wines, flavonoids can occur in the free form or polymerized with sugars (the glycoside form), non-flavonoids (acyl derivatives) and other flavonoids (proanthocyanidins) (Jackson, 2000a). Actually, flavonoids are found in grapes mainly in the glycoside form which are hydrolysed during wine fermentation (Ribéreau-Gayon, et al., 2006). The most common wine flavonoids are flavonols, flavan-3-ols and anthocyanins in red wines. Flavanonols and flavones usually appear in smaller amounts (Monagas, et al., 2005). Flavonols are relatively intense yellow pigments while anthocyanins are red pigments responsible for the colour of red wines (Ribéreau-Gayon, et al., 2006).



Anthocyanidins (= anthocyanin aglycone) Flavan-3-ols



Flavones and flavonols Flavanones and flavanonols

Flavonoids			
Anthocyanidins	**R_1**	**R_2**	
Cyanidin	OH	H	
Delphinidin	OH	OH	
Peonidin	OCH_3	H	
Petunidin	OCH_3	OH	
Malvidin	OCH_3	OCH_3	
Flavan-3-ols	**R_1**	**C-2**	**C-3**
(+)-Catechin	H	R	S
(+)-Gallocatechin	OH	R	S
(-)-Epicatechin	H	R	R
(-)-Epigallocatechin	OH	R	R
Flavones	**R_1**	**R_2**	**R_3**
Apigenin	H	H	H
Luteolin	OH	H	H
Flavonols	**R_1**	**R_2**	**R_3**
Kaempferol	H	H	OH
Quercetin	OH	H	OH
Myricetin	OH	OH	OH
Isorhamnetin	OCH_3	H	OH
Flavanonols	**R_1**	**R_2**	**R_3**
2,3-Dihydrokaempferol	H	H	OH
2,3-Dihydroquercetin	OH	H	OH

FIGURE 2B.6 – Chemical structures of flavonoids in wines.

2B.3.1 *Anthocyanins*

As mentioned above, anthocyanins are water-soluble coloured pigments. Depending on the pH, they may present the following colours: red (low pH), purple and blue (high pH). They are abundantly located in the grape skins of the red varieties, but in *teinturier* varieties they also occur in the pulp (Monagas & Bartolomé, 2009; Ribéreau-Gayon, et al., 2006). Grape

anthocyanins are accumulated during ripening and are extracted to musts during fermentation, especially when maceration is privileged. Indeed, anthocyanins are largely responsible for the colour of red wines. Anthocyanins are glycoside derivatives of anthocyanidins (flavonoid component). Actually, the sugar component enhances the anthocyanidins (anthocyanin's aglycone) chemical stability and therefore their water solubility (Jackson, 2000a). The most common glycosyl moiety in wines is *D*-glucose and is frequently positioned at carbons 3, 5, and 7 (da Costa, Horton, & Margolis, 2000). The aglycones can be distinguished by the number of the hydroxyl groups and by the degree of methylation of these groups (Kosir et al., 2004). The most common anthocyanins in grapes and wines are the 3-*O*-monoglucosides and the 3-*O*-acylated monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin (Figure 2B.7) (Monagas & Bartolomé, 2009). The acylated anthocyanins include the esterification of the glucose molecule with acetic, lactic, *p*-coumaric and caffeic acids. The anthocyanins are quite unstable, except the acylated anthocyanins which are relatively more stable (da Costa, et al., 2000). 3,5-Diglucoside anthocyanins can also occur in grapes, mainly in non *V. vinifera* species. Nevertheless, some *V. Vinifera* grapes have presented trace amounts. In fact, the different proportion of diglucoside anthocyanins between *Vitis* species has been used to ensure that *V. Vinifera* is being used in the preparation of European wines.

The wine anthocyanin composition depends on several factors, but primarily on the grape profile. In this sense, becomes essentially affected by the grape variety, grape maturity and climatic conditions (Monagas & Bartolomé, 2009). In general, it has been found that the most abundant anthocyanin in red grape varieties is malvidin-3-glucoside (oenin or malvin). Its proportion varies according to the variety: represents up to 90% in Grenache and less than 50% in Sangiovese (Ribéreau-Gayon, et al., 2006). Furthermore, the amount of acylated anthocyanins present in grapes also depends on the grape variety. Actually, some varieties may not have this kind of anthocyanins, such as Pinot noir (Monagas & Bartolomé, 2009).

Once extracted and dissolved in wine, anthocyanins can be in equilibrium between several forms: flavylium cation (red), quinoidal base (blue violet), chalcone (pale yellow), carbinol pseudo-base (colourless) and flavene (colourless) (Jackson, 2000a). However, in wines, they essentially occur in the colourless carbinol pseudo-base form (Monagas & Bartolomé, 2009). If a small proportion of anthocyanins (20 to 25%) occur in the flavylium state wines exhibit red colours (Jackson, 2000a). In fact, the proportion of anthocyanins in this state is favoured by low pH, usually from 3.4 to 3.6. The pH increase as well as great sulphur dioxide concentrations causes the decrease of the colour density and the diminishment of the anthocyanins proportion in the flavylium state. Actually, the colours of anthocyanins are sensitive to pH, temperature, light and the presence of metals (da Costa, et al., 2000).

Anthocyanins are quite unstable, therefore their concentration in wine severely decreases during ageing. This decrease is, in part, associated with their breakdown into benzoic and cinnamic acids, and other simpler phenolic compounds (Ribéreau-Gayon, et al., 2006). Thermal processing and oxidative conditions promote this effect. Moreover, wine anthocyanins also polymerize, by reaction with other wine constituents, during winemaking, especially during ageing, generally forming more stable pigments (Monagas & Bartolomé, 2009). Anthocyanins reactions can include: the reaction with enzymatically generated *o*-

quinones of caftaric and coutaric acids; the reaction with small compounds (pyruvic and phenolic acids, acetaldehyde, *p*-vinylphenol) giving rise to pyranoanthocyanin pigments, such as vitisins and portisins; and the direct and acetaldehyde-mediated anthocyanin-flavanol and anthocyanin-anthocyanin condensation reactions (de Freitas & Mateus, 2006; Monagas & Bartolomé, 2009). These reactions are dependent from the reactants concentration, pH, temperature, metal ions and oxygen (Monagas & Bartolomé, 2009). Additionally, wine anthocyanins can develop copigmentation, increasing their stability as well as colour.

2B.3.2 *Flavan-3-ols*

Flavan-3-ols are found in grape seeds, skins and stems in monomeric, oligomeric, or polymeric forms (Monagas, et al., 2005). Generally, the grape seeds enclose higher amounts of flavan-3-ols than skins. The most relevant flavan-3-ol monomeric units present in grapes, and therefore in wines, are *(+)*-catechin and *(-)*-epicatechin. *(+)*-gallocatechin and *(-)*-epigallocatechin can also be found.

The oligomeric and polymeric flavan-3-ols are also known as proanthocyanidins. They are called proanthocyanidins because their heating under acidic conditions liberates anthocyanidins (cyanidin or delphinidin) (Terrier, et al., 2009). If proanthocyanidins are composed by *(+)*-catechin and *(-)*-epicatechin monomers they are classified as procyanidins. On the other hand, prodelphinidins are composed of *(+)*-gallocatechin and *(-)*-epigallocatechin monomers. The proanthocyanidins are also denoted as condensed tannins, since, like all the tannins, they interact or react with proteins promoting their precipitation.

As mentioned above, flavan-3-ols react with anthocyanins through an ethyl bridge by acetaldehyde mediation, especially during wine ageing, to form higher molecular compounds. Acetaldehyde can also mediate the polymerization between flavan-3-ols (Es-Safi, Fulcrand, Cheynier, & Moutounet, 1999; Fulcrand, Doco, Es-Safi, Cheynier, & Moutounet, 1996). Besides acetaldehyde, flavanols are also known to react with other aldehydes, such as glyoxylic acid (Es-Safi et al., 1999; Saucier, Guerra, Pianet, Laguerre, & Glories, 1997). Additionally, flavan-3-ols can react with ellagic acid derived from wood-lignin hydrolyses.

Flavanols are the main polyphenolic compounds associated with white wine oxidative browning. In wines exposed to oxidative conditions, molecular oxygen is reduced to hydrogen peroxide, which in the presence of trace metal ions (iron(II) or copper(II)), can originate the powerful oxidants hydroxyl radicals (Clark, 2008). Ascorbic acid is known to potentiate the hydrogen peroxide production (Barril, Clark, & Scollary, 2008). In turn, hydroxyl radicals oxidise tartaric acid to form an aldehyde, the glyoxylic acid (Clark, 2008). This aldehyde is known to react with flavanols, especially with *(+)*-catechin, and can initiate a sequence of reactions leading to the formation of coloured compounds known as xanthylium cations (absorbance maximum at 440 nm, which corresponds to a yellow colour) (Es-Safi, Guernevé, et al., 1999). These reactions are accelerated by high temperatures or pH values (Clark, 2008). Ascorbic acid, furfural and 5-hydroxymethylfurfural are also known to react with *(+)*-catechin to develop the same products (Barril, et al., 2008; Es-Safi, Cheynier, & Moutounet, 2000).

2B.3.3 *Flavonols*

Flavonols are yellow pigments mainly located in the grape skins of both red and white grapes (Ribéreau-Gayon, et al., 2006). In grapes, they are present in the glycoside form, 3-*O*-glycosides, of the main aglycones: myricetin, quercetin, kaempherol and isorhamnetin (Monagas, et al., 2005). Actually, the most common are the quercetin glycosides (Terrier, et al., 2009). Grape flavonols prevent some damaging effects of ultraviolet (UV) exposure since they absorb this kind of radiation (Jackson, 2000a). Indeed, flavonols synthesis mainly occurs during ripening and is stimulated by direct exposure to UV and blue radiation (Jackson, 2000a; Terrier, et al., 2009). In this sense, grapes highly exposed to daylight are capable of increase the flavonol biosynthesis and thereby produce wines with high flavonol levels. Generally, white grapes do not contain derivatives of myricetin and usually present lower levels of flavonols than red grapes (Terrier, et al., 2009). Wines, not only have glycoside flavonols but also aglycones, derived from the hydrolysis of the glycoside forms during vinification, maturation, and/or ageing of wine (Monagas, et al., 2005). Once more, red wines present higher levels of both flavonol glycosides and aglycones relative to white wines, not only by differences in composition between red and white grapes, but also by the implementation of different vinification technologies (Makris, Kallithraka, & Kefalas, 2006). Traditionally, the vinification of white wines usually does not include contact of must with grape skins. Consequently, the extraction of flavonol glycosides, which are essentially located in skins, is very limited.

Wine flavonols usually decrease during winemaking (Makris, et al., 2006). Firstly, several post-fermentative treatments such as fining usually cause significant reduction of flavonols in wines. Additionally, flavonols during wine ageing and storage are also able to react with anthocyanins to form copigments. Finally, oxidative conditions and temperature also seem to play a central role in flavonols decrease during wine storage and ageing.

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CHAPTER 2C

Organic acids

2C.1 Introduction

Organic acids are compounds with acidic properties. Generally, organic acids acidity comes from their ionization capacity, releasing hydrogen ions (H^+) into aqueous systems. Indeed, these substances are responsible for wine acidity, representing one of the major contributors for wines composition. Besides organic acids, wines also contain inorganic acids, particularly carbonic acid and sulphurous acids, which do not have great influence on wine pH and are barely perceptible in terms of acidity (Jackson, 2000a; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006b). The most abundant organic acids in wines are the carboxylic acids whose acidity is associated with the functional carboxyl group, -COOH (Jackson, 2000a).

Already present in grapes, organic acids are essential during winemaking because they first induce the growth and vitality of yeasts during fermentation and hence increase the sensory complexity of wine. In fact, they participate in the sensory qualities of wines with fresh, tart and sour attributes. Besides directly affect the fermentation, organic acids also influence the colour extraction, maturation, and therefore, have direct influence on flavour balance and colour of the final product. Moreover, they enhance the effectiveness of sulphur dioxide and ethanol in the microbial stability of wines, suppressing the growth and metabolism of most potential wine-spoilage organisms, and support the stability of tartrates in wine (Jackson, 2000a; Jacobson, 2006). Recently, Batista et al. (2010) show that some organic acids in wines exhibit a stabilising effect upon the haze potential of the wine proteins.

2C.2 Wine acidity

Apart of other effects, wine is greatly affected by acidity: wine with too low acidity is flat or insipid while the opposite promote a sour rather than pleasantly tart taste. Wines from grapes grown in warm climates are generally somewhat bland, soft, high in alcohol and low in acidity. Wines from cooler climates usually do not reach proper maturity before being harvested. Their sugar content may be too low and/or acidity may be too high to produced well balanced wines (Beelman & Gallander, 1979).

There are several kinds of acidity: total acidity, pH and volatile acidity, and together with the alcohol and residual sugar contents, contribute significantly to flavour balance (Ribéreau-Gayon, Glories, et al., 2006b). In wineries, the total acidity of musts and wines is often controlled by the classical measurement of the titratable acidity while the strength of acidity is measured according to pH. Indeed, the total acidity contemplates the fixed and the volatile acidity, taking into account all acid forms present in musts and wines, namely inorganic and organic acids and perhaps amino acids (Ribéreau-Gayon, Glories, et al., 2006b). However,

for practical reasons, titratable acidity, which only considerates the proton concentration of the total acidity, is often referred as the total acidity because it encompasses the majority of the total acidity (Jacobson, 2006). Titratable acidity is often determined by neutralization. Fixed acidity represents the non-volatile acids and is frequently expressed in terms of tartaric acid, whereas volatile acidity is often expressed in terms of acetic acid and refers to free and combined acids that can be readily removed by steam distillation (Jackson, 2000a; Jacobson, 2006), especially acetic, carbonic and sulphurous acids (Grainger, 2009). Wine pH reflects the content in hydrogen ion and is measured using a pH meter conveniently calibrated, checking temperature (Jacobson, 2006; Ribéreau-Gayon, Glories, et al., 2006b).

Regarding total acidity, a range between 5.5 to 8.5 mg/L is commonly desired for wines; however for white wines is preferred higher levels than for red wines (Jackson, 2000a). In terms of volatile acidity, wines should not exceed 1.1 g/L of acetic acid to ensure their quality (Ribéreau-Gayon, Glories, et al., 2006b). Generally, wines present pH values ranging between 2.8 and 4.0, but a pH range between 3.1 to 3.4 is usually desired for white wines and from 3.3 to 3.6 for most red wines (Jackson, 2000a; Ribéreau-Gayon, Glories, et al., 2006b). It is reasonable to think that wines with lower pH have higher acidity but, there is no direct connection between total acidity and pH, because the strength of acidity is related to the type of acids involved. For example, it is frequent wines with a high pH and high acidity (Beelman & Gallander, 1979).

Wine acidity can be dependent from several cultural practices as vine variety and corresponded rootstock, pruning, soil fertility, irrigation and also virus infection (Beelman & Gallander, 1979). In fact, vigorous vines on fertile soils generate grapes, and consequently musts, with elevated acidity. The degree of maturation of grapes can also be a crucial aspect. As can be seen, grape is indeed the main contributor to wine acidity. Tartaric and malic acids the most abundant acids in grapes are also abundant in wines, although there is a decrease in their concentration. Most of the wine acidity is due to these acids.

2C.3 Wine primary acids

The most common organic acids found in wines are tartaric, malic, lactic, acetic, citric and succinic acids. Figure 2C.1 depicts their chemical structure. Some are already present in grapes, namely tartaric, citric and malic acids and others appear during winemaking especially after fermentation, such as acetic, lactic and succinic acids, and in minor quantities formic acid and others (Beelman & Gallander, 1979; Ribéreau-Gayon, Glories, et al., 2006b). Eventually, other acids can occur in small amounts which may be derived from ethanol oxidation (Belitz, Grosch, & Schieberle, 2009). Nevertheless, other acids that do not occur naturally can be added to wines, especially to prevent or correct some wine defects, in particular ascorbic and sorbic acids.

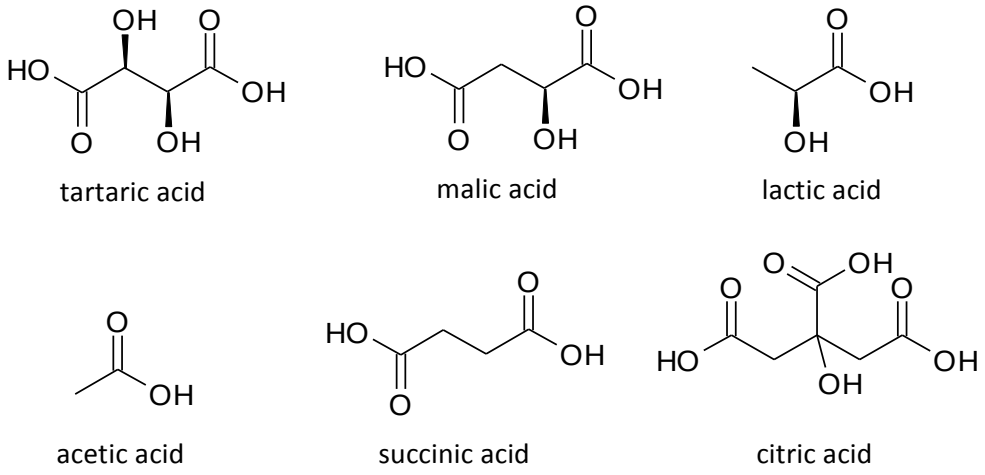

FIGURE 2C.1 – Chemical structure of the main wine organic acids.

2C.3.1 *Tartaric acid*

From a chemical point of view, this fixed acid is considered as the strongest wine acid with a pK_a of 3.01, therefore can greatly influence the wine pH. In fact, tartaric acid lowers the pH of musts to a level where many detrimental bacteria cannot live, and after fermentation acts as a preservative.

Together with malic acid, tartaric acid is the major grape acid. It occurs naturally in many plants, but is produced in appreciable quantities in *Vitaceae* genera, for this reason this acid is considered characteristic of grapes, being called as the "wine acid" (Jackson, 2000a; Ribéreau-Gayon, Glories, et al., 2006b).

Tartaric acid in unripe grapes can be present at very high levels, especially in the skin grapes and in the outer part of pulp, occurring in the natural form *L(+)*-tartaric acid or dextrotartaric acid. As the vine ripens, tartaric is not metabolized like malic acid, therefore the tartaric levels remain quite constant throughout the grape ripening. Consequently, the tartaric acid concentration in wines is deeply dependent from grape variety and vineyard soil.

Tartaric acid is one of the main acids found in wines, occurring as free acid and tartrates salts, mainly monopotassium salts. These dissolved salts tend to precipitate, forming potassium bitartrate crystals (cream of tartar) as the alcohol rises during fermentation and as wine age. Therefore, is common proceed with the cold stabilization of wines before wine bottling, usually at temperatures below -7.2 °C, so that the precipitation process accelerates and, thereby, is avoided the crystal deposition in bottle. Regrettably, this process can continue even after this procedure being applied, particularly due to the conversion of the *L* forms into the less soluble *D* isomers. Additionally, other salts relatively insoluble can contribute for this phenomenon, namely the calcium salts, when used in wine deacidification. The instability induced by calcium tartrate is less frequent but more difficult to control since its precipitation is not activated by refrigeration. The precipitation of potassium and calcium salts of tartaric acid is usually accompanied by the loss of titratable acidity and hence pH increases.

From an organoleptic perspective, tartaric acid along with citric and malic acids influences the taste of the finished wine providing tartness attributes to wines.

2C.3.2 *Malic acid*

Malic acid is one of the most prevalent acids among wines. *L(-)*-Malic acid is the naturally occurring form. Structurally, malic acid is similar to tartaric acid and is an abundant by-product of grape metabolism. Its accumulation is rarely observed in skins berries before the maturation period, but when grapes are ripe the malic acid concentration is well distributed throughout skins and berry pulp (Jackson, 2000c).

In the grape vine, malic acid is implicated in some important processes for the vine vitality. Malic acid content in grapes changes more quickly and noticeably than that of tartaric acid. This fixed acid usually increase up to *véraison* (as tartaric acid) but afterwards declines up to 1 to 9 g/L, since malic acid act as a respiratory substitute for glucose during the later stages of ripening. Notwithstanding, in ripe grapes both acids represent about 70 to 90% of the berry acid content (Jackson, 2000c). The remainder can be composed by other kind of acids, namely citric acid, phenolic acids, amino acids and fatty acids. It is known that grapes grown in hot climates often metabolize most malic before grapes harvest, decreasing its concentration as grapes mature, especially during hot periods at the end of the season. Therefore, at ripening stage these grapes contain small amounts of this acid, implicating musts with low acidity. In contrast, the grapes grown in cold climates may keep great malic acid levels at grape maturity, because at this time they do not synthesized most of their malic acid neither was consumed. Consequently, musts from grapes growth in cold climates may require deacidification to reduce the sour taste in wines, while musts produced from grapes cultivated in warm climates are usually compensated by the manual addition of acid in a process known as acidification. Thus, malic acid content is frequently used as a primary reference to determine harvest dates.

Winemakers usually perform acidification before fermentation by the typical addition of tartaric acid to prevent wines becoming flat and susceptible to microbial spoilage (Jackson, 2000b), up to a maximum of 1.5 g/l in must and 2.5 g/l in wine (European Community (EC) legislation) (Ribéreau-Gayon, Glories, et al., 2006b). Moreover, this acid allows lowering the pH due to the noticeable effect of its dissociation constant (K_a) (Jackson, 2000d). Eventually, citric acid can also be used because improves the iron stabilization but lose efficient with microbial activity than tartaric acid. Alternatively, acidification may also be conducted to wines before bottling, and besides the treatments mentioned above ion exchange can also be used. This procedure involves a column packed with a cation-exchange resin to replace H^+ for the Ca^{2+} or K^+ of tartrate and malate salts (Jackson, 2000d). Relatively to deacidification, musts with high acidity and low pH may be blended with musts with softer acidity and higher pH or be added potassium bicarbonate ($KHCO_3$) or calcium carbonate ($CaCO_3$) (Jackson, 2000b; Ribéreau-Gayon, Glories, et al., 2006b). Once more, winemakers may also choose the use of ion exchangers, but this time anion-exchange resins, to exchange tartrate ions for hydroxyl ions, especially at the final treatments of wines (Jackson, 2000d). Other kind of wine deacidification is malolactic fermentation (MLF). Moreover, malic acid importance in wines is also associated with this secondary fermentation. When wine undergone MLF, the harsher-tasting of malic acid (strong acid with pK_a = 3.46) is decarboxylated enzymatically into a smoother-tasting of

lactic acid, through the metabolic activity of lactic acid bacteria (known as LAB), usually *Oenococcus oeni* specie, via malic acid. Malic acid metabolization actually decreases the wine acidity since it is observed the transformation of a dicarboxylic acid (malic acid) into a monocarboxylic acid (lactic acid). This transformation is usually accompanied by pH increase. This kind of fermentation is very frequent in red wines than in whites, especially those with excessive levels of malic acid. However, some styles of white wines may include MLF in its winemaking. Besides its beneficial effects to wines, MLF can generate turbidity and unpleasant compounds such as off-flavours.

2C.3.3 *Lactic acid*

Lactic acid is produced during the fermentation process, essentially from malolactic fermentation but also from alcoholic fermentation via pyruvic acid. Pyruvic acid is also produced during alcoholic fermentation through the metabolism of carbohydrates, but quickly is transformed into the two isomers of lactic acid, *L(+)* mainly from bacteria activity and *D(-)* mainly originated through yeast (Ribéreau-Gayon, Glories, et al., 2006b). Thus, the predominance of *L(+)*-lactic acid usually indicates that malolactic fermentation was accomplished (Jackson, 2000a). As well as the acids aforementioned, lactic acid also contributes to the overall acidity of wine although more softer (pK_a = 3.81).

L(-)-Lactic acid can be used for wine acidification avoiding some limitations imposed by tartaric acid, namely the metallic mouth-feel encountered in some “hard” wines. The use of this acid for acidification can also prevent the precipitation of its potassium and calcium salts (soluble salts in wine media) and therefore enhances the wine acidification minimizing the pH decrease. Moreover, lactic acid is microbiologically stable, unlike tartaric, malic, and citric acids. However, industrial lactic acid nauseating odour has prevented its use in wineries (Ribéreau-Gayon, Glories, et al., 2006b).

Most of wine acids, including lactic acid can be esterified by ethanol, forming ethyl esters. In the case of lactic acid it is formed ethyl lactate while from others can be formed ethyl acetate, diethyl succinate, monoethyl succinate and others. Ethyl lactate and diethyl succinate have little impact on wine aroma when present in moderate concentrations (Zamora, 2009).

2C.3.4 *Acetic acid*

Acetic acid is known to be very abundant in vinegars. Unlike most acids in wine, acetic acid is volatile, usually pointed out as the main acid responsible for wine volatile acidity.

Small amounts of this acid are formed during the beginning of alcoholic fermentation through the metabolism of yeasts. High levels are usually associated with acetic bacteria or lactic acid bacteria contaminations, especially when fermentation stuck or slows down (Ribéreau-Gayon, Glories, et al., 2006b; Zamora, 2009). Actually, acetic acid bacteria may grow in oxygenated wines, and therefore, produce acetic acid from ethanol. These bacteria, namely the genus *Acetobacter*, greatly develops in wineries with reduced hygiene conditions, at any winemaking stage, especially in old wooden barrels (Grainger, 2009). In fact, acetic acid can also be formed by means of chemical hydrolysis of hemicelluloses during oak- ageing (Jackson, 2000a). In fortified wines, the *Acetobacter* is inhibited by the elevated ethanol concentration

(Steinkraus, 2009). As a result, such wines are quite stable at room temperature for a long time.

Acetic acid can be either beneficial or detrimental based on its concentration. Ideally, the acetic acid concentration in wines should be around 0.3 g/L. At this concentration acetic acid can contribute to wine aroma and taste, adding complexity by itself and through acetic acid reaction to produce acetate esters, which gives to wines a fruity character. However, this compound must be well controlled to avoid that high concentrations compromise wines quality, since this acid gives off a vinegar-like odour to wine and a disagreeable sensation in the mouth if it is much greater than 0.3 g/L. However, some authors support the idea that acetic acid's odour perception depends on wine type and style (Ugliano & Henschke, 2009). In the case of Madeira wines the odour threshold of acetic acid should be higher than previously mentioned, once the sugar levels, higher than in table wines, balance its perception.

Frequently, during wine ageing and even during fermentation, acetic acid can combine with ethanol to form ethyl acetate. This compound may be convenient for wine aroma if present at low concentrations, usually bellow 50 mg/L, but above 150 mg/L may be undesired by producing a sour-vinegar off-odour.

2C.3.5 *Succinic acid*

Succinic acid can appear in *Vitis Vinifera L.* unripen grapes in insignificant amounts (Jackson, 2000c). However, succinic acid is a major component in muscadine young grape berries (*V. Rotundifolia*), but is readily consumed as the vine maturates. The succinic acid in conjunction with fumaric acid is consumed for being involved in the lipid metabolism and the Krebs cycle during grape maturation (Ribéreau-Gayon, Glories, et al., 2006b).

In wines, succinic acid is a common by-product of yeast metabolism during the alcoholic fermentation and is usually found at concentrations close to 1 g/L (Ribéreau-Gayon, Glories, et al., 2006b). This fixed acid is resistant to wine bacteria and originates bitter-salty tastes, causing salivation. Moreover, accentuates the wine's flavour and vinous character.

2C.3.6 *Citric acid*

Citric acid is abundantly found in citrus fruits but it is scarce in ripe grapes. Therefore, usually appears in minor quantities in wines, between 0.5 to 1 g/L (Ribéreau-Gayon, Glories, et al., 2006b). In grapes development plays a crucial role in Krebs cycle, while in musts fermentation slows yeast growing but does not block it. During musts fermentation yeasts have the tendency to convert citric into acetic acid.

As mentioned above, this acid might be used as supplement in wine acidification to increase the wine total acidity, however with less frequency than the tartaric acid since it can add aggressive citric flavours to wine. Indeed, European Union prohibits its use in wine acidification. Furthermore, this acid often forms soluble complexes with ferric iron (Fe^{3+}) (Ribéreau-Gayon, Glories, et al., 2006b). For this reason, citric acid is an authorized additive, even in Europe up to 0.5 g/L, to prevent ferric casse, frequent in white wines when ferric ions

interact with phosphoric acid to form colloidal ferric phosphate, which then interacts with proteins and precipitates (Jackson, 2000d; Ribéreau-Gayon, Glories, et al., 2006b).

2C.3.7 *Other acids*

Ascorbic acid, also known as vitamin C, it is naturally present in grapes but in wines it is rarely present without being added. It has been used as an adjuvant of sulphur dioxide, regularly at a maximum concentration of 150 mg/L, added especially to white wine as an antioxidant agent, capable of preventing wine oxidative browning, promoted by the iron oxidation in aeration processes (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006a). Nowadays, this practice is falling into disuse since only prevents early oxidative browning of the must, regrettably accelerating the wine colouration due to its pro-oxidant activity, initiated by hydrogen peroxide degradation product. Actually, Barril and co-workers (2008) demonstrated that ascorbic acid contributes to the pigment development, by promoting the formation of the coloured xanthylium cations.

Finally, in some countries sorbic acid can be added up to 200 mg/L to wine as an antiseptic agent to enhance the antimicrobial properties of sulphur dioxide (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006).

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CHAPTER 2D

Carbohydrates

2D.1 Introduction

Carbohydrates, precisely sugars, are fundamental constituents on winemaking, given that they are the base substances of the fermentative process which leads to the ethanol formation and various by-products, and therefore the beverage that we call wine. Indeed, yeasts use them as their nutrients. Furthermore they also participate in the wine taste, namely the simpler sugars, contributing to the sweetness, being frequently unnoticed in dry wines and quite evident in sweet wines. On the other hand, sugars also generate derived-products, such as glycerol, flavour volatile compounds, and others, very important for the wine structure and aroma.

2D.2 Chemical structure and properties

Carbohydrates general formula is $C_n(H_2O)_n$. and are classified according to their polymerization degree as monosaccharides or polysaccharides (Sanz & Martínez-Castro, 2009). The smaller carbohydrates, namely monosaccharides, are commonly referred as sugars. This kind of carbohydrates is indeed the most important in wines. Monosaccharides structure with 3 to 8 carbon atoms encompasses several hydroxyl groups and a carbonyl group, a ketone or an aldehyde (Jackson, 2000; Sanz & Martínez-Castro, 2009). If the carbonyl group is a ketone, the monosaccharide is a ketose, but if it is an aldehyde the monosaccharide is an aldose. Monosaccharides can also be grouped in terms of carbon atoms: with 3 carbons are called trioses, with 4 are tetroses, with 5 are pentoses, 6 are hexoses, and so on. The most common in wines are hexoses.

Sugars are very soluble in water (Ribéreau-Gayon, Glories, Maujean, & Dudourdieu, 2006) and once in solution, the carbonyl group (aldehyde or ketone) of the straight-chain monosaccharide reacts reversibly with an intramolecular hydroxyl group to form a hemiacetal or a hemiketal, originating heterocyclic rings with an oxygen bridge between two carbons, until is reached an equilibrium between the two forms (see Figure 2D.1) (Sanz & Martínez-Castro, 2009). During this equilibrium, rings of 5 (furanoses) and 5 (pyranoses) members can be present.

2D.3 Glucose and fructose

Glucose and fructose, in the *D*-form (Figure 2D.1), are the most abundant hexoses in grapes (Ribéreau-Gayon, et al., 2006). Other sugars can also occur but frequently at minor concentrations (Jackson, 2000). The aldose *D*-glucose is often referred as dextrose and the ketose *D*-fructose is also known as levulose (Ribéreau-Gayon, et al., 2006). At full ripening

stage glucose and fructose often occur in almost equal proportions. However, in extremely ripe grapes is common to find higher proportion of fructose.

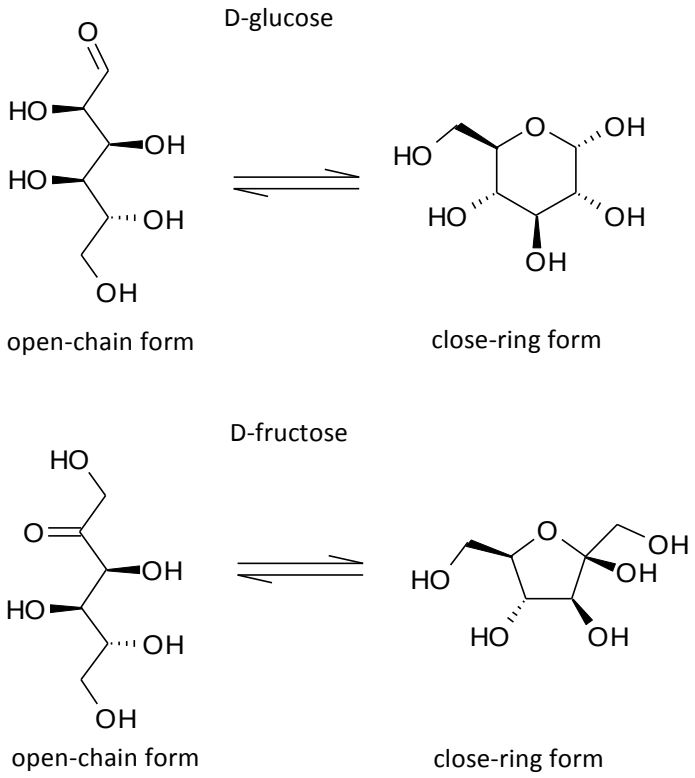

FIGURE 2D.1 – *D*-glucose and *D*-fructose in equilibrium between the open-chain and close-ring forms.

Of course that grape sugar content varies depending on variety, maturity, and fitosanitary conditions, but is frequently encountered juices of ripe grapes containing between 150 to 250 mg/L of sugars, mainly glucose and fructose (Jackson, 2000; Ribéreau-Gayon, et al., 2006). *V. Vinifera* species usually attained a higher percentage of sugars than *V. labrusca* and *V. rotundifolia* (Jackson, 2000).

Glucose and fructose are fermentable sugars, readily consumed by yeast as nutrients during the alcoholic fermentation to produce ethanol and others by-products, such as higher alcohols, fatty acid esters, and aldehydes (Jackson, 2000). Indeed, glucose is preferentially fermented by the great majority of yeasts than fructose (Ribéreau-Gayon, et al., 2006). Therefore, sweet wines contain 2-4 times more fructose than glucose and dry wines, usually completely fermented, still contain small amounts of fructose and others residual sugars such as pentoses (like arabinose, rhamnose, and xylose), which are not fermentable.

From another perspective, fructose and glucose have different effects on wine taste: fructose sweetness is more intense than glucose, with a rating of 1.73 on a scale of sweetness while glucose only rates 0.74 (Ribéreau-Gayon, et al., 2006). Pentoses contribute less to the wine sweetness (rating of 0.4). Moreover these two sugars play different roles on wine flavour, since fructose is more reactive than glucose, thus is more available for the formation of volatile aromas and other derived-products.

2D.4 Chemical reactivity

Sugars are polyfunctional molecules, capable of participating in several reactions with acids, bases and proteins, especially when they appear in aqueous matrices such as wine. Indeed, some of these reactions are quite relevant in wine, especially the sugar degradation in acidic medium and the Maillard reaction.

In acid medium wine sugars, namely glucose and fructose, dehydrate and degrade, being formed furans, pyrans, cyclopentenes, carbonyl compounds and other low-molecular weight compounds (Belitz, Grosch, & Schieberle, 2009; Mottram, 2007; Sanz & Martínez-Castro, 2009). Additionally, brown-coloured compounds are also by-products of the sugar degradation (Sanz & Martínez-Castro, 2009). This reaction can occur with wine ageing, especially in sweet wines and is accelerated by temperature increase, leading to similar products of those of Maillard reaction (van Boekel, 2006). At very high temperatures can take place caramelization.

2D.4.1 *The Maillard reaction*

The Maillard reaction is a type of non-enzymatic browning of extreme importance to food industry, as it affects the quality of food products, particularly the sensory attributes such as colour, aroma and taste, the nutritive value (proteins and amino acids become unavailable for human metabolism) and safety (formation of mutagenic compounds or even potentially carcinogenic as the recently found acrylamide) (Fay & Brevard, 2005; Nunes & Baptista, 2001). Additionally, some Maillard reaction products are also recognize to have antioxidant properties (Kim & Lee, 2009; Morales & Jiménez-Pérez, 2001; Osada & Shibamoto, 2006; Yilmaz & Toledo, 2005). Besides affecting the quality of foods, Maillard reaction can also have effect in biological systems, namely in the *in vivo* protein chemistry (Nunes & Baptista, 2001).

In foods, this complex reaction starts with the condensation of a carbonyl compound, usually a reducing sugar, with an amino compound, usually an amino acid, a peptide, or a protein (Nursten, 2005a). Then, it follows a set of consecutive and parallel reactions being formed a variety of products, from aroma compounds of low-molecular weight to melanoidins with low to high-molecular weight, brown nitrogenous compounds (Martins & Van Boekel, 2005). In this reaction the amino compound firstly acts as a catalyst inducing the dehydration of reducing sugars and in a more advanced stage reacts with other carbonyl compounds producing Strecker's aldehydes (Fay & Brevard, 2005; Jackson, 2000).

This reaction between sugars and amines was named as Maillard reaction in honour of Louis-Camille Maillard that first described it in 1912 (Nursten, 2005a). This discovery prompted the study of this reaction, verifying that develops in heated, dried or stored foods and *in vivo* in mammals, at their pH and physiological temperature (Fay & Brevard, 2005). *In vivo* results from the interaction of glucose with proteins intervening in the ageing of the collagen fiber as well as in the pathological changes of the cornea observed in *diabetes mellitus* (Fay & Brevard, 2005; Nunes & Baptista, 2001).

This chapter does not have intention to review the entire state of knowledge of the Maillard reaction, it just briefly pass through the principal ideas. Figure 2D.2 presents its

general scheme. There are several literatures that discuss in greater detail the chemistry of this reaction and its effects (Ellis, 1959; Jaeger, Janositz, & Knorr, 2010; Mottram, 2007; Namiki, 1988; Nursten, 2005b; Silván, van de Lagemaat, Olano, & del Castillo, 2006; van Boekel, 2001, 2006).

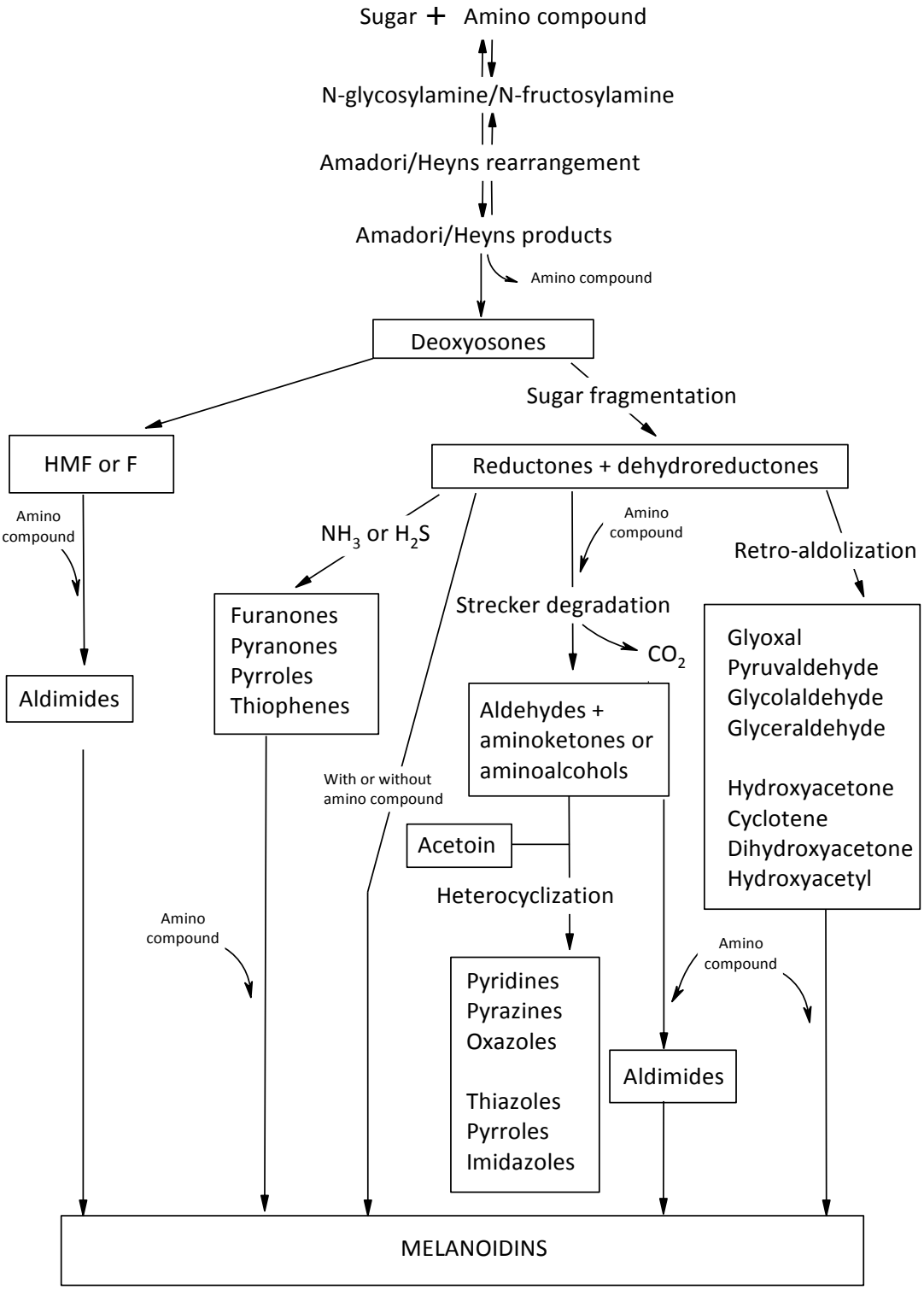

FIGURE 2D.2 - General scheme of Maillard reaction in foods (adapted from (Fay & Brevard, 2005) and (van Boekel, 2006)).

It is very difficult to understand the Maillard reaction, so several attempts have been made to subdivide this reaction into three stages: the initial, the intermediate and the final (Nursten, 2005a). The initial stage involves, in the following order, the sugar-amine condensation, the formation of *N*-glycosylamines (aldose) or *N*-fructosylamines (ketose) and the Amadori/Heyns rearrangement which consequently forms deoxyosones (van Boekel, 2006). At this stage, the reaction steps are well defined and there is no browning, the products are colourless (Nursten, 2005a). At the intermediate stage colourless products or yellow products with strong absorption in the ultraviolet are formed, namely at 280 nm. The most common example is the formation of furfural if the sugar is a pentose or 5-hydroxymethylfurfural (HMF) if sugar is a hexose, through dehydration of 3-deoxyosones, originated via 1,2-enolisation (Mottram, 2007; van Boekel, 2006). Through 2,3-enolisation can be formed 1-deoxyosones, which are transformed into others low molecular weight species such as furanones, pyranones (by reaction with ammonia and hydrogen sulphide) and also dicarbonyl compounds (Fay & Brevard, 2005; Mottram, 2007; van Boekel, 2006). Indeed, deoxyosones suffer fragmentation being formed reductones (carbonyl compounds) and others fragments like acids (formic, acetic, etc.) (van Boekel, 2006). This sugar fragments can undergone several reactions, namely retro-aldolization, leading low-molecular weight compounds such as cyclotene, glyoxal, hydroxyacetone and others. Furthermore, dicarbonyls can degrade amino acids, through oxidative decarboxylation and deamination to lead aldehydes, important to flavour formation and co-operators in the formation of melanoidins (Fay & Brevard, 2005). The dicarbonyl is converted into an α-aminoketone or aminoalcohol. This mechanism pathway is so-called the Strecker degradation. In the final stage of the Maillard reaction takes place the condensation of cyclic subunits, like pyrroles or pyrrole derivatives being developed brown insoluble polymers, designated as melanoidins (Fay & Brevard, 2005).

The formation of brown pigments at the final stage of this reaction is desired in a certain type of food, such as bread, cocoa, roasted coffee, cooked meat, and even some fortified wines, like Madeira wine. However, it is undesirable in others like milk powder, pasteurized milk, in processed products of fruits and tomatoes, etc.. On the other hand, the Maillard reaction results in the development of many odour-active molecules, such as thiazoles, furans, pyrazines, pyrroles, oxazoles, and other heterocyclic compounds, with influence in the aroma of processed food products (Fay & Brevard, 2005). Some of these compounds are off-flavour compounds. Moreover, it should be stated that some heterocyclic compounds, namely those containing amines manifest mutagenic properties.

The Maillard reaction is influenced by several factors such as temperature, heating time, pH, water content and the type and the amount of reagents involved (van Boekel, 2006). Indeed, the sugar type plays an essential role in the development of the Maillard reaction. As already mentioned the sugar in aqueous solution is in equilibrium between the open-chain form and the ring form. The straight-chain form is more reactive, however at room temperature, the amount of sugar in the open-chain form is only 0.5%, thus increasing the pH and/or temperature this percentage can rise, sometimes more than 10%, since the balance shifts in the direction of the more reactive specie (van Boekel, 2001). Pentoses are in general more reactive than hexoses because in solution present higher percentages of acyclic forms. In

the case of glucose and fructose, both are hexoses, but due to the fact that fructose exists in solution in a greater extent in the open-chain form the Maillard reaction happens at faster rate than with glucose (Dills, 1993). On the other hand, is also subjacent the amino acid equilibrium:

$$R\text{-}NH_2 + H^+ \leftrightarrow R\text{-}NH_3^+$$

Deprotonated amino groups are more reactive than protonated species. At low pH (pH < 7), the presence of deprotonated amino groups is low (less than 1%), hence the reactivity is lower. In this sense it is generally accepted that the initial rate and the browning rate increase with pH (van Boekel, 2001). At low pH is favoured the enolization *1,2*, whereas at high pH is more likely to be favoured the pathway *2,3* (Nursten, 2005c).

Several authors have related the appearance of some aromas and browning to the development of Maillard reaction in wines (Cutzach, Chatonnet, & Dubourdieu, 1999; Escudero, Hernandez-Orte, Cacho, & Ferreira, 2000; López de Lerma, Peinado, Moreno, & Peinado, 2010; Pripis-Nicolau, de Revel, Bertrand, & Maujean, 2000). Actually, wine's fermentative processes originate several dicarbonyl compounds susceptible of participating in Maillard reaction, especially in the formation of flavour components, in spite of wine's low pH, low temperature (except some fortified wines), and presence of water (Pripis-Nicolau, et al., 2000; Sanz & Martínez-Castro, 2009). López de Lerma and co-workers (2010) also hypothesize that this reaction may be associated with the antioxidant capacity increase of Pedro Ximénez sweet wines under accelerated oxidative ageing. They also found that volatile Maillard compounds increased throughout the thermal treatment of these wines.

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CHAPTER 2E

Volatile compounds

2E.1 Introduction

Volatile compounds play an important role in the aroma (odour) of wines, determining their character and quality. To date, more than 800 volatile compounds have been found in wines at concentrations ranging from several mg/L to a few ng/L, but only a few can be impact odorants (Aznar, López, Cacho, & Ferreira, 2001; Ferreira, López, Escudero, & Cacho, 1998; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006b). This reflects the complexity of wine aroma. Some compounds are present in trace amounts, still that does not mean they are insignificant to the wine aroma, indeed some may be crucial in characterizing the aroma of certain wines because of their very low sensory thresholds (ng/L), whereas others, much more abundant, may only generate a slight contribution (Ribéreau-Gayon, Glories, et al., 2006b). Other than alcohol, wines generally contain about 0.8 to 1.2 g/L aroma compounds, usually accounting 1% of the ethanol concentration (Rapp & Mandery, 1986). The profile and concentration of volatiles in wines can be influenced by several factors: environment (climate, soil), ripeness and grape variety, fermentative step (pH, temperature, juice nutrients, type of yeast and bacteria), post-fermentation treatments (such as clarification), and at last ageing conditions.

Wine volatile components can be categorized in several ways, but the most common has to do with the time they are formed. In this sense, they can be essentially divided into: primary aromas, compounds already present in grapes and persisting through vinification; secondary aromas, which are those arising from the vinification process, principally those generated during fermentation; and tertiary aromas, derived from wine storage and/or ageing, either in casks, vats, tanks or in bottle (Clarke & Bakker, 2007).

2E.2 Primary aromas

The primary aromas, also known as varietal aromas, are secondary products of plant metabolism and are distributed between the skins and the berry pulp, although most abundant in the skins. The varietal aromas are mainly dependent on climatic factors and cultivation practices, increasing during ripeness parallel to sugar content.

Varietal aromas encompass four distinct classes of chemical families: monoterpenes, C_{13}-norisoprenoids, methoxypyrazines and sulphur compounds with thiol functions (mercaptans). The majority of the varietal aromas occurring in grape juice are present in a bound form, mainly glycosylated, making them non-volatile and hence odourless (Fischer, 2007). Consequently, grape juice generally has very little flavour. Grapes, and therefore their juice, can also present aromas in the free form, especially methoxypyrazines.

Most monoterpenes initially occur as non-volatile glycosides, but can be hydrolysed (enzymatically or chemically) to the free form during fermentation and ageing, being released

floral notes (Ebeler, 2001). Indeed, they are largely responsible for wine's primary aroma. Figure 2E.1 depicts the most relevant monoterpenes: linalool, geraniol, nerol, citronellol and α-terpineol (Pisarnitskii, 2001). Actually, these monoterpenes and their oxide, furan and pyran derivatives play an important role in the aroma of Muscat wines.

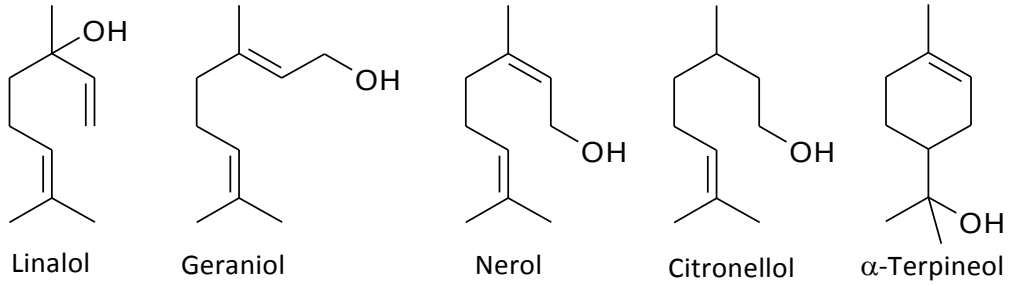
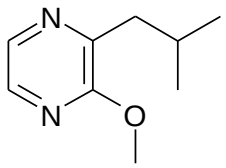


FIGURE 2E.1 – Most common monoterpenes found in wines.

Other wines can also be characterized by primary aromas: Gewürztraminer and Riesling wines by the floral monoterpenic aromas; Cabernet Sauvignon wines by the herbaceous/vegetative aromas of methoxypyrazines; several red and white non-floral *V. Vinifera* varieties such as Chardonnay, and Syrah by the strongly odoriferous pleasant notes of C_{13}-norisoprenoids; and Sauvignon Blanc by thiols (Ebeler, 2001; Ribéreau-Gayon, Glories, et al., 2006b).

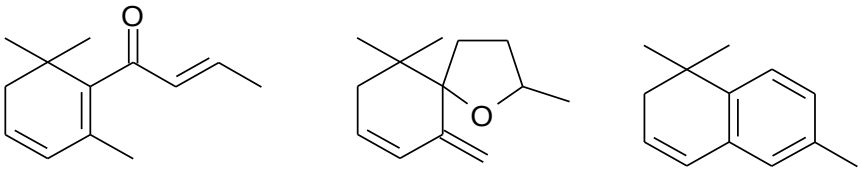
The most common methoxypyrazine encountered in wines is the 2-methoxy-3-isobutyl pyrazine (MIBP, Figure 2E.2), which gives an odour of green bell peppers and is frequently present at concentrations below 40 ng/L, but usually above the 2 ng/L (in water) of olfactory perception threshold (Ebeler, 2001).



2-Methoxy-3-isobutyl pyrazine

FIGURE 2E.2 – Most relevant methoxipyrazine found in wines.

Regarding to C_{13}-norisoprenoids, the most important are *β*-damascenone, vitispirane and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (Figure 2E.3). C_{13}-norisoprenoids result from the oxidation of carotenoids (structurally related to terpenes).



β-Damascenone Vitispirane TDN

FIGURE 2E.3 – Important C_{13}-norisoprenoids in wines.

β-Damascenone (exotic fruits aroma) is a powerful odorant compound with threshold concentration of 9 ng/L (in water). Vitispirane (eucalyptus/camphor aroma) and TDN (petrol kerosene-like aroma), generally absent in grapes and young wines, can have an important

sensorial impact on the aroma of aged wines as they have very low olfactory perception thresholds, 800 and 20 µg/L, respectively.

4-Mercapto-4-methyl-pentan-2-one (4-MMP, Figure 2E.4) is the most known example of wine thiols and is characteristic of Sauvignon Blanc wines (Fischer, 2007). Presents odours resembling black currant, boxwood and broom and has very low odour threshold of 0.8 ng/L in a wine model solution.

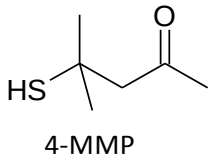


FIGURE 2E.4 – Most relevant example of wine thiols.

2E.3 Secondary aromas

Secondary aromas are the result of the chemical and biochemical reactions developed during the alcoholic and malolactic (if succeeding) fermentations. Among the three main types of volatile compounds found in wines, the fermentative aromas (secondary aromas) are, qualitatively and quantitatively, the largest amount of volatile compounds present in wines (Clarke & Bakker, 2007). The total concentration of secondary aromas can vary between 0.3 to 1.5 g/L (Pisarnitskii, 2001). However, in many cases, the individual concentrations of these volatile compounds are well below the sensory thresholds and often not have great impact on the wine aroma (Ebeler, 2001). Besides ethanol (obviously the most abundant volatile constituent of wine), glycerol and diols, many other volatile compounds are formed by yeast metabolism, essentially higher alcohols and esters, but also acids, aldehydes, ketones, lactones and S-compounds (Rapp, 1998).

Higher alcohols, the so-called fusel alcohols, which contain more than two carbon atoms, are abundant volatile flavour compounds produced either from sugar catabolism or from amino acids decarboxylation and deamination (Ehrlich mechanism, see Figure 2E.5) (Ebeler, 2001; Jackson, 2000).

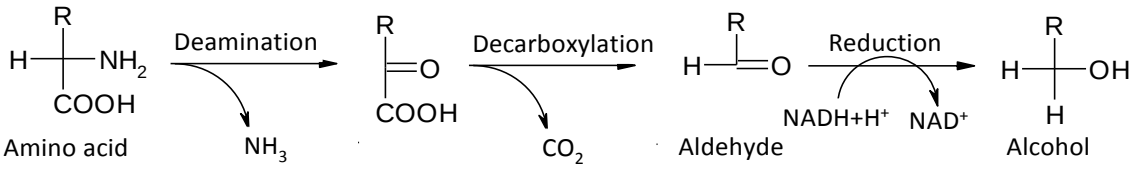
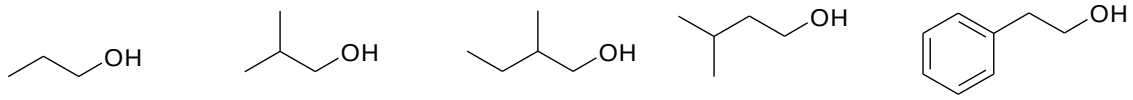


FIGURE 2E.5 – Higher alcohols formation through amino acids via Ehrlich mechanism (adapted from (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006)).

Actually, the total fusel alcohol concentrations range from 140 to 420 mg/L, representing approximately half of the total content of wine volatiles accumulated during fermentation (Ebeler, 2001; Pisarnitskii, 2001). If they arise at concentrations higher than 300 mg/L they can contribute negatively to wine aroma with a strong pungent and fusel-like odour, while optimum levels add desirable complexity to wine, providing a fruity character (Christoph &

Bauer-Christoph, 2007; Ebeler, 2001; Ugliano & Henschke, 2009). The most predominant are 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol (Figure 2E.6). Usually, these fusel alcohols occur at concentrations below their sensory thresholds, with the exception of 2-methylbutanol (30 mg/L in ethanolic solution, malty aroma) and 2-phenylethanol (10 mg/L in ethanolic solution, rose-like aroma) (Christoph & Bauer-Christoph, 2007; Ebeler, 2001). 2-methyl-1-butanol is also named as active amyl alcohol and 3-methyl-1-butanol as isoamyl alcohol. The formation of higher alcohols is affected by several factors: grape variety, composition of grape juice including the initial sugar level, assimilable nitrogen, pH, yeast strains, fermentation temperature, aeration, level of solids, and also skin contact time (Ugliano & Henschke, 2009). Indeed, when fermentation is conducted at high temperatures and in the presence of oxygen and solids, favourable conditions are fulfilled for the formation of higher alcohols (Jackson, 2000).



1-Propanol 2-Methyl-1-propanol 2-Methyl-1-butanol 3-Methyl-1-butanol 2-Phenylethanol

FIGURE 2E.6 – Predominant higher alcohols in wines.

Apart from higher alcohols, various esters are formed during fermentation when alcohols react with acids through enzymatic esterification governed by yeast, with water being released (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006a; Zamora, 2009). Additionally, esters can also be formed through chemical esterification during long-term ageing. Nevertheless, they can also appear already in grapes but at very low quantities which hardly have any impact on wine aroma (Jackson, 2000). In general, they are ubiquitous in wines (over 160 esters have been found in wines) and can have an important impact in the sensory properties of wines, especially in the case of young white wines. Wine esters are essentially ethyl esters of organic acids (e.g. ethyl acetate, ethyl lactate and diethyl succinate), ethyl esters of aliphatic fatty acids (e.g. ethyl butyrate, caproate, caprylate, caprate and laurate) and acetates of higher alcohols (e.g. isoamyl, benzyl and phenylethyl acetates) (Jackson, 2000; Rapp & Mandery, 1986; Zamora, 2009). Figure 2E.7 shows some examples. The most prevalent ester in wine is ethyl acetate since wines contain much more ethanol than any other alcohol, and since the most common volatile organic acid is acetic acid. When present above 150 mg/L, ethyl acetate can generate an undesirable sour-vinegar fragrance in wine (Jackson, 2000). At very low doses (< 50 mg/L) it may contribute to the olfactory complexity of wines and thus have a favourable impact on their quality. Other ethyl esters such as ethyl lactate and diethyl succinate normally they do not appear to be of aromatic significance at normal concentrations, because of their weak odours (Zamora, 2009). Conversely, most wine esters usually contribute positively to wine aroma, essentially with fruity notes, like isoamyl acetate which has a banana-like scent (Ebeler, 2001; Jackson, 2000). The nature and amount of esters formed during fermentation is essentially influenced by the fermentation temperature and by the yeast strain. Low fermentation temperatures (≈ 10 °C) stimulate the synthesis of the fruity acetate esters, such as isoamyl, isobutyl and hexyl acetates, whereas higher temperatures (15 – 20 °C) favour the formation of higher esters, such as the ethyl caprylate, ethyl caprate and phenylethyl acetate (Jackson, 2000). Temperatures higher than 20 °C favour mainly the hydrolysis of ethyl acetates of fatty acids into the corresponding alcohols and acetic

acid. Indeed, the levels of these esters decline during ageing due to their hydrolysis, contrary to most esters.

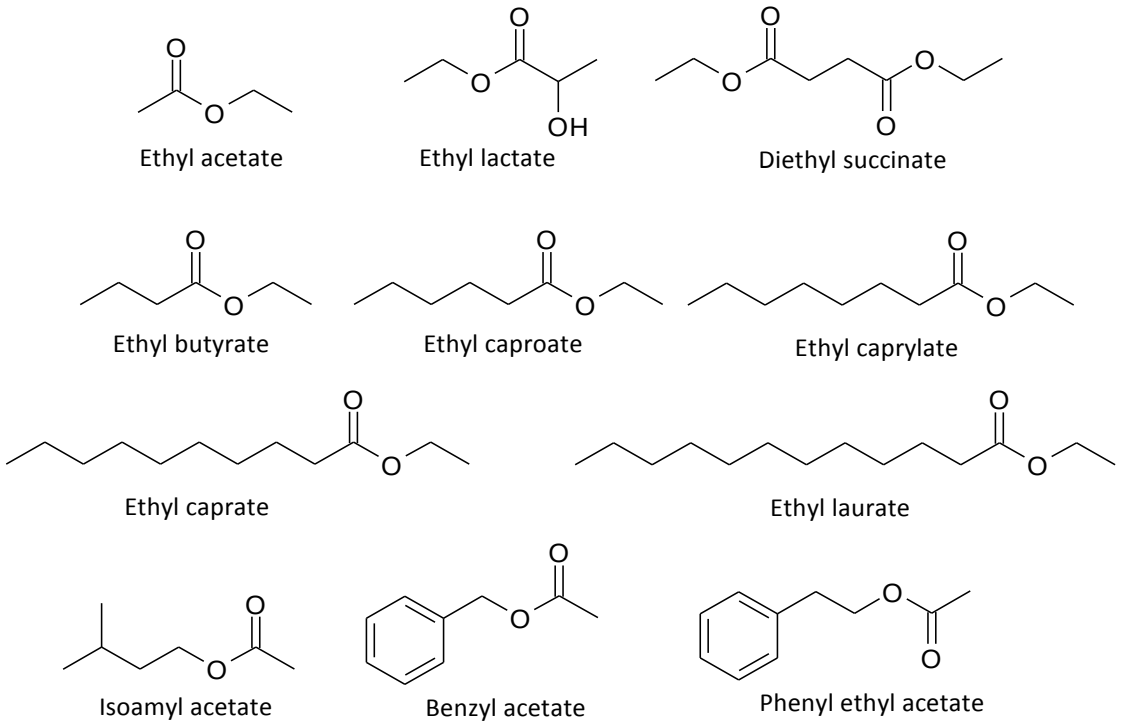


FIGURE 2E.7 – Predominant esters in wines.

Apart from acetic and succinic acids yeasts also synthesize short-chain fatty acids such as propionic, butyric, caproic, caprylic and capric acids as well as long-chain fatty acids, as for instance oleic and linoleic acids, during the alcoholic fermentation (Ribéreau-Gayon, Glories, et al., 2006a). Figure 2E.8 depicts some examples.

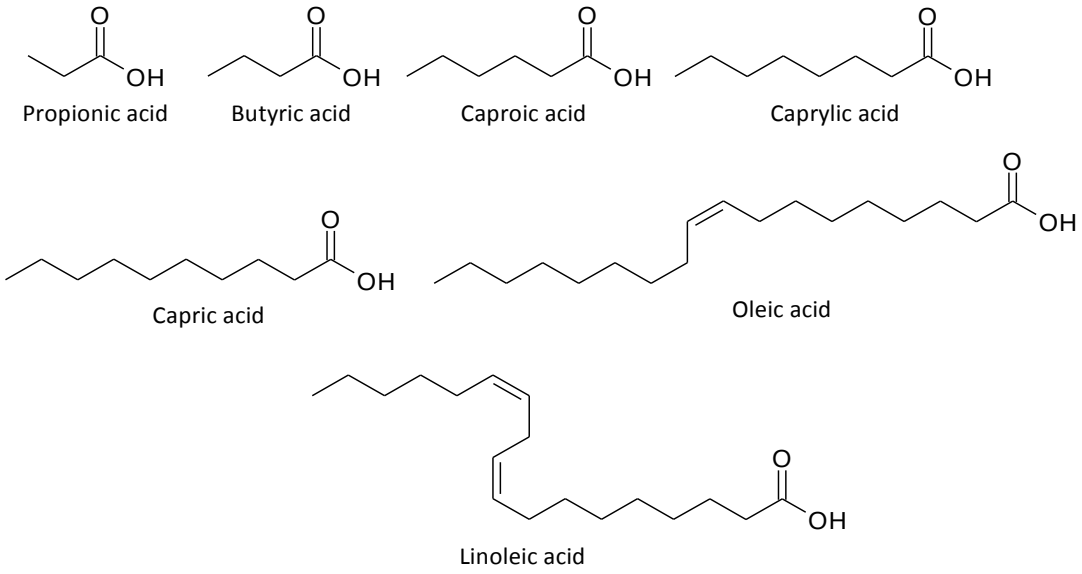


FIGURE 2E.8 – Chemical structures of some acids commonly found in wines.

As already mentioned, the most significant is acetic acid since is the primary responsible for the volatile acidity. Acids are also known to inhibit the fermentation, even when present in traces.

Aldehydes derived from grapes are largely oxidized into the corresponding alcohols during the fermentative stage and usually appear in wines in traces or in very small amounts (Clarke & Bakker, 2007). Acetaldehyde (ethanal), the most important aldehyde found in wines, is obtained by the decarboxylation of pyruvate but is mostly reduced to ethanol than released into the wine during fermentation (Zamora, 2009). Acetaldehyde can also be formed through ethanol by its chemical or biological oxidation, especially during wine ageing. This aldehyde has a nut-like odour, characteristic of oxidized wines. If it is present in wines at high concentrations triggers an unpleasant aroma (butter), which can occur with ageing and under conditions of strong oxidation. This compound is very reactive and has the ability to combine with sulphur dioxide (SO_2) (Ribéreau-Gayon, Glories, et al., 2006a). On the other hand, acetaldehyde also facilitates the copolymerization of phenols (anthocyanins and catechins) forming stable polymeric pigments resistant to SO_2 blocking. Furthermore, acetaldehyde can also react with alcohols to form acetals (herbaceous-like character), namely with ethanol to form 1,1-diethoxyethane (the so-called acetal) (Clarke & Bakker, 2007). Other minor aldehydes derived from fermentation can also be found in wines namely the C_3 to C_7 straight-chain aldehydes (Figure 2E.9). Others can appear or be developed during the long-term ageing such as vanillin (vanilla), cinnamaldehyde (cinnamon) and benzaldehyde (bitter almond) associated with barrel ageing, but also furfural (caramel) and 5-hydroxymethylfurfural (caramel) both arising from carbohydrate degradation (Clarke & Bakker, 2007; Rapp & Mandery, 1986).

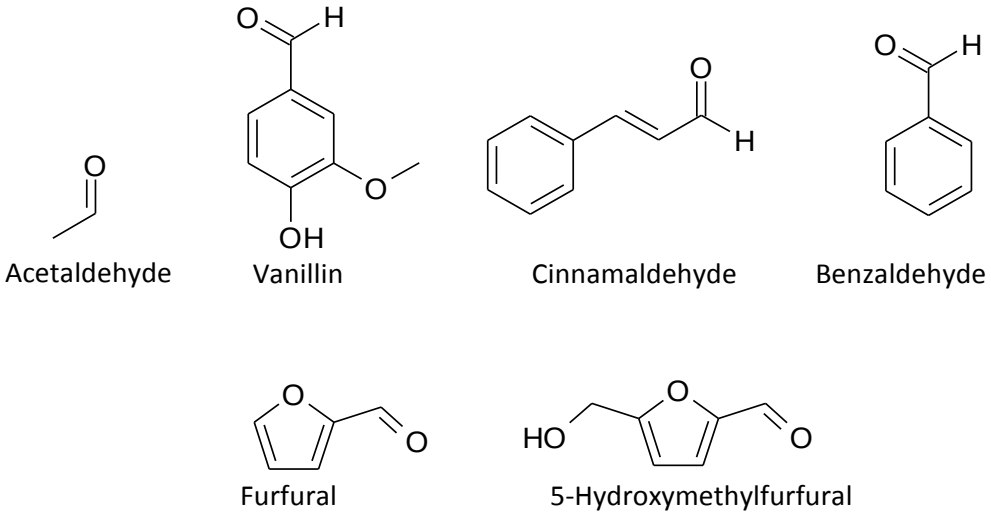


FIGURE 2E.9 – Aldehydes frequently identified in wines.

Ketones also participate in wine aroma, particularly those formed during fermentation such as diacetyl (2,3-butanedione) and acetoin (3-hydroxybutan-2-one) (see Figure 2E.10). Dyacetyl is formed through oxidative decarboxylation of acetolactate, the by-product of the condensation of pyruvate with acetaldehyde, whereas acetoin is produce if the decarboxylation is not oxidative (Zamora, 2009). Additionally, the direct reduction of diacetyl can also originate acetoin. In turn, acetoin can be reduced to form 2,3-butanediol, the most prominent diol in wine however has little influence on wine aroma. Diacetyl has pleasant buttery, hazelnut-like scent which can be perceptible at low concentrations (2 mg/L)

(Ribéreau-Gayon, Glories, et al., 2006a). Nevertheless, when its sensory threshold is exceeded diacetyl generates a buttery, lactic off-odour (Jackson, 2000). Acetoin also gives off a sugary, butter-like smell that may be noticeable when present in wines.

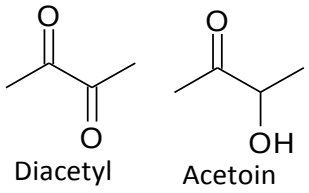


FIGURE 2E.10 – Ketones frequently found in wines.

Lactones are a special subclass of esters, essentially formed by yeast during alcoholic fermentation (Jackson, 2000). They are formed by internal esterification between carboxyl and hydroxyl functions in the same molecule (Ribéreau-Gayon, Glories, et al., 2006a). Lactones with 5-membered rings are known as γ-lactones (or furanones), whereas 6-membered rings are known as δ-lactones (or pyranones) (Clarke & Bakker, 2007). Lactones can also occur already in grapes but the majority have little impact on the aroma. Additionally, lactones can also be synthetized during ageing or even be extracted from oak cooperage. The fermentative lactones do not have a great role in the organoleptic characteristics of wines, namely the most abundant, γ-butyrolactone (Figure 2E.11), usually present at about 1 mg/L (Ribéreau-Gayon, Glories, et al., 2006a). However, there are some lactones that have great impact on wine aroma, especially those accumulated during ageing. The most common example is sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) which has an extremely powerful nutty, sweet, burnt, curry aroma with a very low perception threshold (5 μg/L) (Vicente Ferreira, Jarauta, López, & Cacho, 2003). This compound has been considered a great contributor to the typical aged aroma of oxidative fortified wines, such as Sherry, Port and Madeira (Câmara, Marques, Alves, & Silva Ferreira, 2004; Moreno, Zea, Moyano, & Medina, 2005; Silva Ferreira, Barbe, & Bertrand, 2003). Moreover, sotolon has also been found in wines produced from grapes infected by *Botrytis cinerea* and in *Vin Jaune* from Jura (Ribéreau-Gayon, Glories, et al., 2006a; Sarrazin, Dubourdieu, & Darriet, 2007). The sotolon formation pathway has been studied by several authors (Cutzach, Chatonnet, & Dubourdieu, 1999; Pisarnitsky, Bezzubov, & Egorov, 1987; Pons, Lavigne, Landais, Darriet, & Dubourdieu, 2010; Thuy, Elisabeth, Pascal, & Claudine, 1995). Some authors proposed that sotolon appears via enzymatic/chemical deamination of threonine followed by an aldol condensation with acetaldehyde. Others suggested that it can be produced by an aldol condensation between glutamic and pyruvic acids. Others considered its formation based on the peroxidation of acetaldehyde.

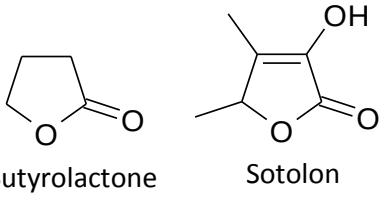
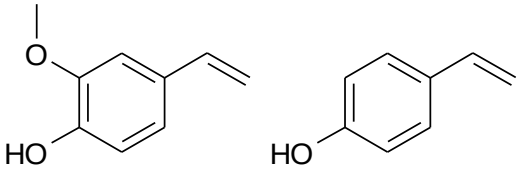


FIGURE 2E.11 – Relevant lactones in wines.

Excepting acetovanillone (already present in grapes) volatile phenols are formed during the fermentation, essentially the vinylphenols (Clarke & Bakker, 2007). The ethyl

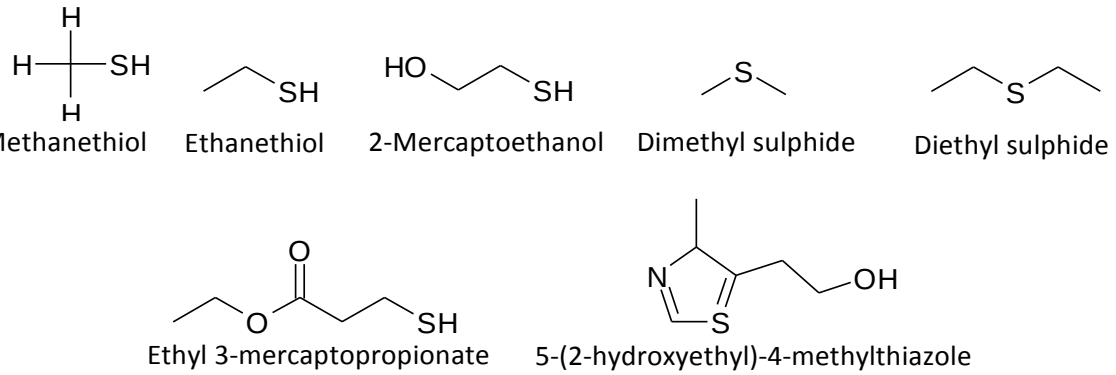
phenols are much more likely to develop during barrel ageing. Volatile phenols derive either from yeast or bacterial metabolism or from hydrolysis of higher phenols (Rapp & Mandery, 1986). As already mentioned in Chapter 2B, the most common volatile phenols found in wines are vinyl guaiacol and vinyl phenol derived from the enzymic or thermal decarboxylation of *p*-coumaric and ferulic acids, respectively (Figure 2E.12). Unfortunately, volatile phenols can negatively influence wine aroma usually with phenolic medicinal scents (Clarke & Bakker, 2007).



Vinyl guaiacol Vinyl phenol

FIGURE 2.12 – Most common volatile phenols found in wines.

At last, sulphur derivatives (S-containing compounds) also encompass the secondary aromas. Almost sulphur-containing compounds are deleterious to wine quality because they are responsible for alliaceous and rubbery aromas (Ebeler & Thorngate, 2009). Although present in trace amounts they can have significant impact in wines since these compounds possess low perception thresholds (usually parts per trillion) (Jackson, 2000). During fermentation, appreciable amounts of the hydrogen sulphide (H_2S) are produced by yeasts. Cystine metabolism can also increase its synthesis. This inorganic compound is characterized for having an unpleasant rotten egg odour and is often associated with the yeasty odour of newly fermented wines. Fortunately, usually appears in trace amounts in finished wines. The metabolism of S-containing amino acids can produce several organosulphur compounds, such as thiols (or mercaptans, e.g. methanethiol, ethanethiol and 2-mercaptoethanol), thioethers (e.g. dimethyl sulphide and diethyl sulphide), thiolanes (e.g. 2-methylthiolane-3-one and 2-methylthiolane-3-ols), thioesters (e.g. ethyl 3-mercaptopropionate) and thiazoles (e.g. 5-(2-hydroxyethyl)-4-methylthiazole)). Figure 2E.13 depicts some examples. Additionally, the so-called *sun-struck* phenomenon can also produce organosulphur off-odours as well as the yeast cells autolysis.



Methanethiol Ethanethiol 2-Mercaptoethanol Dimethyl sulphide Diethyl sulphide

Ethyl 3-mercaptopropionate 5-(2-hydroxyethyl)-4-methylthiazole

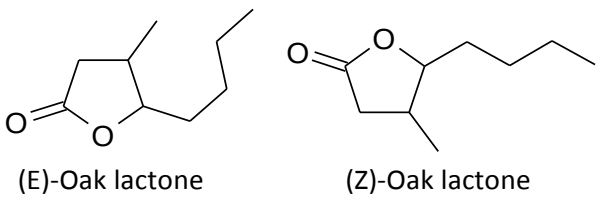
FIGURE 2E.13 – Some examples of sulphur-containing compounds encountered in wines.

2E.4 Tertiary aromas

Tertiary aromas are those developed during post-fermentation treatments, i.e. through wine ageing either in wood barrel or in bottle. During this process the wine volatile composition changes due to several slow chemical reactions, transforming the aroma into the bouquet. The bouquet of oxidation is generally formed during ageing in wooden casks while the bouquet of reduction is developed along bottle-ageing (Rapp & Mandery, 1986). Depending on storage conditions, there are many factors that influence the composition of volatile constituents of wine, mostly temperature and duration. Generally speaking, under wine acidic medium several changes take place, namely the decrease of monoterpenes (linalool, geraniol and citronellol decrease) and subsequent transformation (linalool and nerol oxides increase), the production of new by-products of carotenoids (e.g. vitispiranes) and carbohydrates (e.g. furfural, HMF, 2-acetylfuran, furfuryl alcohol and others), the decline of the levels of acetic esters of aliphatic alcohols, the increase of ethyl esters of carboxylic acids and aliphatic aldehydes (Pisarnitskii, 2001).

As previously denoted, acetates (e.g. isoamyl acetate, isobutyl acetate, hexyl acetate) enzymatically produced in excess during fermentation gradually hydrolyse until they approach to the equilibrium with the corresponding acids and alcohols, a decrease that may be responsible for the loss of freshness and fruity notes characteristic of the young wines. On the other hand, the increase of the concentration of ethyl esters of diprotic acids (e.g. diethyl and monoethyl succinate and diethyl malate) arises from chemical esterification during ageing.

Oak barrel ageing firstly promotes the oxidation of the primary and secondary aromas, since wine oxygenation occur due to the entry of oxygen across the pores of the wood (Clarke & Bakker, 2007). Thus, this process promotes the arising of the oxidative bouquet. Secondly, the release of oak wood aromas also takes place producing the so-called oak aroma. This process promotes the occurrence of several important aromas and the increase of aldehydes such as acetaldehyde due to ethanol oxidation and vanillin produced from the degradation of lignin extracted from wood is often observed. As already mentioned acetaldehyde contributes to the typical nutty flavour of many aged wines. Other essential compounds are the isomers *cis* and *trans* of *β*-methyl-γ-octalactone (Figure 2E.14), commonly known as oak or whiskey lactone (Ebeler, 2001). These two isomers have low sensory threshold, specially the *cis* isomer (92 μg/L), contributing with woody, oaky, coconut-like aromas to wine. During maturation is also observed the increase of acetals, resulting from the reaction between aldehydes and alcohols. In opposition, there is no rise in acetal concentration during bottle ageing.



(E)-Oak lactone (Z)-Oak lactone

FIGURE 2E.14 – The two isomers of oak-lactone.

After some years of ageing in oak casks some wines gain their aromatic tipicity, such as Madeira. Indeed, it is during the oxidative ageing that sotolon is formed, one of the most important aromas of Madeira wine flavour. However, for other wines a slow ageing is

undesired to their organoleptic characteristics, as in the case of white table wines. For example, the excess of aliphatic aldehydes (mainly acetaldehyde), originated from the excessive ageing of white wines, associated with the auto-oxidation of phenolic compounds causes browning, undesirable in this type of wine (Pisarnitskii, 2001).

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PART 2

Developed methodologies

CHAPTER 3

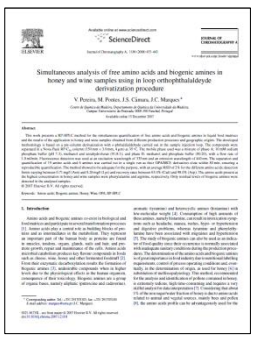
Simultaneous analysis of free amino acids and biogenic amines

This chapter is based on the following publication:

Simultaneous analysis of free amino acids and biogenic amines in honey and wine samples using in loop orthophthalaldeyde derivatization procedure

V. Pereira, M. Pontes, J.S. Câmara, J.C. Marques

Journal of Chromatography A, 1189 (2008) 435–443



Abstract

This chapter presents a RP-HPLC method for the simultaneous quantification of free amino acids and biogenic amines in wines obtained from different production processes and geographic origins. The methodology was also applied to honey samples. The developed methodology is based on a pre-column derivatization with *o*-phthaldialdehyde carried out in the sample injection loop. The compounds were separated in a Nova-Pack RP-C_{18} column (150 × 3.9 mm, 4 µm) at 35 °C. The mobile phase used was a mixture of phase A: 10mM sodium phosphate buffer (pH 7.3), methanol and tetrahydrofuran (91:8:1); and phase B: methanol and phosphate buffer (80:20), with a flow rate of 1.0 mL/min. Fluorescence detection was used at an excitation wavelength of 335 nm and an emission wavelength of 440 nm. The separation and quantification of 19 amino acids and 6 amines was carried out in a single run as their OPA/MCE derivatives elute within 80 min, ensuring a reproducible quantification. The method showed to be adequate for the purpose, with an average RSD of 2% for the different amino acids; detection limits varying between 0.71 mg/L (Asn) and 8.26 mg/L and recovery rates between 63.0% (Cad) and 98.0% (Asp). The amino acid present at the highest concentration in wine samples was arginine. Only residual levels of biogenic amines were detected in the analysed samples.

3.1 Introduction

Amino acids microbial catabolism produces key flavour compounds in foods such as cheese, wine, honey and other fermented foodstuff (Özcan & Senyuva, 2006). As already mentioned, from their enzymatic decarboxylation results the formation of biogenic amines, undesirable compounds when in higher levels, due to the physiological effects in the human organism, consequence of their toxicology. Indeed, amino acids and biogenic amines co-exist in several biological and food matrices and participating in numerous transformation processes (Kutlán & Molnár-Perl, 2003).

The determination of the amino acids and biogenic amines is of great importance in food industry due to nutritional labelling requirements, control of process operating conditions and, eventually, in the determination of origin (Iglesias, de Lorenzo, Polo, Martín-Álvarez, & Pueyo, 2003). Additionally, the amino acids profile can be advantageously used for the characterization of wines, indeed, according to several authors (Nouadje et al., 1997; Soufleros, Barrios, & Bertrand, 1998; Soufleros, Bouloumpasi, Tsarchopoulos, & Biliaderis, 2003; Vasconcelos & Chaves das Neves, 1989), their composition may be a suitable method for the classification of wines according to variety, geographical origin, wine-making technologies and vintage. In recent years, new trends in food safety, together with the consumer's demand for quality and healthier products, have encouraged several authors to study these compounds in several wines (Gloria, Watson, Simon-Sarkadi, & Daeschel, 1998; Herbert, Santos, & Alves, 2001; Mafra, Herbert, Santos, Barros, & Alves, 1999; Vazquez-Lasa, Iniguez-Crespo, Gonzalez-Larraina, & Gonzalez-Guerrero, 1998). They have study of biogenic amines as food quality

indicators since their occurrence is normally associated with inadequate sanitary conditions during the production procedures.

Diverse analytical methods have been proposed for the analysis of amino acids and biogenic amines, including gas chromatography (GC) (Kim, Kim, Cheong, & Jeong, 1996; Nozal, Bernal, Toribio, Diego, & Ruiz, 2004; Pätzold, Nieto-Rodriguez, & Brückner, 2003; Yamamoto, Itano, Kataoka, & Makita, 1982), high-performance liquid chromatography (HPLC) (López-Cervantes, Sánchez-Machado, & Rosas-Rodríguez, 2006; mo Dugo, Vilasi, La Torre, & Pellicanò, 2006; Pripis-Nicolau, de Revel, Marchand, Beloqui, & Bertrand, 2001) and capillary electrophoresis (EC) (Bjergegaard, Pilegaard Hansen, Møller, Sørensen, & Sørensen, 1999; Kovács, Simon-Sarkadi, & Ganzler, 1999). More recently, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been shown to be a very specific and sensitive technique for the determination of underivatized amino acids and biogenic amines (de Person, Chaimbault, & Elfakir, 2008; Millán, Sampedro, Unceta, Goicolea, & Barrio, 2007; Özcan & Senyuva, 2006; Petritis, Elfakir, & Dreux, 2002). Besides involving shorter analysis times the LC–MS/MS technique is expensive and is not available in many research laboratories. There are some methods used for the simultaneous determination of amino acids and biogenic amines by HPLC (Alberto, Arena, & Manca de Nadra, 2002; Bauza, Blaise, Daumas, & Cabanis, 1995; Krause, Bockhardt, Neckermann, Henle, & Klostermeyer, 1995; Lozanov, Petrov, & Mitev, 2004), micellar electrokinetic capillary chromatography (MECC) (Wang et al., 2003) and micellar liquid chromatography (MLC) (Gil-Agustí, Carda-Broch, Monferrer-Pons, & Esteve-Romero, 2007). Traditionally, the determination of amino acids has been conducted by ion-exchange chromatography, followed by post-column derivatization with ninhydrin. So far, the analysis of amino acids and biogenic amines using pre-column derivatization and reversed-phase HPLC separation of the derivatives has become widely accepted and usually shows great sensitivity. Typical derivatization reagents include 9-fluorenylmethyl chloroformate (FMOC-Cl) (Bauza, et al., 1995; Einarsson, 1985), N-(9-fluorenylmethoxycarbonyloxy)succinimide (FMOC-OSu) (Lozanov, et al., 2004), carbazole-9-yl-acetyl chloride (CRA-Cl) (You, Sun, Lao, & Ou, 1999), ortho-phthaldialdeyde (OPA) (Klein & Dudenhausen, 1995; Kutlán & Molnár-Perl, 2003; Mafra, et al., 1999; Sanders & Ough, 1985), phenyl isothiocyanate (PITC) (Fierabracci, Masiello, Novelli, & Bergamini, 1991; Irvine, 1996), 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Kochhar & Christen, 1989; Scaloni, Simmaco, & Bossa, 1995) and dansylchloride (mo Dugo, et al., 2006; Simmaco, De Biase, Barra, & Bossa, 1990).

This study indented to develop a simple RP-HPLC methodology for the simultaneous identification and quantification of amino acids and biogenic amines in liquid food matrices, based on a pre-column OPA derivatization carried out in the chromatograph injection loop. The OPA/MCE reagent was selected due to its high sensitivity, responding to minor amino, acids, good selectivity and simplicity. OPA in the presence of 2-mercaptoethanol (MCE) reacts with amino acids and biogenic amines and proceeds to isoindolic derivatives, at room temperature, in a quick and simple reaction, in spite of OPA/MCE derivatives being considered quite instable (Hanczkó & Molnár-Perl, 2003; Simons & Johnson, 1976). The secondary amino acids, such as proline and hydroxyproline, cannot be determined because they do not react. Some derivatives are unstable making crucial an appropriate control of the reaction and injection time (Hanczkó & Molnár-Perl, 2003). Furthermore, this derivatization reagent allows

the simultaneous analysis of these compounds without extraction and purification processes preceding the derivatization with fluorescent functional group detection (Bjergegaard, et al., 1999; Kutlán & Molnár-Perl, 2003).

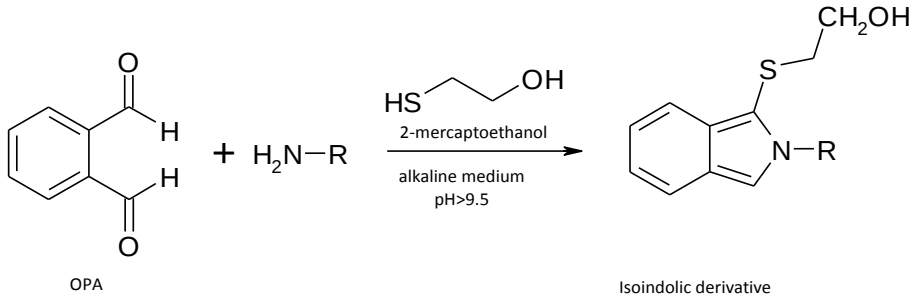


FIGURE 3.1 – Primary amino compounds derivatization with OPA/MCE reagent (Simons & Johnson, 1976).

In order to simplify the derivatization procedure and maintaining the reproducibility of the results, this work was focused in the derivatization operating conditions to be accomplished in the sample injection loop. This methodology was applied to wine samples obtained from different production processes and geographic origins. The application method was extended to other liquid food matrices as honey.

3.2 Experimental

3.2.1 *Standards and reagents*

Ultra-pure water was obtained from a Milli Q-System (Millipore, Milford, MA, USA) while HPLC-grade methanol was obtained from Sigma–Aldrich (St. Louis, MO, USA). Tetrahydrofuran (99.5%), ethanol (99.9%), sodium hydroxide (98%), sodium phosphate monobasic monohydrate (98%) were from Panreac Quimica SA (Barcelona, Spain). *o*-Phthaldialdehyde (p.a.), 2-mercaptoethanol (99%) were supplied by Acros Organics (Geel, Belgium), hydrochloric acid (p.a.) by Riedel-de Häen (Seelze, Germany) and boric acid (99.5%) by Merck Co. (Darmstadt, Germany).

A kit of high purity *L*-amino acids (>98%) was supplied by Sigma–Aldrich (St. Louis, MO, USA) and consisted of 1 g of each of the following standards: aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), methionine (Met), tryptophan (Trp), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu) and lysine (Lys).

The following standards were supplied by Fluka BioChemika AG (Buchs, Switzerland): γ-aminobutyric acid (GABA) and the biogenic amines: histamine (Him), tyramine (Tym), phenylethylamine (Phm) isopenthylamine (Ism), and cadaverine (Cad). Tryptamine (Trm) was purchased from Acros Organics (Geel, Belgium).

A concentrated 10 g/L stock solution of each amino acid and biogenic amine was prepared in 0.1M HCl. Calibration standards (ranging from 0.5 to 60.0 mg/L) were prepared in

0.1M HCl from the concentrated standard solution. Finally, they were filtered through a 0.45 µm filter (Acrodisc® CR-PTFE, Ann Arbor, SOM, USA), stored in a refrigerator and protected from light.

3.2.2 *Equipment*

Amino acids and biogenic amines were simultaneously separated in a HPLC system using a Waters (Milford, MA, USA) liquid chromatograph controlled by the Empower Pro software and equipped with an auto-injector (Waters 2695, separations module) and a Multi λ Fluorescence detector (Waters 2475). Chromatographic analysis were performed using an analytical scale (3.9mm × 150 mm) Nova-Pack RP-C_{18} column, with a particle size of 4 µm, purchased from Waters (Milford, MA, USA).

3.2.3 *Chromatographic conditions*

HPLC conditions were as follow: mobile phase A: 1% of tetrahydrofuran, 8% methanol and 91% phosphate buffer (10 mM, pH 7.3). Mobile phase B: 80% methanol and 20% phosphate buffer (10 mM, pH 7.3). The flow rate was set at 1.00 mL/min and the column maintained at 35 °C.

TABLE 3.1 – Gradient program employed for the separation of amino acids and biogenic amines.

Time (min)	Flow (ml/min)	Eluent A (%)	Curve
0	1.00	100	6
6	1.00	100	6
17	1.00	85	6
25	1.00	80	6
33	1.00	70	6
45	1.00	60	6
61	1.00	20	6
67	1.00	0	6
70	1.00	0	6
71	1.00	100	1
80	1.00	100	6

The eluted OPA derivatives were detected by monitoring their fluorescence at 335 and 440 nm as excitation and emission wavelengths, respectively. The injections were performed in less than 80 min, including column regeneration and stabilization during the last 13 min. The gradient program used is shown in Table 3.1.

3.2.4 *Samples*

A total of 21 samples were analysed with the developed method (12 honeys and 9 wines – see Table 3.2). The honey samples include nine multifloral and three monofloral, from different origins namely Madeira Islands (H1–H5), Portugal mainland (H6–H8) and Canary Islands (H9–H12), purchased in local stores. The wine samples include four Madeira fortified

wines (W1–W4), three Madeira table wines (W5–W7) and two Canarian table wines (W8 and W9) from the following grape varieties: *Malvasia*, *Tinta Negra Mole* and *Sercial*.

Before the derivatization procedure, 200 µL of the sample were added to 1.5 mL of a 0.4 M borate buffer solution (pH 10.5), homogenized in a vortex agitator and then filtered through 0.45 µm PTFE filter. In case of honey samples, 5 g were diluted with ultra-pure water into a 10 mL volumetric flask and filtered.

TABLE 3.2 – Samples analysed by the developed HPLC method.

Samples			Characteristics	Origin
Honey		H1	Multifloral	Madeira Island
		H2	Multifloral	
		H3	Multifloral	
		H4	Multifloral	
		H5	Multifloral	
		H6	Monofloral	Portugal Mainland
		H7	Multifloral	
		H8	Monofloral	
		H9	Multifloral	Canary Island
		H10	Monofloral	
		H11	Multifloral	
		H12	Multifloral	
Wine	*Fortified*	W1	*Malvasia* - Sweet	Madeira Island
		W2	*Sercial* - Dry	
		W3	*Tinta Negra Mole* - Sweet	
		W4	*Tinta Negra Mole* - Dry	
	Table	W5	*Malvasia*	Madeira Island
		W6	*Tinta Negra Mole* - Rosé	
		W7	*Tinta Negra Mole* - Red	
	Table	W8	*Malvasia*	Canary Island
		W9	*Malvasia*	

3.2.5 *Derivatization*

OPA derivatization solution was prepared in a 10 mL volume flask by dissolving 50 mg of reagent in 1.5 mL of ethanol and making up the volume with 0.4 M borate buffer (pH 10.5). Finally 200 µL of 2-mercaptoethanol was added.

At last, the reagent solution was left to settle for 90 min, stored in dark glass vials at 4 °C and freshly prepared every 9 days. The derivatization procedure was performed in the sample injection loop according to the following sequence: 10 µL of buffered sample mixture were aspired to the injection loop followed by 10 µL of OPA solution and maintained for 3 min to promote the derivatization reaction. During this period, the flow was maintained at 0 mL/min to keep the reagent into the loop. Then, the loop content (20 µL) was forced to enter into the column by changing the mobile phase flow to 1 mL/min.

3.2.6 *Calculations*

The concentration of each analyte was obtained by direct interpolation of the peak area in the correspondent linear calibration curve (peak area vs. concentration, ranging from 0.5 to 60.0 mg/L). Samples were diluted when needed to comply with the working range.

3.3 Results and discussion

3.3.1 *Derivatization procedure*

Derivatization of the standard amino acid and biogenic amines mixture was performed by OPA/MCE in boric buffer (0.4M sodium borate, pH 10.5). The first experiments were carried out using an injection volume of 50 µL (25 µL of buffered sample mixture and 25 µL of OPA/MCE reagent) but the volume was reduced in order to extend column life without compromising the good response.

Much of the published methods require sample pre-treatment before derivatization (Busto, Guasch, & Borrull, 1995; Iglesias, et al., 2003; Paramás, Bárez, Marcos, García-Villanova, & Sánchez, 2006). Paramás et al. (2006) developed an OPA/MCE derivatization method for the determination of amino acids in honey which includes a clean-up step and an extraction procedure before derivatization. The current developed method has the advantage of being a simpler methodology, not requiring any complex pre-treatment for liquid food matrices and only a dilution is carried out, if necessary. OPA-derivatization times were short (3 min) when compared with other derivatization reagents used for the simultaneous determination of these compounds (Bauza, et al., 1995; Krause, et al., 1995; Lozanov, et al., 2004). The proposed method by Bauza et al. (1995) using FMOC as derivatization reagent needed 6 min for reaction development, while Krause et al. (1995) used a dansyl method and the derivatization time was 20 min. Lately, Lozanov et al. (2004) proposed the use of FMOC-OSu reacting during 20 min. OPA derivatization did not show the presence of excess reagent, interfering with the analytes resolution, as detected when using FMOC derivatization methods (Lozanov, et al., 2004). Furthermore, the derivatization reaction was automatic, occured in the injection loop and showed sensitive and consistent results.

3.3.2 *Method validation*

The sample analytes were identified by comparison with the retention times of amino acid standard solutions. For the determination of retention times, the reference standards were injected both individually and as a mixture. Quantification was performed by the external standard method based on peak areas of the eluted amino acid and biogenic amines derivatives. The linearity was evaluated by the construction of calibration curves, using the chromatographic peaks areas of the fluorescence response, from triplicate injections of standards, at six increasing concentrations in the 0.5–60.0 mg/L range for all amino acids and biogenic amines. The linear relationship between concentrations and peak area is given by *a*, *b*

and r^2—see Table 3.3, where *a* and *b* are the coefficients of the regression equation $y = ax + b$, *x* being the concentration of the analyte, *y* the peak area and r^2 the coefficient of determination. For this calculation, all obtained values were used instead the average of the three injections. In all cases, the relationship between concentrations and peak areas were linear over the tested range, with coefficients of determination greater than 0.990.

TABLE 3.3 – Retention times, calibration curves ($y = ax + b$), correlation coefficient (r^2), limits of detection (LOD) in mg/L, repeatability (RSD %) and recovery (Rec %) of amino acids and biogenic amines.

Amino acids	**Retention times (min.)**	***a*** **(x10^5)**	***b*** **(x10^5)**	r^2	**sd (x10^5)**	**LOD**	**Rep (RSD%)**	**Rec (%)**
Aspartic acid	1.33 ± 0.28	23.30	27.40	0.998	14.68	0.71	0.60	98
Glutamic acid	2.52 ± 0.30	21.20	13.40	0.999	14.45	1.41	1.65	91
Asparagine	8.60 ± 0.52	19.10	6.94	0.999	11.89	1.50	1.49	90
Serine	11.10 ± 0.35	32.00	12.30	0.998	28.69	2.30	0.14	85
Glutamine	13.74 ± 0.25	2.29	0.58	0.994	4.07	5.08	4.14	90
Histidine	14.25 ± 0.45	9.54	-3.67	0.998	8.57	2.73	1.92	90
Glycine	17.25 ± 0.38	45.50	-11.70	0.997	52.92	3.75	1.09	87
Threonine	19.15 ± 0.30	24.10	8.92	0.999	18.73	1.96	2.55	88
Arginine	21.89 ± 0.65	17.40	1.72	0.998	15.79	2.62	0.58	85
Alanine	26.15 ± 0.25	36.20	-0.14	0.998	36.15	3.00	0.72	85
GABA	28.50 ± 0.25	30.50	-22.40	0.996	42.26	4.89	0.89	83
Tyrosine	32.50 ± 0.40	17.60	4.87	0.999	12.14	1.79	0.51	91
Methionine	45.20 ± 0.25	21.40	-0.89	0.991	45.14	6.37	7.92	98
Tryptophan	46.25 ± 0.35	31.20	16.70	0.999	16.98	1.10	0.39	91
Valine	47.50 ± 0.20	13.60	-1.56	0.998	12.49	2.87	0.56	82
Phenylalanine	50.20 ± 0.30	19.10	2.21	0.999	15.97	2.39	0.63	83
Isoleucine	52.50 ± 0.45	29.90	10.30	0.999	19.27	1.59	0.88	91
Leucine	53.90 ± 0.25	27.30	3.42	0.999	21.30	2.21	0.67	88
Lysine	59.50 ± 0.40	6.78	-8.75	0.990	15.74	8.26	5.41	82
Biogenic amines								
Histamine	49.10 ± 0.45	29.50	-25.30	0.996	4209260	5.14	0.40	87
Tyramine	58.90 ± 0.25	28.90	-24.30	0.994	4972310	6.00	6.04	82
Tryptamine	63.50 ± 0.30	21.90	-25.10	0.994	3815051	6.37	0.66	81
Phenylethylamine	64.75 ± 35	24.20	-18.10	0.995	3963053	5.66	0.24	78
Isopenthylamine	65.80 ± 0.20	35.00	1.75	0.994	4184279	3.54	5.14	91
Cadaverine	67.00 ± 0.20	20.50	-19.80	0.992	4182516	7.09	1.09	63

The repeatability of the method was evaluated by nine consecutive injections of the same sample during a working day. Detection limits (defined as three times the signal-to-noise ratio) ranged from 0.71 mg/L (Asp) to 8.26 mg/L (Lys)—Table 3.3.

To determine intra-sample and inter-day precisions, three identical samples were run on three separate days. Intra-samples precisions for individual measurements of amino acids range from 1.9 to 4.8% and the inter-day precisions range from 4.2 to 9.4% (RSD). Biogenic amines were not considered as only vestigial quantities were found. The calculated concentrations of individual amino acids showed residual standard deviations (RSD) of about 2% in the analyses of wine and honey samples.

The accuracy was estimated by means of the recovery tests. For the evaluation of the recovery rate, H3 honey and W7 wine (n = 5) were spiked with 10 mg/L standard solution,

derivatizated and quantified. The recovery rate averages obtained were acceptable, with values ranging from 82% (Lys) to 98% (Asp) for amino acids, and 63% (Cad) to 91% (Ism) for biogenic amines—Table 3.3.

3.3.3 *Chromatographic analysis*

The proposed HPLC method allowed the simultaneous determination of 19 amino acids and 6 biogenic amines in 83 min, including the column regeneration (9 min) and derivatization time (3 min), slightly higher than the methodology suggested by Alberto et al. (Alberto, et al., 2002), 63 min, but ensuring better separation. The applied methodology allowed the total separation of all amino acids and biogenic amines in the standards solutions and analysed matrices, overcoming some peak overlay obtained by several authors (Cometto, Faye, Di Paola Naranjo, Rubio, & Aldao, 2003; Conte, Miorini, Giomo, Bertacco, & Zironi, 1998; Davies & Harris, 1982; Hermosín, Chicón, & Dolores Cabezudo, 2003) namely Asn + Ser (Davies & Harris, 1982; Hermosín, et al., 2003), Gln + Thr (Davies & Harris, 1982), Asp + Asn (Conte, et al., 1998), Glu + Gln (Conte, et al., 1998) and Thr + Ala (Cometto, et al., 2003). Figure 3.2 shows the separation obtained for the amino acids and biogenic amines present in a 20 mg/L standard solution, together with typical chromatograms obtained for honey (H5) and wine (W4).

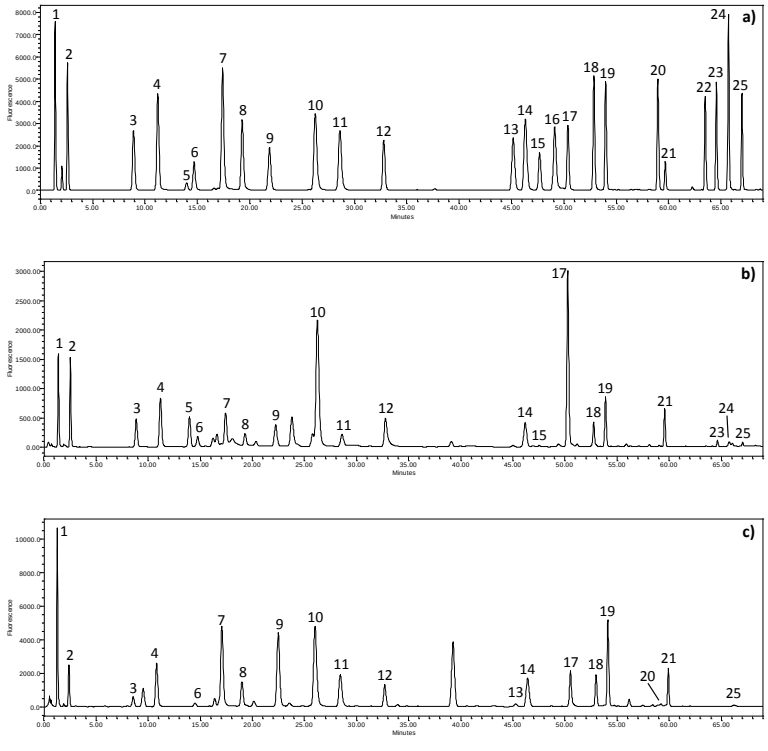


FIGURE 3.2 – Typical chromatogram profile of amino acids and biogenic amines in: **a)** 20 mg/L standard mixture, **b)** H5 honey and **c)** W4 wine. **Peak identification:** (1) aspartic acid, (2) glutamic acid, (3) asparagine, (4) serine, (5) glutamine, (6) histidine, (7) glycine, (8) threonine, (9) arginine, (10) alanine, (11) gaba, (12) tyrosine, (13) methionine, (14) tryptophan, (15) valine, (16) histamine, (17) phenylalanine, (18) isoleucine, (19) leucine, (20) tyramine, (21) lysine, (22) tryptamine, (23) phenylethylamine, (24) isopenthylamine and (25) cadaverine.

TABLE 3.4 – Levels (mg/L) of free amino acids in studied honey and wine samples with the respective standard deviation of the mean value (n = 3 for each data point)

	Asp	Glu	Asn	Ser	Gln	His	Gly	Thr	Arg	Ala	GABA	Tyr	Met	Trp	Val	Phe	Ile
HONEYS																	
Madeira island																	
H1	3.73 ± 0.080	4.65 ± 0.11	2.83 ± 0.01	< LOD	39.99 ± 0.30	3.20 ± 0.20	< LOD	< LOD	3.36 ± 0.03	3.69 ± 0.04	< LOD	4.77 ± 0.35	n.d.	3.31 ± 0.00	< LOD	76.38 ± 0.72	2.24 ± 0
H2	3.89 ± 0.01	5.64 ± 0.01	2.85 ± 0.03	2.67 ± 0.00	55.19 ± 0.48	4.71 ± 0.00	< LOD	< LOD	2.99 ± 0.00	7.77 ± 0.01	< LOD	8.44 ± 0.02	n.d.	5.56 ± 0.00	< LOD	146.65 ± 1.65	4.07 ± 0
H3	3.33 ± 0.04	4.94 ± 0.04	3.01± 0.00	3.44 ± 0.03	32.71 ± 0.37	2.73 ± 0.18	< LOD	< LOD	3.61 ± 0.03	12.41 ± 0.05	< LOD	5.47 ± 0.01	n.d.	2.03 ± 0.01	n.d.	19.99 ± 0.02	< LOD
H4	6.26 ± 0.27	11.77 ± 0.27	3.29 ± 0.00	4.77 ± 0.11	34.06 ± 0.86	6.73 ± 0.21	< LOD	3.21 ± 0.04	< LOD	6.41 ± 0.05	< LOD	20.65 ± 0.28	n.d.	3.37 ± 0.00	6.45 ± 0.00	97.76 ± 0.08	2.36 ± 0
H5	3.54 ± 0.04	6.36 ± 0.01	5.51 ± 0.05	2.73 ± 0.00	32.56 ± 0.22	5.07 ± 0.00	< LOD	< LOD	3.67 ± 0.00	4.45 ± 0.09	< LOD	6.49 ± 0.02	n.d.	2.45 ± 0.01	< LOD	20.37 ± 0.06	< LOD
Portugal mainland																	
H6	3.82 ± 0.04	2.84 ± 0.06	4.95 ± 0.05	3.56 ± 0.01	20.08 ± 0.11	4.77 ± 0.05	< LOD	< LOD	4.27 ± 0.15	3.72 ± 0.08	< LOD	33.84 ± 0.35	n.d.	1.65 ± 0.05	< LOD	109.61 ± 0.29	1.62 ± 0
H7	5.49 ± 0.13	6.58 ± 0.14	3.81 ± 0.10	3.33 ± 0.09	26.93 ± 1.19	4.92 ± 0.03	< LOD	< LOD	4.00 ± 0.03	8.02 ± 0.01	< LOD	8.91 ± 0.14	n.d.	2.37 ± 0.00	< LOD	47.69 ± 0.39	< LOD
H8	23.65 ± 0.28	39.23 ± 0.45	15.17 ± 0.89	7.76 ± 0.53	154.42 ± 0.91	4.07 ± 1.36	4.15 ± 0.18	3.26 ± 0.16	9.98 ± 0.26	11.36 ± 0.29	8.70 ± 0.17	9.07 ± 0.06	n.d.	6.79 ± 0.07	< LOD	145.28 ± 1.53	3.70 ± 0
Canary island																	
H9	2.29 ± 0.10	4.74 ± 0.04	2.66 ± 0.04	3.19 ± 0.03	31.34 ± 0.06	6.21 ± 0.07	< LOD	< LOD	3.43 ± 0.05	3.58 ± 0.00	< LOD	40.67 ± 0.01	n.d.	2.06 ± 0.02	< LOD	159.68 ± 0.17	< LOD
H10	17.04 ± 0.39	9.28 ± 0.27	17.93 ± 0.36	3.85 ± 0.03	57.08 ± 1.26	5.77 ± 0.05	< LOD	< LOD	4.58 ± 0.03	5.24 ± 0.02	< LOD	6.10 ± 0.05	n.d.	3.44 ± 0.04	n.d.	10.44 ± 0.05	1.85 ± 0
H11	43.85 ± 0.91	45.58 ± 0.47	4.42 ± 0.26	7.36 ± 0.82	91.68 ± 2.74	3.05 ± 0.20	8.42 ± 0.03	< LOD	< LOD	19.99 ± 0.06	< LOD	2.64 ± 0.18	n.d.	5.21 ± 0.09	n.d.	15.74 ± 0.36	< LOD
H12	6.84 ± 0.06	7.48 ± 0.06	7.63 ± 0.54	3.83 ± 0.09	61.35 ± 1.41	9.64 ± 0.38	< LOD	2.62 ± 0.07	< LOD	5.29 ± 0.15	< LOD	19.66 ± 0.13	n.d.	3.58 ± 0.04	< LOD	74.62 ± 1.84	2.09 ± 0
WINES																	
Madeira fortified																	
W1	14.62 ± 0.08	12.16 ± 0.10	2.32 ± 0.04	21.23 ± 0.09	< LOD	4.38 ± 0.09	14.03 ± 0.10	19.39 ± 0.60	313.46 ± 1.62	85.74 ± 0.22	95.60 ± 1.16	5.69 ± 0.04	< LOD	10.61 ± 0.00	n.d.	8.51 ± 0.02	3.49 ± 0
W2	20.90 ± 0.01	6.46 ± 0.00	3.03 ± 0.02	6.98 ± 0.02	1.17 ± 0.05	3.49 ± 0.13	10.54 ± 0.02	6.88 ± 0.10	15.46 ± 0.05	16.51 ± 0.20	9.41 ± 0.07	6.37 ± 0.05	1.39 ± 0.00	6.52 ± 0.09	n.d.	9.83 ± 0.19	4.81 ± 0
W3	30.44 ± 0.41	11.47 ± 0.15	4.89 ± 0.04	16.77 ± 0.04	< LOD	4.73 ± 0.09	11.85 ± 0.07	21.96 ± 0.40	459.56 ± 6.29	87.23 ± 0.56	33.57 ± 0.21	18.17 ± 0.09	n.d.	11.40 ± 0.07	n.d.	11.33 ± 0.07	6.72 ± 0
W4	29.52 ± 0.19	8.45 ± 0.08	3.88 ± 0.12	12.20 ± 0.01	< LOD	3.69 ± 0.10	18.84 ± 0.52	9.10 ± 0.08	47.42 ± 0.32	28.67 ± 0.15	13.91 ± 0.34	11.78 ± 0.07	< LOD	10.58 ± 0.07	n.d.	15.40 ± 0.03	6.72 ± 0
Madeira table																	
W5	10.67 ± 0.06	26.48 ± 0.11	11.03 ± 0.29	4.86 ± 0.01	< LOD	5.29 ± 0.21	5.61 ± 0.12	3.47 ± 0.00	19.43 ± 0.50	17.58 ± 0.03	5.18 ± 0.01	4.70 ± 0.03	< LOD	4.16 ± 0.01	n.d.	6.64 ± 0.03	3.17 ± 0
W6	10.35 ± 0.02	29.6 4± 0.01	5.39 ± 0.36	4.35 ± 0.02	28.37 ± 0.05	6.78 ± 0.28	8.41 ± 0.21	2.47 ± 0.18	24.73 ± 0.15	14.90 ± 0.02	31.27 ± 0.11	8.61 ± 0.02	< LOD	3.74 ± 0.15	n.d.	7.13 ± 0.05	2.35 ± 0
W7	1.55 ± 0.01	9.49 ± 0.04	1.39 ± 0.01	3.60 ± 0.06	21.36 ± 0.17	2.03 ± 0.07	5.43 ± 0.05	0.85 ± 0.02	5.58 ± 0.08	8.31 ± 0.09	6.27 ± 0.11	2.05 ± 0.04	0.15 ± 0.01	1.50 ± 0.11	n.d.	1.93 ± 0.06	0.64 ± 0
Canarian table																	
W8	30.13 ± 0.62	46.52 ± 0.62	3.20 ± 0.10	12.09 ± 0.14	< LOD	84.26 ± 0.71	25.14 ± 0.32	11.69 ± 0.02	278.09 ± 6.29	66.12 ± 0.67	101.65 ± 3.55	29.83 ± 0.20	6.51 ± 0.04	23.45 ± 0.07	n.d.	17.90 ± 0.62	7.92 ± 0
W9	37.17 ± 0.56	53.21 ± 1.01	4.42 ± 0.30	12.65 ± 0.06	6.18 ± 0 .56	71.18 ± 0.44	25.80 ± 2.06	10.24 ± 0.61	181.55 ± 1.16	48.28 ± 1.08	98.85 ± 1.26	25.92 ± 0.38	6.99 ± 0.00	13.44 ± 0.26	< LOD	17.51 ± 0.43	6.44 ± 0

Besides determining the amino acid composition, this methodology is a useful tool for the control of biogenic amines with known toxic activity, like histamine (maximum recommended levels of 5–6 mg/L in Belgium and 10 mg/L in Switzerland (Lehtonen, Saarinen, Vesanto, & Riekkola, 1992)) and tyramine, considered toxic to human health (25–40 mg/L) (Moreno-Arribas & Polo, 2009).

The results obtained for the honey and wine samples are summarized in Table 3.4, where only amino acid concentrations are shown as biogenic amines were found only in vestigial quantities, usually below the LOD. The main amino acids found in honey samples were phenylalanine, glutamine and lysine and in wines were arginine, alanine and GABA. Methionine showed very low level in wines and was not detected in honeys.

The total amount of the primary amino acids found is described in Figure 3.3. The values range from 76.89 mg/L (W7) to 802.40 mg/L (W8). From the analysed honeys, Madeira multifloral honey (H2) presents the highest amount of amino acid found, 286.00 mg/L. Analysing Madeira wines from similar grape varieties, it was observed that the fermented ones showed lower values (about 172.94 mg/L) than the wines submitted to partial fermentation, like sweet wines (684.73 mg/l in average for W1 and W3). This was expected since amino acids are catabolized in several reactions during fermentation.

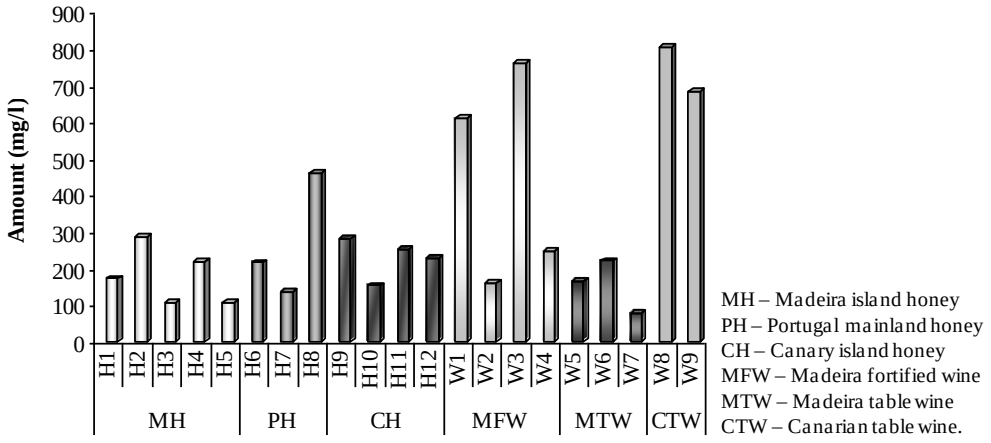


FIGURE 3.3 – Amount of amino acid in the liquid food matrices studied.

Comparing the results obtained for the same variety submitted to a different fermentation process (W3 and W4), the content of the following acids decreased at least 50% during fermentation: arginine, alanine, γ-aminobutyric acid and threonine, showing the importance of these amino acids in the formation of typical aromas present in Madeira wines (together with cysteine, not determined with this method, due to low sensitivity). The total amino acid content in Madeira fortified dry wines is similar to Madeira table wines, since the fermentation is almost complete. Canarian table wines presented higher levels of these compounds (741.64 mg/L in average), close to Madeira submitted to partial fermentation, explained by the fact that those wines were produced from over-maturated *Malvasia* grapes.

TABLE 3.5 – Relative abundance (%) of each amino acid in honeys and wines under study.

	Madeira island honeys					Portugal mainland honeys			Canary island honeys			
Amino Acids (%)	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**
Asp	9	9	8	14	8	9	13	54	22	39	100	16
Glu	10	12	11	26	14	6	14	86	10	20	100	16
Asn	16	16	17	18	31	28	21	85	15	100	25	43
Ser	---	34	44	61	35	46	43	100	41	50	95	49
Gln	26	36	21	22	21	13	17	100	20	37	59	40
His	33	49	28	70	53	49	51	42	64	60	32	100
Gly	---	---	---	---	---	---	---	49	---	---	100	---
Thr	---	---	---	98	---	---	---	100	---	---	---	80
Arg	34	30	36	---	37	43	40	100	34	46	---	---
Ala	18	39	62	32	22	19	40	57	18	26	100	26
GABA	---	---	---	---	---	---	---	100	---	---	---	---
Tyr	12	21	13	51	16	83	22	22	100	15	6	48
Met	---	---	---	---	---	---	---	---	---	---	---	---
Trp	49	82	30	50	36	24	35	100	30	51	77	53
Val	---	---	---	100	---	---	---	---	---	---	---	---
Phe	48	92	13	61	13	69	30	91	100	7	10	47
Ile	55	100	---	58	---	40	---	91	---	45	---	51
Leu	47	100	14	8	---	48	10	13	17	---	17	---
Lys	60	59	46	45	60	41	45	40	74	42	---	100
Total compounds	13	14	13	16	12	14	13	17	13	13	12	13

	Madeira island wines							Canary island wines	
	Fortified				Table			Table	
	W1	**W2**	**W3**	**W4**	**W5**	**W6**	**W7**	**W8**	**W9**
Asp	39	56	81	79	28	28	4	80	100
Glu	23	12	21	16	49	55	18	86	100
Asn	21	27	44	35	100	49	13	29	42
Ser	100	33	79	57	23	20	17	57	60
Glu	2	4	8	1	7	100	75	5	20
His	5	4	6	4	6	8	2	100	85
Gly	56	42	47	75	22	33	22	100	97
Thr	88	31	100	41	16	11	4	53	45
Arg	68	3	100	10	4	5	1	61	39
Ala	98	19	100	33	20	17	10	76	54
GABA	94	9	33	14	5	31	6	100	96
Tyr	19	21	61	39	16	29	7	100	88
Met	9	20	---	22	29	22	2	93	100
Trp	45	28	49	45	18	16	6	100	58
Val	---	---	---	---	---	---	---	---	100
Phe	48	55	63	86	37	40	11	100	96
Ile	44	61	85	85	40	30	8	100	81
Leu	38	61	54	93	50	41	9	100	97
Lys	10	42	40	90	54	54	7	93	100
Total compounds	18	18	17	18	18	18	18	18	19

The percentage of the amino acid relative to abundance is exposed in Table 3.5. The samples with the highest relative abundance of amino acids were H8 (honey) due to serine, glutamine, threonine, arginine, GABA and tryptophan and W8 (wine) due to histidine, glycine, GABA, tyrosine, tryptophan, phenylalanine, isoleucine and leucine. Wines besides having higher amounts of amino acids also possess the richest amino acid profile.

The applicability of the reported procedure for simultaneously analysis of amino acids and biogenic amines has been demonstrated for the analysis of wine and honey samples.

3.4 Conclusions

A simple RP-HPLC analytical method for the simultaneous analysis of amino acids and biogenic amines in liquid food matrices is proposed based on a pre-column derivatization with OPA, performed in the sample injection loop, and fluorescence detection. The separation and quantification of 19 amino acids and nine amines was carried out in a single run as their OPA/MCE derivatives elute within 80 min, ensuring a reproducible quantification. The practical utility of the proposed chromatographic procedure was shown by the analysis of the amino

acid and biogenic amine contents in honey and wine samples without any preliminary separation or clean-up steps. The method showed high sensitivity and response to minor compounds with the exception to proline, cysteine and hydroxyproline. Future trends pass through the use of shorter columns to reduce the analysis and the application of this procedure to other food matrices.

Relatively to the analysed samples, the amino acid present at the highest concentration in honeys was phenylalanine and in wines was arginine. The biogenic amines suspected to cause toxicological effects (histamine, tyramine and phenylethylamine) were no cause for concern in the analysed honey and wine samples since they are present in vestigial quantities.

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CHAPTER 4

Analysis of organic acids, furans and polyphenols

This chapter is based on the following publication:

HPLC-DAD methodology for the quantification of organic acids, furans and polyphenols by direct injection of wine samples

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Abstract

This chapter presents a simple and sensitive HPLC method with photo-diode array detection for the analysis of organic acids, monomeric polyphenols and furanic compounds in wine samples by direct injection. The chromatographic separation of 8 organic acids, 2 furans and 22 phenolic compounds was carried out with a buffered solution (pH 2.70) and acetonitrile as mobile phases and a difunctionally bonded C_{18} stationary phase, Atlantis dC_{18} (250 x 4.6 mm, 5 µm) column. The elution was performed in 12 min for the organic acids and in 60 min for the phenolic compounds, including phenolic acids, stilbenes and flavonoids. Target compounds were detected at 210 nm (organic acids, flavan-3-ols and benzoic acids), 254 nm (ellagic acid), 280 nm (furans and cinnamic acid), 315 nm (hydroxycinnamic acids and trans-resveratrol) and 360 nm (flavonoids). The RSDs for the repeatability test (n = 5) of peak area and retention times were below 3.1 and 0.3%, respectively, for phenolics and below 1.0 and 0.2% for organic acids. The RSDs expressing the reproducibility of the method were higher than for the repeatability results but all below 9.0%. Method accuracy was evaluated by the recovery results, with average values between 80 and 104% for polyphenols and 97–105% for organic acids. The calibration curves, obtained by triplicate injection of standard solutions, showed good linearity with regression coefficients higher than 0.9982 for polyphenols and 0.9997 for organic acids. The LOD was in the range of 0.07–0.49 mg/L for polyphenols (cinnamic and gallic acids, respectively) and 0.001–0.046 g/L for organic acids (oxalic and lactic acids, respectively). The method was successfully used to measure and assess the polyphenolic fingerprint and organic acid profile of red, white, rosé and fortified wines.

4.1 Introduction

The analytical characterization of wines is usually a time-consuming process, but it yields the necessary information for the elaboration and control of a quality product and definition of suitable conditions for adequate preservation. The profile and evaluation of the organic acid and polyphenol contents are important parameters in wineries, and hence it is essential to have a rapid and precise methodology for quantification. The determination of organic acids, mainly tartaric, malic and lactic acids, is important for the fermentation process monitoring, as they contribute to flavour balance, chemical stability and microbiologic control and frequently subject to control in food to accomplish law and regulations. In addition, polyphenols also have effects on the organoleptic characteristics (colour, flavour and taste), thus their profile and content are also significant (Ibern-Gomez, Andres-Lacueva, Lamuela-Raventos, & Waterhouse, 2002; Romero & Munoz, 1993). These two types of chemical species are very common in wines and both are affected by several factors such as ripening, variety, growing region, atmospheric conditions as well as production techniques (Goldberg et al., 1995; Lamikanra, Inyang, & Leong, 1995; McDonald et al., 1998).

One of the most used technologies to detect and quantify organic acids is HPLC method with photo-diode array detection (HPLC-DAD) and there is a number of published methods (Cabrita et al., 2008; Cunha, Fernandes, Faria, Ferreira, & Ferreira, 2002; Kerem, Bravdo, Shoseyov, & Tugendhaft, 2004; Mato, Suárez-Luque, & Huidobro, 2005; Soyer, Koca, &

Karadeniz, 2003; Valentão et al., 2007). Indeed, Mato and co-workers (Mato, et al., 2005) reviewed the analytical methods to determine organic acids in grape juices and wines.

TABLE 4.1 – HPLC-DAD methods reported in the literature for the analysis of polyphenols and organic acids in wines and/or similar matrices.

Samples	Analytes	Stationary phase	Eluents	Detection wavelenght (nm)	LOD (mg/L)	Ref.
Red wines						
LLE	15 polyphenols	ODS-Hypersil (2.1 i.d. × 100 mm, 5 µm), T = 40ºC	Gradient: A: acidified water (0.6% perchloric acid); B: methanol; Flow: 0.3	280 nm		[44]
Red wines						
DI	20 polyphenols including anthocyanins	LiChrospher RP-18 (4.0 i.d. × 250 mm, 5 µm), T = 40ºC	Gradient: A: 9 mM aqueous orthophosphoric acid, pH 2.5; B: solvent	280, 320, 360, and 520 nm		[45]
Red wines						
SPE	12 polyphenols	Hypersil ODS (4.6 i.d. × 200 mm, 5 µm)	Gradient: A: acetic acid in water, 2% v/v; B: water/acetonitrile/acetic acid, 78:20:2 v/v/v;	254, 280 and 340 nm	0.05 - 1.95	[49]
Red wines						
LLE	16 polyphenols	ODS-Hypersil (2.1 i.d. × 200 mm, 5 µm)	Gradient: A: water/formic acid, 99:1 v/v; B:	280, 320 and 350		[40]
Red wines						
DI	35 polyphenols including anthocyanins	Spherisorb C18 (4.6 i.d. × 250 mm, 5 µm), T = 40ºC	Gradient: A: 50 mM aqueous ammonium hydrogenphosphate, pH 2.6; B: solvent	280, 320, 360, and 520 nm		[50]
Red wines						
LLE	47 polyphenols	Nova-Pak C18 (3.9 i.d. × 300 mm, 4 µm)	Gradient: A: acetic acid in water, 2% v/v; B: water/acetonitrile/acetic acid, 78:20:2 v/v/v;	280, 340, and 310 nm		[51]
Red wines						
DI	30 polyphenols including anthocyanins	Atlantis dC18 (2.1 i.d. × 250 mm, 5 µm), T = 30ºC	Gradient: A: formic acid in water, 5% v/v; B: acetonitrile/water/formic acid, 80:15:5	280, 320, 360 and 520 nm		[56]
Red wines						
SPE	6 organic acids	Nucleogel Ion 300 OA (7.7 × 300 mm),	Isocratic: 0.01 N sulfuric acid; Flow: 0.2	214 nm	0.01 - 1.67	[12]
Red wines						
DI for hydroxycinnamic acids	38 polyphenols including anthocyanins	Waters symmetry C18 (4.6 i.d. × 150 mm, 5 µm), T = 35ºC	Gradient: A: formic acid in water, 5% v/v; B: methanol; Flow: 1.0 mL/min	280, 320,360 and 520 nm		[38]
LLE followed by SPE for hydroxybenzoic acids, catechins and flavonols		ODS Hypersyl (4.6 i.d. × 250 mm, 5 µm), T = 35ºC	Gradient: A: formic acid in water, 2.5% v/v; B: methanol; Flow: 1.0 mL/min			
Red wines						
DI	48 polyphenols including anthocyanins	Ace® 5 C18 (4.6 i.d. × 250 mm), T = 20°C	Gradient: A: 50 mM aqueous ammonium hydrogenphosphate, pH 2.6; B: solvent	280, 320, 360, and 520 nm	0.088 - 0.711	[53]
Red wines						
DI	6 polyphenols	LC18 reversed phase packing (Supelco) (2.1 i.d. × 150 mm, 5 µm)	Gradient: A: 5% formic acid in water; B: acetonitrile; Flow: 0.3 mL/min to 0.8	285, 306 and 270 nm	0.16 - 1.50	[54]
Red wines						
SPE for organic acids	11 polyphenols and 2 organic acids	LichroCART® 250-4 Superspher® RP 18 (4.6 i.d. × 250 mm, 5µm)	Isocratic: 5 mM fosforic acid; Flow: 0.7 mL/min	210 nm		[13]
LLE for polyphenols		Superpher® 100, C18 (4.6 i.d. × 250 mm, 5µm)	Gradient: A: water/acetic acid, 98:2 v/v; B: water/methanol/acetic acid, 68:30:2 v/v/v;	254, 280 and 320 nm		
Musts and wines from red grapes						
DI	7 organic acids 6 polyphenols	Synergi™ Polar-RP™ (4.6 i.d. × 250 mm), T = 30°C	Gradient: A: trifluoroacetic acid in water, 0.2% v/v, pH 1.9; B: acetonitrile; Flow: 1.5	210 and 280 nm		[11]
White wines						
DI	17 polyphenols	Nova-Pak C18 (3.9 i.d. × 300 mm, 4 µm), T = 20ºC	Gradient: A: acetic acid in water, 2% v/v; B: water/acetonitrile/acetic acid, 58:40:2 v/v/v;	280 and 320 nm		[52]
White grapes and their juices						
DI	3 organic acids	Bio Rad Aminex HPX-87(300 × 7.8 mm^2)	Isocratic: 0.01 N sulfuric acid; Flow: 0.6	214 nm	15.0 - 30.0	[10]
Red and white wines						
DI	17 polyphenols	Chromolith Performance RP-18e (4.6 i.d. × 100 mm), T = 30°C	Gradient: A: methanol/double-distilled water, 2.5:97.5 v/v, at pH 3 with H_3PO_4; B: methanol/double-distilled water, 50:50 v/v,	256, 280, 308, 324 and 365 nm	0.010 - 0.160	[46]
Red and white wines						
SS-LLE	13 polyphenols	Agilent Zorbax Eclipse XDB-C18 (4.6 i.d. × 250 mm, 5 µm)	Gradient: A: water/methanol/formic acid, 97:2.5:0.5 v/v/v; B: methanol; Flow: 1.0	280, 305 and 370 nm	0.073 - 0.164	[41]
Red, white and rosé wines						
LLE	17 polyphenols	Nova-Pak C18 (3.9 i.d. × 150 mm, 4 µm)	Gradient: A: water/acetic acid/methanol, 88:2:10 v/v/v; B: water/acetic	270, 307 and 360 nm	0.03 - 11.5	[55]
Musts and fortified wines						
SPE followed by NBDI derivatization	6 organic acids	Spherisorb C18 (4.6 i.d. × 150 mm, 3 µm)	Gradient: A: water; B: acetonitrile; Flow: 1.5 mL/min	265 nm	5.0 - 98.0	[9]
Wines						
LLE	16 polyphenols	Phenomenex Luna C18 (4.6 i.d. × 150 mm, 5 µm)	Gradient: A: formic acid in water, 0.1 % v/v; B: methanol; Flow: 0.7 mL/min	λ with lowest energy (λ_{max})	0.01 - 0.03	[48]
Brandies						
DI	13 polyphenols	Lichrospher RP18 (4.0 i.d. × 250 mm, 5 µm), T = 40ºC	Gradient: A: formic acid in water, 2% v/v; B: methanol/water/formic acid, 70:28:2	280 and 320 nm	0.01 - 1.15	[47]

LLE - Liquid - liquid extraction; SPE - Solid phase extraction; DI - Direct injection; SS-LLE - Solid-supported liquid - liquid extraction

Some methods are based on ion-exclusion separations (López-Tamames, Puig-Deu, Teixeira, & Buxaderas, 1996; Valentão, et al., 2007), which normally require the removal of

polyphenols before sample analysis, others involve ion-exchange (Falqué-López & Fernández-Gómez, 1996) and reversed-phase (RP) separations (Llorente, Villarroya, & Gómez-Cordovés, 1991; Pazo, Traveso, Cisneros, & Montero, 1999; Samanidou, Antoniou, & Papadoyannis, 2001). Most methods are not applicable to wine due to the alcohol content or low resolution (Dong, 1998). Frequently, isocratic elutions are described using an acidified aqueous solvent and separation time is no longer than 20 min (Walker, Morris, Threlfall, & Main, 2003). It is also common to find in the literature that organic acid analysis includes a sample pre-treatment which increases analysis time and affects the reliability of the results.

A variety of techniques have been used for the determination of phenolic compounds in wines based on GC (Goldberg et al., 1994; Luan, Li, & Zhang, 2000; Minuti, Pellegrino, & Tesei, 2006; Soleas, Dam, Carey, & Goldberg, 1997) and CE (Capote, Rodríguez, & Castro, 2007; Gu, Chu, O'Dwyer, & Zeece, 2000; Pazourek et al., 2005), but RP-HPLC has been elected and considered the most appropriate technique to analyse wine polyphenols, often used to give product composition and differentiation (García-Falcóna, Pérez-Lamela, Martínez-Carballo, & Simal-Gándara, 2007; Makris, Kallithraka, & Mamalos, 2006; Silva et al., 2005). Generally, studies make use of RP C_{18} columns (Abad-García et al., 2007; Nave, Cabrita, & Costa, 2007) and binary solvent systems consisting of a solvent A, usually acidified water, and a polar organic solvent B, such as acetonitrile or methanol (Vitrac, Monti, Vercauteren, Deffieux, & Mérillon, 2002). DAD methods are the most common (Arnous, Makris, & Kefalas, 2001; Cabrita, et al., 2008; Canas, Belchior, Spranger, & Bruno-de-Sousa, 2003; Castellari, Sartini, Fabiani, Arfelli, & Amati, 2002; García-Falcóna, et al., 2007; Garcia-Viguera & Bridle, 1995; Gomez-Alonso, Garcia-Romero, & Hermosin-Gutierrez, 2007; Gutiérrez, Lorenzo, & Espinosa, 2005; Kerem, et al., 2004; Monagas, Suárez, Gómez-Cordovés, & Bartolomé, 2005; Nave, et al., 2007; Paixão, Pereira, Marques, & Câmara, 2008; Preys et al., 2006; Recamales, Sayago, González-Miret, & Hernanz, 2006; Robbins & Bean, 2004; Sanza, Domínguez, Cárcel Cárcel, & Gracia, 2004; Silva, et al., 2005; Tarola & Giannetti, 2007; Valentão, et al., 2007), but other detection methods as electrochemical (Bravo et al., 2008; Kaoutit et al., 2008) and MS (Bilbao, Andrés-Lacueva, Jáuregui, & Lamuela-Raventós, 2007; Borbalán, Zorro, Guillén, & Barroso, 2003; Pérez-Magariño, Revilla, González-SanJosé, & Beltrán, 1999) have also been used. The use of LC-MS and LC-MS/MS has become the best option for the analysis of these compounds in several matrices as well as their derived products (Griffith & Collison, 2001; Kao, Huang, Inbaraj, & Chen, 2008; Seeram, Lee, Scheuller, & Heber, 2006), but the opportunity of access to these advanced technologies is still restricted for most laboratories. In Table 4.1, several published methods are summarized for the determination of these compounds in wine and similar matrices.

Therefore, the aim of this study was to develop a simple and sensitive methodology using RP-HPLC-DAD chromatographic separation, allowing a single run determination of organic acids and monomeric polyphenols in the same wine sample, with no sample pre-treatment, covering the compounds normally found in wines. RP separation mechanism was chosen since it is frequent in polyphenol analysis and performs organic acids faster analysis (Ding, Koizumi, & Suzuki, 1995). Other HPLC procedures have also been developed for the simultaneous analysis of organic acids and polyphenols in wines and grapes (Dopico-García, Valentão, Guerra, Andrade, & Seabra, 2007; Kerem, et al., 2004), but these studies were

developed for a restricted number of polyphenolic compounds. For the purpose of the study, the method was extended to two furanic compounds, 5-hydroxymethylfurfural (HMF) and furfural, as they are usually detected in fortified wines. Considering the elution conditions, both furans are presented in tables associated with polyphenols. The current project intends to apply the developed methodology for the assessment of these compounds in several wine types: fortified, red, white and rosé wines.

4.2 Experimental

4.2.1 *Standards and reagents*

Polyphenol standards: gallic acid, gentisic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, ellagic acid, cinnamic acid, *p*-hydroxybenzoic acid, *(+)*- catechin, *(-)*-epicatechin, *(-)*-epigallocatechin, myricetin, sinapic acid, rutin and kaempferol were supplied by Fluka Biochemika AG (Buchs, Switzerland), protocatechuic acid, vanillin, syringic acid and *trans*-resveratrol by Sigma-Aldrich (St. Louis, MO, USA), whereas syringaldehyde, HMF and furfural were acquired from Acros Organics (Geel, Belgium) and quercetin from Riedel-de-Haën (Seelze, Germany). The purity of all polyphenolic standards was greater than 95%. Polyphenol stock solutions of 1 g/L were prepared by dissolving the appropriate amount of each compound in ethanol. These solutions were stored at 4 °C and diluted before use with Milli-Q water to prepare the working standard solutions.

Acids standards were obtained from different suppliers: *L*-tartaric (99.5%), *L*-malic (99.5%) and succinic (99.5%) from Merck (Darmstadt, Germany); lactic (85%) and acetic (99.7%) from Panreac Química S.A. (Barcelona, Spain); citric (99.5%) from Fluka BioChemika AG; formic (99.7%) and oxalic (99%) were obtained from Fisher Scientific (Loughborough, UK) and Acros Organics, respectively. Stock standard solutions of 10 g/L were prepared by dissolving each acid in Milli-Q water and stored at 4 °C for 1 month. Working standard solutions were prepared by dilution with Milli-Q water.

HPLC-grade acetonitrile was obtained from Sigma-Aldrich and ultra-pure water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). Disodium hydrogen phosphate dihydrate (99%) was supplied by Panreac Química S.A., sulphuric acid (95–97%) was supplied by Riedel-de-Haën. The eluents were previously filtered with membrane filters obtained from Pall (0.20 µm, Ann Arbor, MI, USA).

4.2.2 *Apparatus and operating conditions*

Chromatographic analyses were carried out using a Waters Alliance liquid chromatograph (Milford, MA, USA) equipped with an auto-injector (Waters 2695, separations module) and a photodiode array detector (Waters 2996). To separate organic acids and polyphenols, an Atlantis dC_{18} column (250 x 4.6 mm id; 5 µm; Milford, MA, USA) was selected as the analytical column, using the following mobile phases: A: 10mM of phosphate solution buffered at pH 2.70 with concentrated sulphuric acid; B: 100% acetonitrile.

As polyphenols are present in wine in minor quantities (about mg/L) when compared with organic acids content (up to g/L), the separation method was divided into two steps, maintaining the general operation conditions but allowing the correct evaluation of the different concentration ranges. Organic acids chromatographic separation was carried out using an isocratic elution, 100% A during 8 min followed by 12 min of washing and re-equilibration period, while polyphenols and the two furans require a gradient elution applied as follows: 0–30 min, 0–20% B, linear; 30–50 min, 20–50% B, linear; 50–60 min, washing and re-equilibration of the column. The mobile phase was set to a flow rate of 1.0 mL/min and the column thermostated at 30 °C. Injection volume was set to 10 mL and all standards and wine samples were injected in triplicate, after being filtered through membrane filters Acrodisc® CR PTFE from Waters (0.45 μm). Target compounds were detected at 210 nm (organic acids, flavan-3-ols and benzoic acids), 254 nm (ellagic acid), 280 nm (furans and cinnamic acid), 315 nm (hydroxycinnamic acids and trans-resveratrol) and 360 nm (flavonoids). The detector signals were recorded on a chromatography data system controlled by the Empower Pro software. Chromatographic peaks were identified by comparison of elution order, retention times, the spectral UV–Vis with those of standards and spiking samples with pure compounds. The quantification of the studied compounds was carried out using the external standard method.

4.2.3 *Samples*

This methodology was applied to different types of wines: four fortified wines (F wines), four red table wines (R wines), four white table wines (W wines) and one rosé wine (Rs wine). All wines were produced from *Vitis vinifera L.* grape varieties. Red and white wines were bought in local stores and fortified wines were supplied by a local producer. Samples were filtered (0.45 μm) and diluted with mobile phase A when needed to comply with the working range.

4.2.4 *Method validation*

Retention times were previously determined using individual standards dissolved in mobile phase A. The working range for each compound was estimated from the expected results for this type of samples and the higher concentration working standard solution was accordingly prepared from the stock solution of each compound (10 g/L for organic acids and 1 g/L for polyphenolic and furanic compounds) and diluted with Milli-Q water. Five other working solutions were prepared by successive dilutions and injected for the linearity range test.

Wide concentration ranges were used as the amount of the studied compounds depends on the wine variety. Quantification was carried out by the external standard method based on peak areas of the eluted compounds.

Method sensitivity was assessed by the determination of LOD and LOQ of each compound. These parameters were calculated on the basis of linear regression, LOD = $3.3\sigma/b$

and LOQ = 10σ/b, σ is the y-intercept standard deviation and b is the slope of the linear regression.

The precision was evaluated by inter- and intra-day repetition method. Intra-day repeatability was assessed by five successive replicate determinations of three standards. Inter-day reproducibility was assessed by analysing, on three distinct occurrences, five replicates of three standards.

Recovery was determined by the addition of known amounts of organic acids, furans and polyphenols to the wine samples, tested for two concentration levels and replicated three times. Average recovery was calculated by comparing mean values of replicates with theoretical concentrations of each replicate.

4.3 Results and discussion

4.3.1 *Method development*

Usually, the chromatographic analysis of organic acids is carried out using ion-exchange columns, requiring phenolic compounds to be previously removed from the sample, whereas the polyphenol separation is frequently performed by reversed-phase. The present method was developed to allow the sequential analysis of 8 organic acids, 22 monomeric phenolic and 2 furanic compounds commonly found in wines (Table 4.2), using the same RP column, a difunctional-bonded C_{18} stationary phase, Atlantis dC_{18} column.

Initial HPLC working conditions were selected based on the organic acids method published in Waters application notebook for Atlantis columns (Waters, 2004). Then, the method was optimized in order to achieve good resolution for the maximum number of peaks in the shortest analysis time, considering the following parameters: injection volume, wavelength detection, the solvents used and the elution program. As summarized in Table 4.1, the separation of polyphenols usually involves the use of acid additives, aiming to suppress ionization, namely acetic and formic acids. Besides being target compounds, these additives absorb at 210 nm, affecting the use of this wavelength in the measurement of polyphenols, namely flavan-3-ols, which have higher absorptivity at 210 nm than at 280 nm. Avoiding the use of these acid additives, the alternative was the use of buffered mobile phase for acid pH adjustment. The initial concentration of the buffered mobile phase (20 mM) was decreased to 10 mM to avoid problems with precipitation and the abrasive effect of phosphate buffers on pump seals, but ensuring pH control. As phosphate buffers higher then pH 7 are known to accelerate the dissolution of silica and shorten severely the lifetime of silica-based HPLC columns, the resolution degradation was monitored and the column seemed to be unaffected at the low pH used in this method (2.70). The method was developed with the intention of simultaneous analysis of organic acids and polyphenols, in a single run, but for calibration purposes and considering their disproportionate concentration ranges in wines, it was preferred to perform their analysis separately. However, as organic acids elute at low

retention times (up to 9 min) and furans and polyphenols elute at higher retention times, a single run analysis can be carried out without losing separation.

TABLE 4.2 – Retention times, peak identification, spectral bands (λ_{max}, in bold), detection wavelength ($\lambda_{detection}$) and linearity parameters of organic acids, furans and polyphenols obtained using the proposed methodology.

#	t_R (min)	Compound	Chemical family	UV bands (nm)	$\lambda_{detection}$ (nm)	Linear range	*a*	*b*	R^2	LOD	LOQ	Recovery (%)
						g/L						
1	3.06	Oxalic acid	Organic acid	**199**	210	0.012 - 0.307	-25918	7060916	0.9999	0.001	0.003	105
2	3.48	Tartaric acid	Organic acid	**198**	210	0.060 - 1.512	-7230	954374	0.9997	0.010	0.031	97
3	3.71	Formic acid	Organic acid	**200**	210	0.120 - 3.001	-11242	562545	0.9997	0.021	0.064	104
4	4.33	Malic acid	Organic acid	**198**	210	0.122 - 3.045	-7765	489490	0.9998	0.017	0.052	100
5	5.08	Lactic acid	Organic acid	**198**	210	0.239 - 5.976	-3134	133420	0.9997	0.046	0.138	103
6	5.37	Acetic acid	Organic acid	**200**	210	0.239 - 5.985	-4889	151930	0.9998	0.042	0.127	102
7	7.03	Citric acid	Organic acid	**197**	210	0.090 - 2.252	-9930	644315	0.9998	0.012	0.037	105
8	8.61	Succinic acid	Organic acid	**208**	210	0.062 - 1.542	-14435	1262365	0.9998	0.008	0.024	100
						mg/L						
9	12.40	Gallic acid	Hydroxybenzoic acid	**216**, 271	210	2.70 - 54.00	-51608	77647	0.9995	0.49	1.48	95
10	13.48	HMF	Furan	226, **284**	280	1.50 - 30.00	-31960	92726	0.9995	0.27	0.82	93
11	15.01	Furfural	Furan	228, **277**	280	0.75 - 15.00	-20602	82660	0.9986	0.23	0.69	82
12	17.33	Protocatechuic acid	Hydroxybenzoic acid	**205**, 219, 259, 293	210	0.80 - 15.90	-17951	66943	0.9993	0.18	0.53	80
13	21.35	Gentisic acid	Hydroxybenzoic acid	**210**, 324	210	0.80 - 16.05	-26461	86293	0.9992	0.19	0.59	81
14	22.46	*p*-Hydroxybenzoic acid	Hydroxybenzoic acid	**196**, 254	210	0.75 - 15.00	-17117	61694	0.9996	0.13	0.39	83
15	23.15	*(-)*-Epigallocatechin	Flavan-3-ol	**206**, 271	210	0.75 - 15.00	-26412	107423	0.9983	0.26	0.78	89
16	24.51	*(+)*-Catechin	Flavan-3-ol	**203**, 279	210	0.75 - 15.00	-22836	95136	0.9993	0.17	0.50	101
17	25.60	Vanillic acid	Hydroxybenzoic acid	208, **218**, 260, 292	210	0.79 - 15.75	-16325	56327	0.9996	0.13	0.40	104
18	26.99	Caffeic acid	Hydroxycinnamic acid	218, 238, **324**	315	0.84 - 16.80	-11537	52286	0.9996	0.14	0.43	90
19	27.49	Syringic acid	Hydroxybenzoic acid	**217**, 274	210	0.75 - 15.00	-17089	71916	0.9995	0.15	0.45	91
20	28.46	*(-)*-Epicatechin	Flavan-3-ol	**203**, 279	210	0.80 - 16.05	-21504	94208	0.9994	0.17	0.51	96
21	29.77	Vanillin	Hydroxybenzaldehyde	**204**, 230, 279, 307	210	0.75 - 15.00	-16877	48269	0.9996	0.12	0.37	98
22	32.22	Syringaldehyde	Hydroxybenzaldehyde	**216**, 307	210	0.78 - 15.60	-24925	56216	0.9982	0.27	0.82	102
23	32.86	*p*-Coumaric acid	Hydroxycinnamic acid	212, 226, **310**	315	0.79 - 15.75	-14506	73759	0.9994	0.16	0.47	88
24	35.29	Ferulic acid	Hydroxycinnamic acid	217, 234, **323**	315	0.79 - 15.75	-10514	51221	0.9996	0.14	0.43	92
25	35.91	Sinapic acid	Hydroxycinnamic acid	200, 237, **323**	315	0.77 - 15.30	-10273	48126	0.9990	0.20	0.61	102
26	36.31	Rutin	Flavonol	**204**, 255, 354	360	0.83 - 16.50	-3310	12905	0.9990	0.23	0.69	99
27	37.22	Ellagic acid	Hydroxybenzoic acid	**254**	254	0.86 - 17.10	-41982	75271	0.9988	0.28	0.84	86
28	40.81	Myricetin	Flavonol	**207**, 253, 370	360	0.77 - 15.30	-10951	23849	0.9983	0.27	0.82	87
29	42.47	*trans*-Resveratrol	Stilbene	216, **305**	315	0.77 - 15.45	-17009	69118	0.9994	0.15	0.46	97
30	44.18	Cinnamic acid	Cinnamic acid	204, 216, **277**	280	0.80 - 16.05	-8569	79329	0.9999	0.07	0.22	98
31	44.77	Quercetin	Flavonol	**203**, 254, 370	360	0.75 - 15.00	-16388	43127	0.9996	0.12	0.37	96
32	48.44	Kaempferol	Flavonol	**200**, 265, 364	360	0.85 - 16.95	-21875	47608	0.9998	0.11	0.34	96

a - *y*-axis intercept
b - slope of the regression line

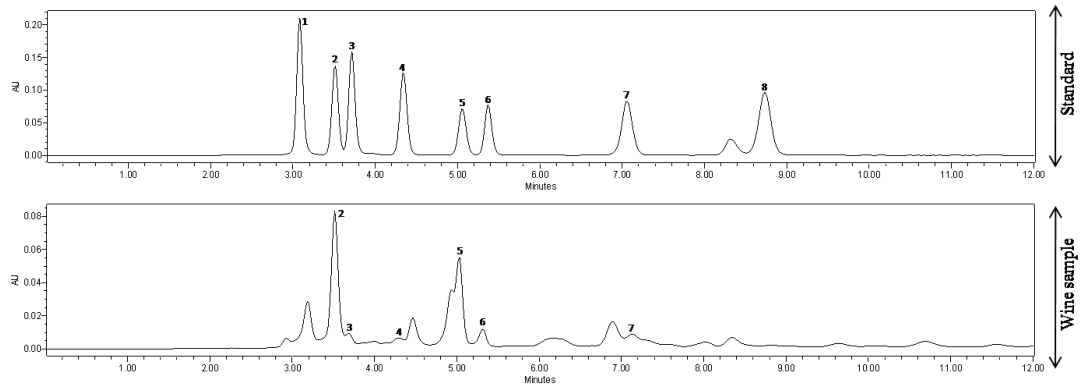

FIGURE 4.1 – Representative chromatograms obtained with the proposed method for the determination of organic acids at 210 nm, when applied to the standard solution and a wine sample. See Table 4.2 for peak identification.

Therefore, an isocratic elution was carried out for organic acids with the buffered mobile phase at pH 2.70 (Figure 4.1) and a gradient elution was used for monomeric polyphenols and furans (Figure 4.2). The gradient elution, described in Section 4.2.2, was performed during 60 min, including washing and re-equilibration stage, starting with 100% of aqueous mobile phase and requiring a maximum of 50% of organic solvent to elute the analytes under study, avoiding high consumption of the organic phase, which frequently represents a significant cost in laboratories. Figure 4.2 shows typical chromatograms obtained applying this gradient to a polyphenols and furans standard solution and a wine sample.

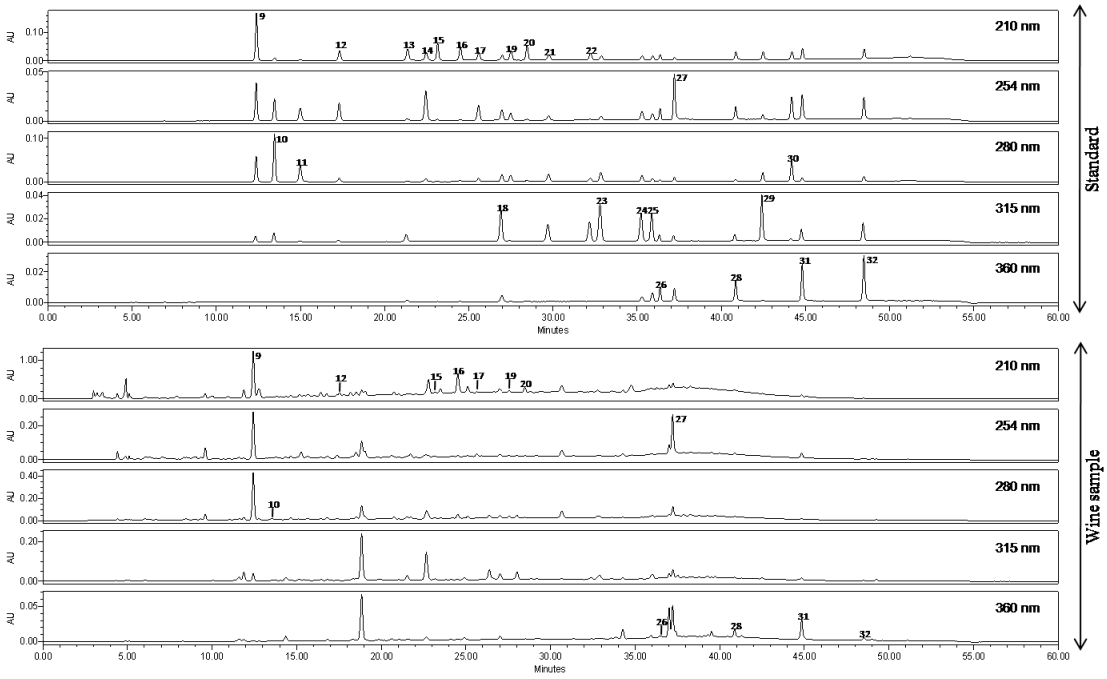

FIGURE 4.2 – Representative chromatograms obtained with the proposed method for the determination of polyphenols and furans at the selected detection wavelengths: 210, 254, 280, 315 and 360 nm, when applied to a standard solution and a wine sample. See Table 4.2 for peak identification.

Phenolic acids are currently detected at 280 nm, even if most of them have higher absorption at wavelengths close to 210 nm, as flavan-3-ols. The spectral bands of the studied compounds were obtained by their spectral array between 190 and 600 nm and are summarized in Table 4.2. The detection wavelength was chosen near to the absorption maximum, except for the compounds which elute at the final stage of the analysis, as the influence of the acetonitrile absorption increases at lower wavelengths. The use of different detection wavelengths ensured the compromise between selectivity and sensitivity. As published analytical methods usually require sample pre-treatment and long-time analysis, this study intended to overcome this, in order to obtain an easier methodology.

Wine phenolic composition was then determined by direct injection of wine samples, after being filtered through 0.45 µm membrane filters. The direct injection of the samples was selected after testing other pre-sample treatments, including SPE, without losing selectivity and resolution of the compounds of interest due to wine matrix (including the high alcohol content). Thus using the optimized conditions, well-resolved chromatograms of wine samples

were obtained as shown in Figure 4.2. This method also upgrades other previously proposed methods for the simultaneous analysis of organic acids and polyphenols (Kerem, et al., 2004), maintaining the basic principles but improving sensitivity and chromatographic resolution as well as the number of target compounds (up to 32).

4.3.1 *Validation procedure*

In order to validate the developed methodology, several parameters such as linearity, analytical determination limits, recovery, precision and accuracy were considered.

The linearity was evaluated by the analysis in triplicate of six standards solutions. The obtained validation parameters are listed in Table 4.2. Good correlation coefficients (R^2) were observed, higher than 0.9982 for polyphenols and furans and 0.9997 for organic acids, confirming the linearity of the method.

TABLE 4.3 – Repeatability (intra-day) and reproducibility (inter-day) of the developed method, expressed in terms of the variation (RSD%) of retention times (t_R) and areas.

Compounds	S1	Intra-day[a]		Inter-day[b]		S2	Intra-day[a]		Inter-day[b]		S3	Intra-day[a]		Inter-day[b]	
		t_R RSD %	Area RSD %	t_R RSD %	Area RSD %		t_R RSD %	Area RSD %	t_R RSD %	Area RSD %		t_R RSD %	Area RSD %	t_R RSD %	Area RSD %
	g/L					**g/L**					**g/L**				
Oxalic acid	0.077	0.2	0.1	0.2	1.9	0.154	0.1	0.1	0.2	4.0	0.230	0.1	0.1	0.2	0.8
Tartaric acid	0.378	0.2	0.5	0.2	1.5	0.756	0.1	0.4	0.2	3.6	1.134	0.2	0.3	0.3	0.6
Formic acid	0.750	0.1	0.3	0.1	2.2	1.501	0.1	0.3	0.2	3.9	2.251	0.1	0.3	0.2	1.3
Malic acid	0.761	0.1	0.3	0.2	1.5	1.523	0.1	0.1	0.2	3.4	2.284	0.2	0.1	0.4	0.3
Lactic acid	1.494	0.1	0.5	0.2	5.7	2.988	0.1	0.7	0.1	9.0	4.482	0.1	1.0	0.3	5.9
Acetic acid	1.496	0.1	0.5	1.7	0.3	2.993	0.1	0.6	1.4	2.8	4.489	0.2	1.0	1.6	0.7
Citric acid	0.563	0.1	0.1	1.1	1.8	1.126	0.1	0.1	1.1	3.5	1.689	0.1	0.1	1.4	0.5
Succinic acid	0.386	0.0	0.1	0.4	1.6	0.771	0.1	0.1	0.5	3.5	1.157	0.2	0.1	0.8	0.6
	mg/L					**mg/L**					**mg/L**				
Gallic acid	5.40	0.0	0.3	0.1	0.1	18.90	0.3	0.3	1.2	0.4	40.50	0.0	0.2	1.2	0.1
HMF	3.00	0.0	0.2	0.1	0.1	10.50	0.2	0.2	0.8	0.2	22.50	0.1	0.1	0.8	0.3
Furfural	1.50	0.0	0.6	0.1	1.1	5.25	0.1	0.4	0.7	2.2	11.25	0.1	0.1	0.7	1.1
Protocatechuic acid	1.59	0.0	0.2	0.1	0.5	5.57	0.3	0.5	1.1	1.3	11.93	0.1	0.3	1.3	0.2
Gentisic acid	1.61	0.1	0.5	0.1	4.6	5.62	0.3	0.3	1.0	2.9	12.04	0.1	0.2	1.3	3.4
p-Hydroxybenzoic acid	1.50	0.0	1.2	0.1	4.0	5.25	0.2	0.8	0.9	3.7	11.25	0.1	0.2	1.1	3.4
(-)-Epigallocatechin	1.50	0.0	0.2	0.1	1.3	5.25	0.2	0.4	0.6	0.8	11.25	0.1	0.4	0.9	1.4
(+)-Catechin	1.50	0.0	0.4	0.2	2.3	5.25	0.2	0.3	0.7	0.3	11.25	0.1	0.1	1.0	0.1
Vanillic acid	1.58	0.0	0.6	0.0	1.4	5.51	0.2	0.4	0.6	0.4	11.81	0.1	0.2	0.8	0.5
Caffeic acid	1.68	0.0	0.5	0.2	1.3	5.88	0.2	0.3	0.7	0.3	12.60	0.1	0.2	0.9	0.5
Syringic acid	1.50	0.0	0.8	0.0	0.7	5.25	0.2	0.3	0.5	0.6	11.25	0.1	0.2	0.7	0.2
(-)-Epicatechin	1.61	0.0	0.2	0.1	0.9	5.62	0.2	0.3	0.6	1.1	12.04	0.1	0.2	0.8	0.5
Vanillin	1.50	0.0	1.2	0.0	1.2	5.25	0.2	0.3	0.6	1.6	11.25	0.1	0.1	0.8	1.0
Syringaldehyde	1.56	0.0	0.9	0.0	2.7	5.46	0.1	0.6	0.4	1.2	11.70	0.0	0.3	0.6	0.2
p-Coumaric acid	1.58	0.0	0.4	0.1	2.6	5.51	0.2	0.5	0.6	0.5	11.81	0.1	0.1	0.9	0.4
Ferulic acid	1.58	0.0	0.9	0.2	1.2	5.51	0.1	0.5	0.4	0.6	11.81	0.0	0.3	0.6	0.6
Sinapic acid	1.53	0.0	0.5	0.3	0.5	5.36	0.1	0.6	0.3	0.5	11.48	0.0	0.3	0.5	1.5
Rutin	1.65	0.0	2.9	0.4	7.4	5.78	0.1	0.5	0.2	1.3	12.38	0.0	0.9	0.3	1.4
Ellagic acid	1.71	0.0	1.1	0.2	7.2	5.99	0.1	0.8	0.2	4.5	12.83	0.0	1.0	0.4	2.0
Myricetin	1.53	0.0	3.1	0.3	6.0	5.36	0.1	1.3	0.3	3.1	11.48	0.0	0.6	0.3	1.8
trans-Resveratrol	1.55	0.0	0.7	0.1	2.3	5.41	0.1	0.4	0.3	0.8	11.59	0.0	0.1	0.4	0.1
Cinnamic acid	1.61	0.0	0.4	0.0	0.3	5.62	0.1	0.2	0.3	0.5	12.04	0.0	0.1	0.3	0.1
Quercetin	1.50	0.0	1.4	0.0	1.1	5.25	0.1	0.9	0.3	1.9	11.25	0.0	0.4	0.3	1.5
Kaempferol	1.70	0.0	1.4	0.3	4.8	5.93	0.1	0.9	0.3	1.4	12.71	0.0	0.4	0.3	1.2

a - n = 5; b - 3 different days n = 15

S1, S2 and S3 are standards at different concentrations; t_R - retention time

Method sensitivity was evaluated by LOD and LOQ determinations, calculated on the basis of the linear regression curves. The LODs were in the range of 0.07–0.49 mg/L for polyphenols (cinnamic and gallic acids) and furans and 0.001–0.046 g/L for the organic acids (oxalic and lactic acids). Given that the LODs and LOQs are considerably low (Table 4.2), it is reasonable to conclude that this method can be used for quantitative analysis in wines. The

LODs results are comparable or lower than those found in the literature (Gomez-Alonso, et al., 2007; Paixão, et al., 2008; Sanza, et al., 2004; Tarola & Giannetti, 2007).

Recovery studies were carried out to determine the accuracy of the method. A wine sample was analysed before and after the addition of different known amounts of organic acids, furans and polyphenols, and recoveries ranged between 80 and 104% for furans and polyphenols and 97–105% for organic acids were found. These results reveal that the matrix composition complexity does not compromise selectivity and sensitivity of the method, allowing the direct analysis of wines.

The method precision (repeatability and reproducibility) was evaluated by the assessment of five successive analyses of standard working solutions, at three different concentrations, by intra- and inter-day (three different days) repetition method. The precision is expressed in terms of the variation (RSD%) of retention times (t_R) and areas obtained for the repeatability and reproducibility tests (Table 4.3). The small variation of t_R (with a maximum of 1.7%) is very important in order to avoid misidentification of peaks in wine samples (Figure 4.2). The area variation is, in general, small but higher for the reproducibility tests, with maxima at 7.4% for phenolics and 9.0% for organic acids as summarized in Table 4.3.

4.3.2 *Wine sample analysis*

In order to test the developed methodology in red, white, rosé and fortified wines, the samples were simply filtered (0.45 µm) and diluted, when necessary, to apply to the constructed calibration curves. For the purpose of this study, quantified results slightly below the previous validated working range were confirmed by increasing the injection volume. The obtained results are summarized in Table 4.4.

Regarding the organic acid analysis, the attained results vary from 0.055 to 6.273 g/L in fortified wines for oxalic and lactic acids, 0.063 to 9.839 g/L in red wines for succinic and lactic acids, 0.043 to 3.118 g/L in white wines and 0.031 to 3.642 g/L in rosé wine, for oxalic and malic acids, respectively. As can be shown, the concentration of organic acids found in wines varies significantly between wine type and also from one sample to another, suggesting that it is strongly dependent on wine nature and therefore on the vinification process applied. Cunha et al. (2002) and Esteves et al. (2004) also report variable concentrations when they analysed tartaric, malic, lactic, succinic and acetic acids in fortified wines, with values between 0.219 and 1.442 g/L and between 0.041 and 2.752 g/L, respectively. The same result was obtained by Villiers et al. (2003) when determining the same compounds in red and white wines.

Polyphenols in fortified wines ranged between 0.53 and 6.13 mg/L, between 0.46 and 37.26 mg/L in red wines, between 0.43 and 16.12 mg/L in white wines and between 0.38 and 11.64 mg/L in the rosé wine. These values are in the range of the amounts found in other red (Castellari, et al., 2002; Monagas, et al., 2005; Silva, et al., 2005; Tarola & Giannetti, 2007), white (Castellari, et al., 2002; Darias-Martín, Andrés-Lacueva, Díaz-Romero, & Lamuela-Raventós, 2008) and fortified (Ho, Hogg, & Silva, 1999) wine varieties, showing that the results obtained in this study are acceptable and coherent.

TABLE 4.4 – Concentrations found in the studied wine varieties by application of the developed method

Wines	F1		F2		F3		F4		R1		R2		R3		R4		W1		W2		W3		W4		Rs	
Compound	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**	**Cc**	**RSD %**
g/L																										
Oxalic acid	0.061	0.8	0.055	6.0	n.d.		0.066	3.7	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.043	1.0	0.031	0.6
Tartaric acid	1.726	0.1	1.613	0.3	2.193	0.1	2.734	0.4	1.704	0.5	0.545	0.3	2.422	0.7	1.682	2.0	1.274	1.0	1.508	0.4	1.328	0.8	1.681	0.4	0.725	2.4
Formic acid	0.246	3.6	0.449	1.5	0.372	1.5	0.363	2.4	0.346	5.4	0.452	3.8	0.404	0.6	0.431	1.9	0.514	3.4	0.508	3.3	0.440	4.2	0.595	2.9	0.417	3.5
Malic acid	3.551	0.5	3.506	2.3	2.652	1.1	0.765	6.1	0.351	5.4	0.373	3.6	0.252	4.7	0.249	1.2	3.118	2.7	1.859	3.6	2.256	1.9	2.845	1.2	3.642	0.2
Lactic acid	1.070	4.4	1.469	5.3	2.102	5.5	6.273	3.5	7.406	3.7	6.248	5.0	9.839	1.9	7.562	2.6	n.d.		1.331	6.6	1.175	6.3	1.424	3.3	0.821	6.0
Acetic acid	0.666	3.3	1.627	5.0	0.974	3.4	1.734	8.4	1.512	0.4	1.599	1.9	2.207	0.4	2.090	2.5	0.826	2.8	0.871	4.3	0.929	5.2	0.956	2.1	1.067	0.4
Citric acid	n.d.		n.d.		0.319	1.6	n.d.		0.421	2.7	n.d.		n.d.		0.509	3.3	0.236	0.7	0.291	0.5	0.333	0.4	0.362	1.3	0.300	2.3
Succinic acid	n.d.		n.d.		n.d.		n.d.		n.d.		0.063	1.2	n.d.		0.071	3.8	0.124	2.1	0.152	1.8	0.109	1.2	0.131	4.0	0.137	0.6
mg/L																										
Gallic acid	5.18	0.5	6.13	0.6	5.82	0.1	5.96	0.3	31.94	0.2	24.07	0.5	17.57	4.8	37.26	1.5	7.42	0.3	6.51	0.2	1.84	0.6	2.59	4.3	11.64	0.2
HMF	15.47	0.2	338.76	0.1	8.71	0.5	118.96	0.4	1.11	11.4	n.q.		n.q.		n.q.		0.84	0.2	n.q.		1.76	0.3	n.q.		n.q.	
Furfural	1.61	0.5	10.40	0.4	2.01	0.4	8.75	0.5	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Protocatechuic acid	2.67	2.5	3.46	0.2	3.80	0.8	4.66	2.9	2.95	7.9	2.89	7.4	n.d.		3.32	2.7	2.67	1.1	2.78	0.4	2.25	0.4	1.83	0.2	2.84	4.3
Gentisic acid	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		1.45	5.2	1.29	0.6	n.d.		1.26	0.5	n.d.	
p-Hydroxybenzoic acid	n.d.		1.61	0.6	1.83	2.2	3.36	2.2	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
(-)-Epigallocatechin	1.05	1.5	1.41	0.1	1.31	2.6	1.89	2.1	4.53	7.6	3.42	8.0	7.76	1.8	3.45	3.7	1.51	0.9	n.q.		0.95	1.2	n.q.		1.95	0.5
(+)-Catechin	n.d.		n.d.		0.90	5.8	0.76	7.9	13.68	2.0	13.59	0.3	17.32	0.3	8.88	2.8	5.18	2.1	16.12	0.1	7.40	0.4	7.47	0.1	8.22	1.3
Vanillic acid	n.d.		0.76	0.7	0.76	4.9	n.d.		5.40	4.1	6.23	0.6	5.16	0.5	8.92	2.7	0.75	1.1	n.d.		1.37	2.2	n.d.		1.71	1.1
Caffeic acid	1.95	0.5	1.34	0.2	2.53	2.3	0.76	3.2	3.34	1.0	1.41	2.8	3.03	0.5	4.00	0.2	4.36	0.6	2.48	0.2	5.62	0.2	2.38	0.2	3.15	0.2
Syringic acid	4.62	0.8	0.70	1.8	0.53	2.6	1.00	1.2	6.14	3.8	5.35	1.3	1.34	1.9	7.59	5.0	n.d.		n.d.		n.d.		n.d.		1.33	2.0
(-)-Epicatechin	n.d.		n.d.		n.d.		n.d.		3.57	8.6	7.33	3.2	7.63	1.9	3.15	1.1	1.47	1.3	10.16	0.4	2.97	3.7	3.73	0.3	3.03	0.6
Vanillin	n.d.		0.72	1.7	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Syringaldehyde	1.69	1.0	2.95	1.0	2.49	4.4	1.91	2.9	n.d.		n.d.		n.d.		2.42	2.4	n.d.		n.d.		n.d.		n.d.		n.d.	
p-Coumaric acid	1.23	0.5	1.64	4.3	1.29	3.3	1.04	3.2	2.59	2.2	0.89	1.7	1.83	1.4	1.22	3.2	1.90	0.2	2.05	0.1	2.27	0.3	2.24	0.2	2.08	0.5
Ferulic acid	n.q.		n.q.		n.q.		n.q.		n.q.		n.d.		0.61	6.7	n.d.		0.64	0.7	n.q.		0.86	0.5	n.q.		n.q.	
Sinapic acid	1.76	2.2	1.16	2.8	2.27	0.4	1.32	1.5	2.79	3.0	3.09	4.6	4.86	7.6	n.d.		1.44	0.6	n.q.		0.61	1.5	0.64	0.7	4.08	0.8
Rutin	n.d.		n.d.		n.d.		n.d.		1.86	10.5	n.d.		n.d.		n.q.		n.d.		n.d.		n.d.		n.d.		n.d.	
Ellagic acid	3.05	2.7	2.33	0.7	2.42	1.1	3.52	1.0	14.67	1.2	4.64	0.1	5.28	0.6	7.94	0.8	n.q.		n.d.		n.q.		n.d.		n.d.	
Myricetin	n.d.		n.d.		n.d.		n.d.		2.46	5.2	0.82	4.7	1.71	3.1	1.48	4.5	n.q.		n.d.		n.d.		n.d.		n.q.	
trans-Resveratrol	n.d.		n.d.		n.d.		n.d.		0.70	5.3	1.28	0.4	1.05	7.1	0.84	0.9	n.q.		0.49	1.1	n.q.		n.q.		0.82	1.1
Cinnamic acid	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Quercetin	n.d.		n.d.		n.d.		n.d.		3.37	0.4	1.29	2.4	3.61	1.1	2.58	0.2	n.d.		n.d.		0.44	2.1	0.43	2.0	0.38	3.8
Kaempferol	n.d.		n.d.		n.d.		n.d.		0.72	1.0	n.q.		0.69	2.0	0.46	1.3	n.d.		n.d.		n.d.		n.d.		n.d.	

Cc - concentration; n.d. - not detected, lower than LOD; n.q. - not quantified, lower than LOQ

In addition, furans were also determined as they are present mainly in fortified wines, showing maximum results of 338.76 and 10.40 mg/L for HMF and furfural, respectively. As similar results were obtained by Ho et al. (1999), the above application demonstrates the effectiveness of the developed method for the determination of these compounds in fortified wines.

4.4 Conclusions

A simple and rapid method was developed for the sequential determination of organic acids, furans and phenolic compounds in different wine matrices by HPLC technology. This method combines sensitivity with time-effectiveness and was successfully used to measure and assess the polyphenolic fingerprint and organic acids profile of red, white, rosé and fortified wines. The determination of two furanic compounds, HMF and furfural, frequently detected in fortified wines, was also performed by the present method. Furthermore, the methodology provides the potential to analyse wine samples in a single chromatographic column and avoiding tedious and time consuming sample preparation procedures. Therefore, 22 of the most common phenolic compounds and furans in wines were separated in 60 min and eight organic acids in 12 min, allowing simultaneous quality control analysis. The methodology can be extended to the determination of other wine polyphenols if additional calibrating standards are used.

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PART 3

Impact of the *estufagem* process on the Madeira wine composition

CHAPTER 5

Evolution of 5-hydroxymethylfurfural and furfural in Madeira wines submitted to *estufagem*

This chapter is based on the following publication:

Evolution of 5-hydroxymethylfurfural (HMF) and furfural (F) in fortified wines submitted to overheating conditions

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Abstract

As furfural (F) and 5-hydroxymethylfurfural (HMF) are essentially formed from sugars dehydration, especially in food submitted to heat, they can be found in beverages, as well as in fortified sweet wines. In order to assess the impact of temperature on Madeira winemaking, three traditional varieties of Madeira wines (*Malvasia*, *Sercial* and *Tinta Negra Mole*) were studied to evaluate the F and HMF content. The wines were produced by two vinification processes, following traditional and modern methodologies, heated at standard conditions (30 °C and 45 °C, for 4 months) and compared with the same wines submitted to overheating conditions (55 °C, for 4 months). The RP-HPLC-DAD methodology used for the control of F and HMF during the process showed no significant changes in the wines maintained at 30 °C (*canteiro*) and a noticeable but controlled increase in the wines heated at 45 °C (*estufagem*) where values up to about 150 mg/L of HMF can be found in sweet wines. The strong relation of this compound with the sugar content and baking temperature standed out in the wines submitted to overheating conditions, where values higher that 1 g/L could be found for sweeter wines, with HMF level being in general higher than F.

The results clearly suggest that the amount of HMF in these fortified wines can be easily controlled when submitted to adequate conditions of heating during *estufagem* and storage. Furthermore, different temperatures for the baking of sweet and dry wines may be considered.

5.1 Introduction

As already mentioned, Madeira fortified wines hold alcoholic strengths between 17 and 22% (v/v) and sweetness levels ranging from 0 (dry) up to about 130 g/L (sweet). *Malvasia* and *Sercial* grapes are two of the traditional white varieties used for the preparation of high quality sweet and dry wines, respectively and *Tinta Negra Mole* is a red grape versatile variety, used for the production of different types of Madeira. Sweet wines, traditionally not fermented, are currently obtained by a partial fermentation, in order to ensure 4% of alcohol exclusively derived from alcoholic fermentation, maintaining the high content of residual sugars. In contrast, Madeira dry wines can be completely fermented to sugar levels close to 0 g/L (traditional method) or be fermented to low sugar levels (less than 1.5 °Be). Modern vinification techniques, following recent studies carried out to improve the typicity characteristics (Oliveira e Silva et al., 2008), have been introduced with the purpose of stabilizing the total sugar content in sweet wines to about 80 g/L and maintaining some residual sugars in dry wines. When the required sweetness level is attained the fermentation is stopped by the addition of a natural grape spirit (containing 95% (v/v) of ethanol). Then, two ageing processes can be followed: the *canteiro*, usually applied to the finest wines, namely those produced from *Malvasia* and *Sercial* grapes, where the wines are maintained under mild heating storage conditions (heating rooms not exceeding 30 °C); and the *estufagem*, where the wines are heated to about 45 °C for 3 months. The *Tinta Negra Mole* red variety, the most prolific variety in Madeira, used for the production of wines with different sweetness, is usually submitted to the practice of *estufagem* before undergoing a normal maturation

process in oak casks, for a minimum period of 3 years. During the heating stage, a premature ageing process occurs, originating the typical colour and bouquet of these wines and contributing to their exceptional longevity.

The current concern with the alimentary quality increases the necessity of using chemical markers, which evaluate possible damages in the foodstuffs submitted to overheating and drawn out storage. The heating process can be used advantageously to preserve foods, destroying the spoilage organisms, but holding back the nutritional and organoleptic properties. In the case of Madeira wines, the heating process, used in the preparation of these wines since the 18th century, is generally associated with the toasted aroma and typical brownish colour. Among the aromas formed during this period (Oliveira e Silva, et al., 2008), the current study has focused its attention on the formation of two furanic compounds, furfural (F) and 5-hydroxymethylfurfural (HMF). These are the main degradation products of carbohydrates and their occurrence in foods is generally related to non-enzymatic browning reactions, namely Maillard type reactions (MR), sugar degradation in acid medium and caramelization (Antonelli, Chinnici, & Masino, 2004; Granados, Mir, Serrana, & Martinez, 1996). Indeed, they are currently used as heat-treatment markers of foods.

In acidic medium, the heating of pentoses and hexoses originates F and HMF, respectively, after a slow enolization and a fast *β*-elimination of three water molecules (Belitz, Grosch, & Schieberle, 2009). Indeed, the acid-catalysed degradation mechanism of fructose and glucose produces in a first step 1,2- or/and 2,3-enediolic intermediates, which rapidly eliminates water molecules before producing HMF (see Figure 5.1).

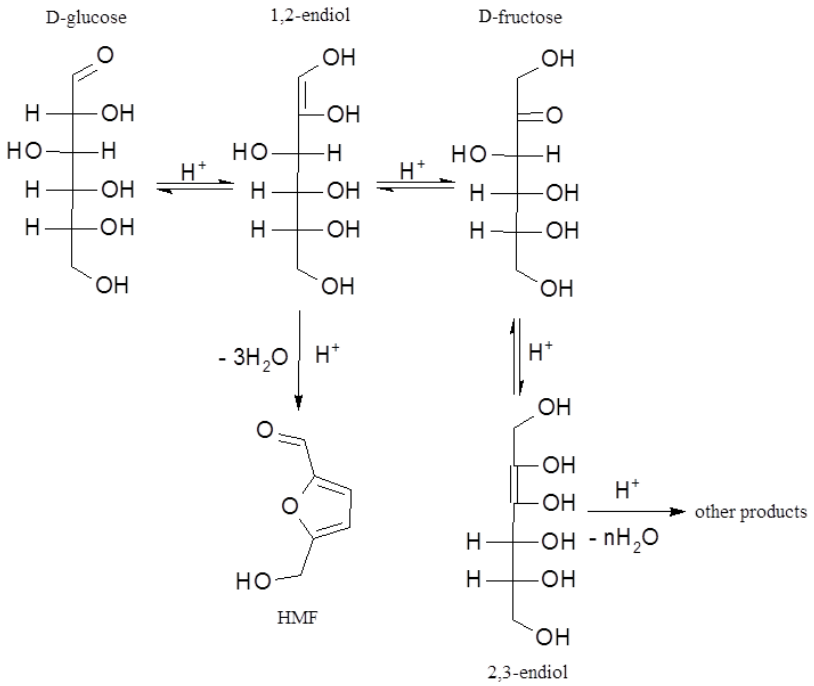

FIGURE 5.1 – HMF formation pathway by sugar acid-catalysed dehydration (adapted from Antonelli et al. (2004)).

At wine pH (about 3.5) the formation route for F and HMF in Madeira wines can be explained almost entirely by acid-catalyzed sugar degradation, since Maillard chemistry is not favoured in the acidic media. The analytical control of F and HMF has received some importance and its occurrence has been reported in several food products, including fruit juices (Gökmen & Acar, 1999), beers (Lo Coco, Valentini, Novelli, & Ceccon, 1995), brandies (Granados, et al., 1996) and fortified wines (Cutzach, Chatonnet, & Dubourdieu, 1999; Ho, Hogg, & Silva, 1999). From a safety perspective and for food quality assurance, HMF legal limits were already issued for some foodstuffs, namely for concentrated rectified grape must: EC Regulation No. 1493/99 sets up a limit of 25 ppm (Falcone, Tagliazucchi, Verzelloni, & Giudici, 2010). The F content is also useful as an off-flavour indicator and HMF is frequently correlated with browning reactions (Lo Coco, et al., 1995).

Being essentially considered as indicators of overheated foodstuff, the presence of HMF and F in foods has raised some toxicological concerns in recent years. Some authors considered that they are natural components of traditional foods, posing no risk to human health (Adams et al., 1997; Janzowski, Glaab, Samimi, Schlatter, & Eisenbrand, 2000), while others say that HMF can be poisonous to the nervous system due to accumulation in the body when combined with proteins, eventually causing damages in the muscles and viscera (Li & Lu, 2005). HMF derivatives, such as 5-chloromethyl- and 5-sulfoxymethylfurfural (SMF) have been associated with cytotoxic, genotoxic, and tumoral effects (Nassberger, 1990; Surh, Liem, Miller, & Tannenbaum, 1994; Zhang et al., 1993). In recent studies, especial attention has been given to HMF-related carcinogenicity (Durling, Busk, & Hellman, 2009; Monien, Frank, Seidel, & Glatt, 2009).

The growing attention of the scientific community towards the potentially toxic effects of HMF and F has triggered the current interest on the formation of these compounds in Madeira wines, especially because sweet wines have a rather high content of carbohydrates and are submitted to a quite long heating process (at least 3 months).

The study was focused on their determination in wines with different sweetness levels, produced under diverse fermentation and heating conditions, in order to simulate different ageing processes. To do so, three traditional varieties of Madeira wines, *Malvasia*, *Sercial* and *Tinta Negra Mole*, were produced by two different vinification processes and heated under overheating conditions (at 55 °C for 4 months), and compared with wines submitted to standard heating conditions (30 and 45 °C). F e HMF levels were determined by direct RP-HPLC-DAD analysis of the wines under study.

5.2 Experimental

5.2.1 *Standards and reagents*

HMF and F analytical standard-grade (both with assay > 98%) were obtained from Acros Organics (Geel, Belgium). *D*-fructose and *D-(+)*-glucose were supplied by Himedia (Mumbai, India) with assays higher than 99%. The hydroalcoholic solutions were prepared with

ethanol (96%) from Sigma–Aldrich (St. Louis, MO, USA) and ultra-pure water (Milli-Q System, Millipore, Bedford, MA, USA). The chromatographic mobile phases were prepared with ultra-pure water, methanol HPLC grade (Sigma–Aldrich, St. Louis, MO, USA) and acetic acid (JMGS, Portugal, > 99%). All solvents used were previously filtered through 0.45 µm membranes from Pall Corporation (Ann Arbor, MI, USA) to remove any impurities.

5.2.2 *Wines*

Traditionally, the vinification process of sweet Madeira wines is often characterized by short fermentative steps or even by its absence, originating wines with high sugar levels, whereas dry Madeira wines are often completely fermented (traditional methods). Nowadays, there is a tendency to extend the fermentation of sweet wines (lowering the amount of residual sugars) and shorten the fermentation of dry wines (modern methods).

For the purpose of the present study, about 600 L of must were obtained from *Malvasia* grapes (2003 harvest) and equal amounts were fermented according to different methods: traditional and modern. One was almost not fermented, the *Malvasia* traditional wine (*M*t), containing 125 g/L of residual sugars. The other one was slightly fermented (4 days at 21 °C), getting a sugar level of about 78 g/L, denominated as *Malvasia* modern wine (*M*m). The same procedure was applied to produce sweet wines from *Tinta Negra Mole* grapes: *Tinta Negra Mole* modern sweet (*T*mS) and *Tinta Negra Mole* traditional sweet (*T*tS). Two *Sercial* wines (equal amounts) were produced from 600 L of must. One was fermented until complete transformation of sugars (*Sercial* traditional, *S*t). The other was fermented maintaining a low level of residual sugars (*Sercial* modern, *S*m). Similarly, *Tinta Negra Mole* was used for the production of two dry wines: *Tinta Negra Mole* modern dry (*T*mD) and *Tinta Negra Mole* traditional dry (*T*tD). All wines were industrially elaborated in stainless steel tanks of local Madeira wine-producing cellars and the alcoholic fermentation was carried out by indigenous yeast under controlled temperature, while malolactic fermentation was not encouraged. Sulphite was added to musts up to 150 mg/L. After vinification, all wines were placed in stainless steel vats and heated at three different temperatures, 30, 45 and 55 °C, for 4 months. For the purposes of the study, *Sercial* and *Malvasia* musts were also processed by the modern methods to obtain a sweet (*Sercial* modern sweet, *S*mS) and a dry wine (*Malvasia* modern dry, *M*mD), respectively, being heated at 45 °C during the same period. As *Malvasia* is almost exclusively used for sweet wines and *Sercial* for dry wines, this experiment was carried out in order to allow a comparison between both varieties when submitted to the same fermentation and heating processes.

Considering that the optimum temperature during *estufagem* is 45 °C (Oliveira e Silva, et al., 2008), the wines under study were also submitted to 55 °C (overheating temperature). This temperature was considered high enough to produce significant differences relative to 45 °C, but not so high to promote the appearance of organoleptic defects. Table 5.1 briefly displays data on the experiment.

The baking step was carried out in a special pilot scale system equipped with 200 L stainless steel vats (Figure 5.2), designed for careful and independent control of temperature

by the circulation of hot raw water. The temperature in each vat was continuously monitored and electronically adjusted with deviations less than 2 °C during the entire experimental period. The system included 10 vats with a similar design to the industrial large vats and was controlled by a PlantWatch software system supplied by CAREL (Padova, Italy).

FIGURE 5.2 – Laboratory *Estufas* used for heating the current Madeira wines.

TABLE 5.1 – Characteristics of the studied wines submitted to the baking step at 30, 45 and 55 °C.

Grape variety	Method	Abbreviation	Conditions of fermentation	Sugar content (g/L)
Malvasia	Modern Sweet	*M*m	Alcohol is added when density reaches 1050 g/cm^3	78
	Traditional Sweet	*M*t	Alcohol is added after the beginning of the fermentation	125
	Modern Dry*	*M*mD	Alcohol is added when the density reaches 1000 g/cm^3	---
Sercial	Modern Dry	*S*m	Alcohol is added when the density reaches 1000 g/cm^3	16
	Traditional Dry	*S*t	Alcohol addition after complete fermentation	0
	Modern Sweet*	*S*mS	Alcohol is added when the density reaches 1050 g/cm^3	---
Tinta Negra Mole	Modern Sweet	*T*mS	Alcohol is added when the density reaches 1050 g/cm^3)	92
	Traditional Sweet	*T*tS	Alcohol is added after the beginning of the fermentation	110
	Modern Dry	*T*mD	Alcohol is added when the density reaches 1000 g/cm^3	3
	Traditional Dry	*T*tD	Alcohol addition after complete fermentation	6

*only heated at 45ºC

The wine samples were collected (about 75 cL) every 30 days and stored at -20 °C before analysis. The determination of the basic chemical parameters including the alcoholic strength, pH and reducing sugars content of the wines in study were determined. The alcoholic strength by volume was carried out according to the usual method of the OIV procedures (OIV, 2000). The pH was also determined according to OIV standard procedure (OIV, 2000) while reducing sugars were determined according to the titration method of Lane-Eynon, as described in the Portuguese Official Standards (NP) for Spirits and Alcoholic Beverages (NP2223).

5.2.3 *Chromatographic analysis*

All samples were analysed by direct injection on a Waters HPLC system equipped with a Waters 1525 Binary HPLC Pump, a Waters 996 DAD and a Waters 717 Plus Autosampler. A Millenium chromatography manager software, version 3.2, was used for data acquisition. Furanic compounds were separated on a Waters 150 mm × 3.9 mm i.d., 4 µm Nova-Pak C_{18} column. The analysis was carried out using an eluent A composed by water-acetic acid-methanol (80:2:18) and an eluent B prepared with the same solvents though comprising the following composition 8:2:90. Gradient elution program was: 6 min at 100% A, to 20% A in 4 min, 5 min at 20% A, to 100% A in 3 min and maintenance at 100% A during 5 min. The flow rate was adjusted to 0.60 mL/min and the injected volume was 10 µL. The DAD was operated with a resolution of 1.2 nm in the wavelength range of 240 – 390 nm. The analytes were detected at 280 nm and identified by superimposing the spectra of each peak with the corresponding spectra of the standards and by comparison of their retention times. Each sample was analysed in triplicate.

5.2.4 *Validation and quantification*

Quantification was established by means of an external calibration curve. Analytical parameters of the validated methodology are summarised in Table 5.2. Standard solutions of HMF and F (1 g/L in methanol) were prepared, from which mixtures at different concentrations were made in the range of 2.5 – 75.0 mg/L, by dilution in ultra-pure water. The curves (five data points, n = 3) were linear with r^2 values higher than 0.999. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as follows: $3.3\sigma/b$ and $10\sigma/b$, respectively, where σ is the y-intercept standard deviation and b is the slope of the linear regression. The obtained LOD values were 1.22 mg/L for both analytes. The method reproducibility and recovery were checked. A RSD of 0.09% for HMF and 0.15% for F, and recoveries above 99% were obtained, when 5 replicates of a *Tinta Negra Mole* modern dry sample, spiked with 50 mg/L of HMF after heating at 55 °C, were injected.

5.2.5 *Statistics*

All determinations were carried out in triplicate and results were expressed as the mean value ± standard deviation. Significant differences between wines along heating and the initial state were assessed with analysis of variance (One-way ANOVA with Holm-Sidak Post Hoc test), using the statistical software SigmaPlot 11.0 for Windows.

5.3 Results and discussion

The initial alcoholic strength of both *Malvasia* wines was similar, about 17.0% (v/v) for *Malvasia* traditional wine and 17.5% (v/v) for *Malvasia* modern wine. It remained almost constant during the period of the experience. This behaviour was expected as the amount of samples taken was small compared to the total volume in the stainless steel vats, in which the evaporation processes is not significant. Analogous results (17% (v/v)) were observed for

Sercial and *Tinta Negra Mole* wines. The initial pH ranged between 3.41 and 3.57 and showed a small increase with the baking time (about 0.05, after 4 months) but independent of the heating temperature. The total amount of carbohydrates in the studied Madeira wines was also evaluated and initial values are presented in Table 5.1. Accordingly, *Malvasia* traditional was the sweetest wine and *Sercial* traditional the driest one, as expected due to their specific fermentation conditions and time of fortification.

After the implementation of the conditions described in 5.2.3 it was evidenced that HMF and F eluted after 3.0 and 4.3 min of the analysis, respectively. The validation procedure and the obtained parameters (Table 5.2) showed that the method was adequate for quantification purposes and could be used to evaluate the F and HMF content during the baking of the Madeira wines under study. The advantage of the applied RP-HPLC-DAD method was that no additional clean-up methodology was necessary.

TABLE 5.2 – Analytical parameters of the working method.

		HMF	**F**
Concentration range (mg/L)		2.5 - 75.0	2.5 - 75.0
Linear regression	*a*	20173	-37983
$y=bx + a$	*b*	116779	152773
r^2		0.999	0.999
LOD		1.22	1.22
LOQ		3.68	3.69
RSD (%) (n = 5)		0.09	0.15
Recovery (%) (n = 5)		100	99

As the thermal procedure applied to foodstuff favours the formation of HMF and F, the same can be expected in Madeira wines even if lower temperatures and longer times are used. Ho et al. (1999) determined F and HMF in several fortified wines including a 10 years old *Verdelho* Madeira wine, and the levels found were 8.8 and 361.0 mg/L, respectively. It was concluded that the high value obtained was probably due to the *estufagem* process. However, little was undertaken both to evaluate the real impact of temperature and sweetness on the Madeira winemaking process and to define operating conditions for minimisation. In a recent study (Oliveira e Silva, et al., 2008), it was determined the optimal temperature and baking time to obtain a Madeira wine considered typical by an expert panel, which were 4 months at 45 °C. Furthermore, on the basis of AEDA results it was observed that several volatiles, usually related to Maillard reactions, such as sotolon, F, 5-methylfurfural, 5-ethoximethylfurfural, methional, and phenylacetaldehyde, were identified as common to both *Malvasia* and *Sercial* wines, conferring their typicity. In that study HMF was not identified as a key odorant of Madeira typical wines. Considering that HMF does not improve the characteristics of these wines and can be of some concern when present in higher concentrations in food or beverages, it is important to perform an adequate control and be able to find out the operating conditions for minimizing its levels in these wines.

5.3.1 *Development of the furanic compounds*

The amount of HMF showed a slight increase (sweet wines) or could not be quantified (dry wines) during the baking conducted at 30 °C. It was evidenced final concentrations lower than 12 mg/L. F was found in trace quantities in all wines baked at this temperature. These results suggested that sugar content was not the determinant factor for HMF and F development in Madeira wines. At higher temperatures, 45 and 55 °C, a continuous growth was verified with heating temperature and baking time (Table 5.3). HMF was reported to appear very high in wines submitted to 55 °C (overheating temperature), mostly in sweet wines, such as in the traditional *Malvasia* and *Tinta Negra Mole* sweet, where 1.2 g/L were reached. These results confirmed the high dependence of HMF levels on temperature and time, as is pointed out in different studies. It was evidenced that higher values were obtained for the sweetest wines, particularly when processed by the traditional method, where the sugar content was higher.

The *estufagem* at standard procedures, up to 45 °C, did not promote HMF levels higher than 150 mg/L, even for non-fermented musts where the content in residual sugars remained high. So, the obtained results indicated that the formation of HMF can be controlled during *estufagem* if the temperature is carefully adjusted and maintained below 45 °C. At higher temperatures the increase of HMF formation in sweet wines was very important, attaining an amount 10 times higher with a 10 °C increase of the heating temperature. F was also reported to increase during the test, yet important changes were only detected at overheating conditions (55 °C), with slight variations observed between the two vinification procedures when the resulting wines were heated at 45 °C (e.g. 8.47 and 5.82 mg/L for *Malvasia* modern and traditional wines, respectively). At overheating conditions, F reached in average 19.65 mg/L in sweet wines and 8.98 mg/L in dry ones. *Malvasia* modern wine baked at 55 °C had the lowest reducing sugar level of the sweet wines, but presented the highest level in F, showing that F amount cannot easily correlated with the sweetness of the wine. Under these conditions, the formation of F and HMF in dry wines remained low, with the exception of *Sercial* modern dry wine (189.05 mg/L and 11.71 mg/L, for HMF and F respectively), explained by the highest level of residual sugars between the dry wines under study. Câmara et al. (2004) showed that furanic aldehydes present a linear behaviour with the ageing of Madeira wines undergone in wood casks. The same seems to be valid for the wines submitted to *estufagem* up to 45 °C, with sweet wines showing an important increase at higher temperatures. This temperature was considered following the organoleptic analysis carried out by an expert panel. It was concluded that the typical characteristics of Madeira wines are achieved by *estufagem* whenever samples are baked at 45 °C during 4 months (Oliveira e Silva, et al., 2008). Therefore, the present study also included the comparison with two experimental wines: a dry *Malvasia* and a sweet *Sercial* (both wines are not commercially produced) heated at 45 °C for 4 months. Results showed that *Sercial* sweet wine presented the same behaviour as other wines produced at similar conditions, though HMF evolution was less extended, not exceeding the 67.91 mg/L. This result is consistent with

TABLE 5.3 – HMF and F concentrations (mg/L) found in the studied wines submitted to heating at 30, 45 and 55 °C.

Means±SD			HMF			F		
			30 ºC	45 ºC	55 ºC	30 ºC	45 ºC	55 ºC
Dry wines	***T*mD**	Initial	tr.	tr.	tr.	n.d.	n.d.	n.d.
		2M	tr.	3.72±0.01*	13.33±0.04*	n.d.	tr.	4.87±0.03*
		4M	**tr.**	**9.02±0.04***	**15.83±0.01***	**n.d.**	**tr.**	**6.85±0.05***
	***T*tD**	Initial	tr.	tr.	tr.	n.d.	n.d.	n.d.
		2M	tr.	4.15±0.02*	23.50±0.12*	n.d.	tr.	5.90±0.04*
		4M	**tr.**	**6.21±0.04***	**42.27±0.19***	**n.d.**	**tr.**	**9.29±0.06***
	***S*m**	Initial	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		2M	5.90±0.01*	21.13±0.16*	83.51±0.09*	tr.	4.19±0.02*	12.56±0.00*
		4M	**6.10±0.04***	**28.78±0.12***	**189.05±0.60***	**tr.**	**7.01±0.07***	**11.71±0.12***
	***S*t**	Initial	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		2M	tr.	5.39±0.01*	14.24±0.05*	n.d.	tr.	5.53±0.03*
		4M	**tr.**	**10.01±0.02***	**19.63±0.27***	**tr.**	**3.82±0.02***	**8.08±0.14***
	***Mm*D**	Initial		tr.			n.d.	
		2M	–	tr.	–	–	tr.	–
		4M		**3.68±0.04***			**tr.**	
Sweet wines	***T*mS**	Initial	5.67±0.03	5.67±0.03	5.67±0.03	tr.	tr.	tr.
		2M	7.63±0.05*	40.06±0.61*	558.05±0.75*	tr.	tr.	11.35±0.03*
		4M	**8.62±0.09***	**95.37±0.16***	**976.32±6.76***	**tr.**	**5.45±0.03***	**18.09±0.06***
	***T*tS**	Initial	5.97±0.22	5.97±0.22	5.97±0.22	n.d.	n.d.	n.d.
		2M	7.63±0.05*	58.00±0.18*	637.97±1.49*	tr.	tr.	11.22±0.14*
		4M	**10.90±0.04***	**141.48±0.29***	**1249.24±0.17***	**tr.**	**4.24±0.06***	**21.29±0.15***
	***M*m**	Initial	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		2M	5.53±0.08*	55.12±0.31*	257.65±0.74*	tr.	3.93±0.03*	8.32±0.07*
		4M	**9.56±0.12***	**136.65±0.08***	**874.23±5.54***	**tr.**	**8.47±0.03***	**22.90±0.36***
	***M*t**	Initial	tr.	tr.	tr.	n.d.	n.d.	n.d.
		2M	2.49±0.08*	39.16±0.09*	354.55±0.14*	tr.	tr.	6.98±0.04*
		4M	**11.54±0.02***	**148.95±0.23***	**1247.80±1.08***	**tr.**	**5.82±0.01***	**16.33±0.15***
	***Sm*S**	Initial		4.11±0.01			n.d.	
		2M	–	29.94±0.03*	–	–	tr.	–
		4M		**67.91±0.05***			**tr.**	

2M - 2 Months of heating
4M - 4 Months of heating
SD - Standard deviation

n.d. – not detected, below LOD
tr.. – tr.ace amounts below LOQ
*p<0.001, significant differences were detected when compared with the initial state

the lower sugar potential of *Sercial* grapes; for this reason is traditionally used for dry wines. In the case of *Malvasia* dry wine, the amount of HMF was rather low 3.68 mg/L, and similar or lower than other dry wines. F amount was not high enough to be quantified in both wines.

The main sugars present in grapes are glucose and fructose (hexoses), usually in similar amounts at harvest time. Although both decrease during fermentation, their ratio in musts depends on the conditions of the process, since glucose is consumed by the great majority of yeasts prior to fructose (Sanz & Martínez-Castro, 2009). Thus, when the fermentation of sweet wines is halted by fortification high amounts in glucose, fructose and others residual sugars are still present. So in this kind of wines the high amounts in HMF can be confirmed by glucose and fructose degradation essentially carried out by acidic dehydration, especially when higher temperatures are used in the winemaking process. F occurrence may indicate the existence of pentoses in these wines. These kind of carbohydrates are not fermentable by yeasts, which may explain the observed formation of F in dry wines heated at higher temperatures. It was also observed that HMF levels were always relatively higher than F, even when wines were completely fermented (traditional dry wines).

To understand which sugar contributes more to the HMF formation, a simple test was carried out: a 18% (v/v) hydroalcoholic solution containing 125 g/L of fructose and other with equal amount of glucose were heated at 50 °C during 75 days. This preliminary test showed that the fructose solution produced 46 times more HMF than the glucose solution, attaining the amount of 226.41 mg/L. This may be due to the fact that fructose naturally exists in higher proportion in the open-chain form than does glucose, and easily dehydrates. Further studies should be conducted taking into account other factors likely to influence sugar degradation, during the *estufagem* of the wines.

5.3.2 *Assessment of the furanic compounds in commercial wines*

The study was also extended to commercially available Madeira wines in order to evaluate F and HMF contents found in the market (from different producers). So it covered not only samples which might be submitted to *estufagem* (most 3-year-old wines) but also those which followed *canteiro* ageing (below 30 °C). Thus, the study analysed 24 samples from dry to sweet wines. Table 5.4 shows the obtained results and evidence points to the fact that commercial wines (under 5 years old) presented relatively low amounts of HMF and F, less than 71 mg/L and 5 mg/L, respectively.

The highest amounts were found in sweet wines but those submitted to *estufagem* (presented in the table without reference to the variety) did not showed significant differences to the wines submitted to *canteiro* ageing (variety indicated in the table). The 10-year-old wines, prepared before current studies were carried out, showed higher amounts of HMF (4 samples with more than 100 mg/L, corresponding to wines submitted to the heating stage before ageing). Even considering that HMF can increase with ageing, the high amounts detected in commercial wines were essentially the result of the initial heating stage, pointing out that its level can be controlled using adequate conditions of *estufagem* (45 °C). This was also confirmed by the lower values obtained in sweet wines aged in casks (*canteiro*).

TABLE 5.4 – HMF and F contents (mg/L) found in commercial Madeira wines.

Commercial samples		**HMF**	**F**
3-years-old	dry 1	27.95±0.31	n.q.
	dry 2	4.80±0.06	n.d.
	medium dry 1	14.36±0.03	n.q.
	medium dry 2	5.85±0.04	n.d.
	medium sweet 1	60.32±0.20	n.q.
	medium sweet 2	6.83±0.30	n.d.
	sweet 1	90.95±0.19	3.82±0.01
	sweet 2	6.71±0.24	n.d.
5-years-old	dry 1 (*Sercial*)	21.95±0.02	n.q.
	dry 2	29.87±0.07	3.97±0.28
	medium dry 1 (*Verdelho*)	30.70±0.29	n.q.
	medium dry 2	36.18±0.05	4.11±0.02
	medium sweet 1 (*Boal*)	38.90±0.20	3.97±0.04
	medium sweet 2	20.45±0.05	n.q.
	sweet 1 (*Malvasia*)	70.83±0.07	4.98±0.06
	sweet 2	39.84±0.07	n.q.
10-years-old	dry 1 (*Sercial*)	40.57±0.16	4.60±0.02
	dry 2	367.39±1.32	8.29±0.01
	medium dry 1 (*Verdelho*)	59.63±0.11	5.40±0.08
	medium dry 2	195.57±0.40	6.87±0.47
	medium sweet 1 (*Boal*)	48.07±0.03	6.65±0.09
	medium sweet 2	491.90±1.72	11.55±0.13
	sweet 1 (*Malvasia*)	150.41±1.11	8.31±0.07
	sweet 2	287.43±2.21	9.77±0.50

n.q.- under LOQ; n.d. - not detected or under LOD

5.4 Conclusions

A validated method was used with success for the evaluation of HMF and F contents in Madeira wines submitted to prolonged heating. The amount of HMF tended to increase with heating and ageing, where important amounts (greater than 1 g/L) were formed in sweet wines submitted to overheating conditions (55 °C), after a 4 month period. The study clearly showed that the amounts of HMF and F formed in sweet wines fermented in order to reduce the amount of residual sugars and baked at temperatures not higher than 45 °C, are under control even for longer ageing periods. On the contrary, dry wines can be fermented in order to maintain a low level of residual sugars, in order to induce the formation of some typical aromas resulting from sugar degradation, being heated up to 45 °C without a significant increase of the final amount of furans.

The heating process known as *estufagem*, used in the production of Madeira wines since 1795, is associated to the bouquet of these fortified wines and may play an important role in their exceptional longevity. Heating conditions can be adjusted in order to maintain

these important characteristics without compromising the final amount of HMF and contributing to improve general quality. The observed tendency to enhance modern wines, resulting from organoleptic analysis and HMF evolution data, clearly suggests the importance of introducing changes in the fermentation process (sweetness) and baking (temperature). The results also showed that dry and sweet wines should not necessarily be heated at the same conditions, with dry wines having lower evolution and supporting higher temperatures. This conclusion can suggest changes in the differentiation of heating conditions applied to different wines, in accordance with the general idea that *Sercial* wines need extended ageing periods for attaining typicity.

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CHAPTER 6

Trend of amino acids and biogenic amines during the Madeira wine heating

This chapter is based on the following publication:

Determination of primary amino acids and biogenic amines in young Madeira wines and their evolution during the traditional heating process

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(Submitted)

Abstract

Giving the role played by amino acids during fermentation, providing nitrogen to yeast and acting as metabolic precursors of higher alcohols, the current chapter presents a study focused on the impact of the subsequent heating, usually followed by Madeira wines. Two grape varieties (one red and one white) were used in the preparation of common sweet and dry wines. The amino acid profiles were obtained during the heating step (45 °C, 3 months) and compared with the profiles of the same wines baked at 70 °C for a month, following a previously described methodology based on a pre-column OPA/MCE derivarization carried out in the injection loop. Data was obtained for 18 amino acids and 4 amines and showed arginine as the most abundant amino acid, presenting a significant reduction during fermentation and heating. A significant decrease of the total amount of amino acids was detected during heating (up to 30% for *Malvasia*), indicating their importance in the formation of ageing products (namely aromas) enhanced by temperature (Stecker degradation). A slight increase of some amino acids, namely asparagine, was detected during the heating step and cysteine was not detected in sweet wines, probably due to the short fermentation. The total concentration of biogenic amines never exceeded 12 mg/L.

6.1 Introduction

Amino acid composition of musts and wines has received special attention in the literature, not only for its importance on fermentative step (as the nitrogen source of yeasts) but essentially for its significant effect on wine flavour development (as metabolic precursors of higher alcohols), as well as for authenticity studies and criteria for differentiation. Without a doubt, is essential to know the amino acid profile of each wine, since their composition varies from one wine to another. As previously pointed out, their composition, and consequently of biogenic amines, depends on the grape variety and technological procedures accomplished, during the entire vinification process, including vineyard practices and some critical winemaking factors and steps.

Little is known about Madeira wine amino acid profile and concentration, in particular, from their evolution during the baking stage. Thus, the main purpose of this work was the determination of amino acids profile of different types of Madeira wines and their behaviour during *estufagem*. Additionally, was also performed the evaluation of amines present in these wines. For this purpose, *Tinta Negra Mole* (*TNM*) dry and sweet wines were heated at 45 °C during 3 months and compared with a sweet wine produced from *Malvasia* variety. For comparison purposes, these wines were also baked at stress conditions, 70 °C during 1 month, to evaluate the temperature effect.

6.2 Experimental

6.2.1 *Wines*

Two *Vitis Vinifera L.* grapes varieties, *Tinta Negra Mole* (red) and *Malvasia* (white), from the 2007 harvest were chosen and the corresponding wines were industrially elaborated in stainless steel tanks of local Madeira wine-producing cellars. The alcoholic fermentation was carried out by indigenous yeast under controlled temperature and malolactic fermentation was not encouraged. Two sweet wines, one from *Malvasia* grapes and other from *TNM* were produced and their fermentation was stopped by the addition of natural grape spirit, when must density attained 1019 and 1025 g/cm^3, remaining 96 and 115 g/L of reducing sugars, respectively. A dry wine from *TNM* grapes was also produced and the fermentation was allowed until the density reached 986 g/cm^3. When all subsequent procedures were accomplished, about 200 L of each wine was then placed in stainless steel vats and heated at 45 °C during 3 months. Additionally, small amounts of the three wines, about 250 mL, were also baked at stress conditions, 70 °C during 1 month. The experiment is schematized in Figure 6.1 and was conducted in a lab oven while the heating at 45 °C was carried out in a special pilot scale system, equipped with 200 L stainless steel vats, designed for careful and independent control of temperature by the circulation of hot raw water.

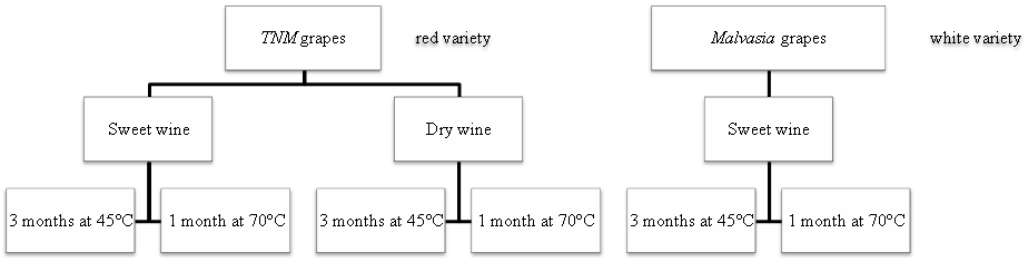

FIGURE 6.1 – Scheme of the conducted experience.

Samples were monthly collected and preserved at -20 °C before being submitted to the analytical control. Some oenological parameters of wines were also determined according to winery current standard procedures, namely reducing sugars content, total and volatile acidity, pH and alcoholic strength.

6.2.2 *Standards and reagents*

The amino acid γ-aminobutyric acid (GABA) was supplied by Fluka BioChemika AG (Buchs, Switzerland) while the others were supplied by Sigma–Aldrich (St. Louis, MO, USA): aspartic acid (Asp), glutamic acid (Glu), cysteine (Cys), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), methionine (Met), tryptophan (Trp), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu) and lysine (Lys). Histamine (Him), tyramine (Tym), phenylethylamine (Phm), isopenthylamine (Ism), and cadaverine (Cad) were from Fluka BioChemika AG (Buchs, Switzerland) while tryptamine (Trm) was from Acros Organics (Geel, Belgium). All standards have a minimum assay of 98%.

Ultra-pure water was obtained from a Milli Q-System (Millipore, Milford, MA, USA) while HPLC-grade methanol was obtained from Sigma–Aldrich (St. Louis, MO, USA). Tetrahydrofuran (99.5%), ethanol (99.9%), sodium hydroxide (98%), sodium phosphate monobasic monohydrate (98%), as well as iodoacetic acid (IDA, 99%) were from Panreac Quimica SA (Barcelona, Spain). The derivatization reagent *o*-phthaldialdehyde (OPA, p.a.) and 2-mercaptoethanol (MCE, 99%) were supplied by Acros Organics (Geel, Belgium). Finally, hydrochloric acid (p.a.) was from Riedel-de Häen (Seelze, Germany) and boric acid (99.5%) from Merck Co. (Darmstadt, Germany).

The OPA/MCE solution was prepared by diluting 50 mg of OPA in 1.50 mL of ethanol and adding 400 mM borate buffer (pH 10.5) up to 10 mL. Finally, after the addition of 200 µL of MCE reagent, the solution settled down for 90 min before use. The IDA solution was prepared by adding 0.583 g of IDA to 10 mL of borate buffer.

6.2.3 *Amino acids and amines determination*

A Waters (Milford, MA, USA) HPLC system, consisting of a separations module with auto-injector (Waters 2695) and a Multi λ Fluorescence detector (Waters 2475), was used for the simultaneous amino acid and amine analysis. The Empower Pro software was used for data storage and integration. The methodology used was based on the previously described (Pereira, Pontes, Câmara, & Marques, 2008) adding an initial step to include cysteine derivatization by carboxymethylation using iodoacetic acid (IDA), as did Pripis-Nicolau and co-workers (2001) in their methodology (see Figure 6.2).

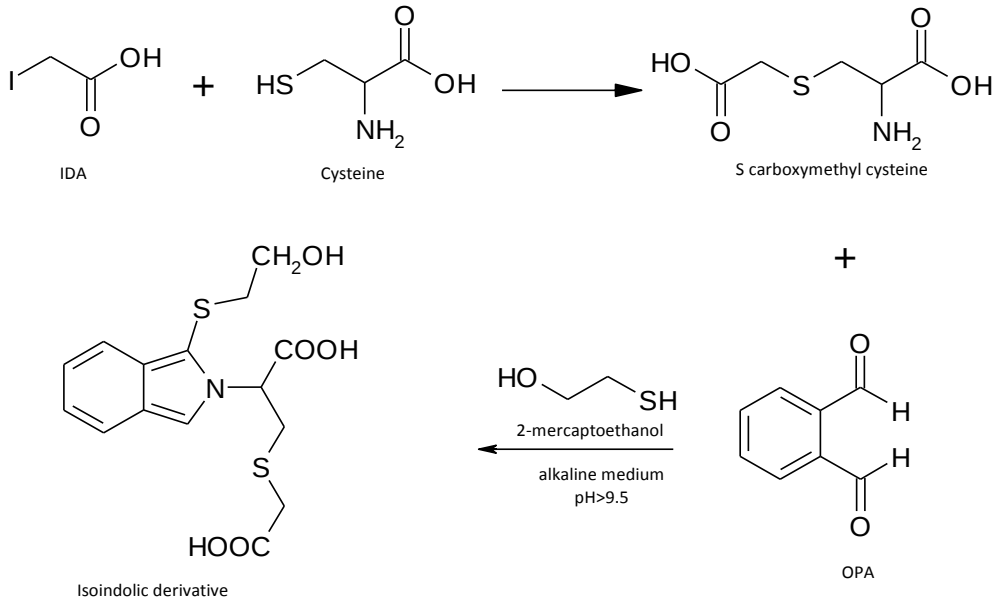

FIGURE 6.2 – Carboxymethylation of cysteine by reaction with iodoacetic acid (IDA) and subsequent reaction with OPA/MCE reagent.

Briefly, the method was based on a pre-column OPA/MCE derivatization procedure carried out in the sample injection loop, according to the following sequence: to 5 µL of filtered (0.45 µm) standard/sample (200 µL of sample/standard are previously diluted in 1.5 mL of borate buffer solution) were added 5 µL of IDA solution and 10 µL of OPA/MCE solution.

This mixture was kept in the injection loop for 2 min to promote the derivatization reaction, before being injected into a Nova-Pak RP-C_{18} column of 3.9 mm i.d. × 150 mm, 4 µm (Waters, Milford, MA, USA), thermostated at 35 °C. The separation of the 20 amino acids and 6 amines was accomplished using a gradient elution with a phase A: 10 mM sodium phosphate buffer (pH 7.3), methanol and tetrahydrofuran (91:8:1); and a phase B: methanol and phosphate buffer (80:20) as follow, with the flow rate set to 1.0 mL/min: 100% A isocratic for 6 min, 85% A in 11 min, 80% A in 8 min, 70% A in the following 8 min, 60% A in 12 min, 20% A in 16 min, 0% A in 6 min, isocratic during 3 min and finally regeneration and equilibrium in the next 10 min. The fluorescence signal was recorded using an excitation wavelength of 335 nm and an emission wavelength of 440 nm. As a consequence of the carboxymethylation step introduction, to derivatize cysteine, Table 6.1 briefly presents the linearity parameters of the current determination.

TABLE 6.1 – Summary of the linearity parameters obtained from the applied method for the determination of the individual amino acids and biogenic amines in Madeira wines submitted to *estufagem*.

			Linearity parameters ($y=ax+b$)				
t_R (min)	**Amino compound**	**Abbrev.**	**Concentration range**	***a***	***b***	**R^2**	**LOD (mg/L)**
2.0	Aspartic acid	Asp	3.01 - 60.18	673980	302623	0.9998	0.45
3.2	Glutamic acid	Glu	3.00 - 60.06	635438	293410	0.9998	0.42
4.9	Cysteine	Cys	3.00 - 60.00	127643	33774	0.9999	0.57
9.4	Asparagine	Asn	3.00 - 60.00	449956	173267	0.9999	0.51
11.5	Serine	Ser	3.01 - 60.24	1036916	827421	0.9994	0.68
14.0	Glutamine	Gln	3.00 - 60.07	182147	456093	0.9994	0.66
14.9	Histidine	His	3.01 - 60.12	324410	218263	0.9998	0.41
17.4	Glycine	Gly	3.00 - 60.06	1629176	337283	0.9999	0.68
19.1	Threonine	Thr	3.00 - 60.06	742561	230380	0.9999	0.49
21.9	Arginine	Arg	3.01 - 120.05	521261	1797214	0.9992	1.17
25.9	Alanine	Ala	3.01 - 60.18	1177658	83468	0.9998	0.51
28.2	γ-aminobutyric acid	GABA	3.00 - 120.24	992290	920153	0.9997	0.87
32.3	Tyrosine	Tyr	3.00 - 60.06	532135	230152	0.9998	0.39
38.5	Unknown	Unk	---	---	---	---	---
44.5	Methionine	Met	3.00 - 60.00	778083	307865	0.9998	0.39
45.7	Tryptophan	Trp	3.02 - 60.48	931939	357251	0.9998	0.40
47.2	Valine	Val	3.02 - 60.36	509248	204224	0.9999	0.40
50.0	Phenylalanine	Phe	3.01 - 60.18	755146	407337	0.9999	0.43
52.5	Isoleucine	Ile	3.01 - 60.18	941243	257488	0.9998	0.41
53.7	Leucine	Leu	3.01 - 60.12	927913	580392	0.9999	0.44
59.6	Lysine	Lys	3.00 - 60.00	177571	-229832	0.9990	2.02
49.3	Histamine	Him	3.00 - 60.00	851424	-284552	0.9996	0.72
59.0	Tyramine	Tym	3.00 - 60.06	897313	-196446	0.9997	0.80
63.6	Tryptamine	Trm	3.04 - 120.48	608809	-1013792	0.9993	1.18
64.7	Phenylethylamine	Phm	3.01 - 120.48	713629	-698822	0.9997	0.79
65.8	Isopentylamine	Ism	2.93 - 117.00	1127761	-1762345	0.9990	1.45
67.1	Cadaverine	Cad	3.13 - 125.28	505228	-3832866	0.9935	3.41

6.2.4 *Statistical analysis*

Regular statistical analysis was performed with Microsoft Office Excel 2007, while Principal Component Analysis (PCA) was carried out with the computational platform MatLab (version 7.6, The Mathworks, Inc.).

6.3 Results and discussion

6.3.1 *Oenological parameters*

Basic oenological parameters of the wine samples in study are presented in Table 6.2. Before the thermal procedure wines pHs were similar (about 3.4) and only irrelevant changes could be observed during the baking step. The same applies to the total acidity but not to the volatile acidity. As observed, the *TNM* dry wine, before baking, showed twice the volatile acidity of the correspondent sweet wine. This parameter increased in sweet wines and decreased in dry wines, tending to similar values after the heating step. The volatile acidity of the current wines never reached the 0.6 g HAc/L, concentration limit above which the sensorial quality of table wines can be modified (Marco, Moreno, & Azpilicueta, 2006). The reducing sugar content was also determined before and after the thermal treatment. The sweet wines differed in 19.41 g/L before the heating step, and after the baking step at standard conditions, the sugar content of *Malvasia* decreased 7.04 g/L, while *TNM* sweet lost 13.52 g/L. In the case of dry wine only decreased 1.07 g/L. These results may indicate that sugars may participate in some reactions namely in Maillard reactions and essentially degrading due to wine acidic conditions, darkening the wines colour.

TABLE 6.2 – Basic oenological parameters of the selected sample set of Madeira wines submitted to *estufagem.*

	0 m	**1 m**	**2 m**	**3 m**
***TNM* sweet**				
Density (g/L)	1025.20	---	---	1024.70
Reducing sugars (g/L)	115.16	---	---	101.64
Alcohol (v/v)	17.5	---	---	17.7
pH	3.52	3.45	3.44	3.50
Volatile acidity (g HAc/L)	0.09	0.12	0.15	0.12
Total acidity (g TarAc/L)	6.53	6.75	6.53	6.53
***TNM* dry**				
Density (g/L)	986.10	---	---	985.00
Reducing sugars (g/L)	3.57	---	---	2.5
Alcohol (v/v)	16.7	---	---	17.7
pH	3.45	3.38	3.36	3.42
Volatile acidity (g HAc/L)	0.21	0.21	0.18	0.15
Total acidity (g TarAc/L)	6.90	7.13	6.90	6.75
Malvasia				
Density (g/L)	1018.68	---	---	1018.18
Reducing sugars (g/L)	95.75	---	---	88.71
Alcohol (v/v)	18.0	---	---	18.1
pH	3.47	3.43	3.47	3.48
Volatile acidity (g HAc/L)	0.09	0.12	0.15	0.18
Total acidity (g TarAc/L)	6.68	6.45	6.38	6.53

HAc – acetic acid; TarAc – tartaric acid

6.3.2 *Amino acids and amines*

The primary amino acids and amines found in the different wine samples at the initial stage (0 m), after 1, 2 and 3 months of baking at 45 °C and after 1 month at 70 °C are exposed in Table 6.3 (A-C). The total concentration of amino acids and amines was calculated as the sum of the concentrations of the individual corresponding compounds. In addition, was also determined the percentage of each compound in samples relative to the total value.

As it can be seen in Table 6.3 (A-C), 18 amino acids were found from the 20 analysed. Arg was always the most abundant amino acid, varying from 23.83 to 355.89 mg/L, followed by Ala (12.84 - 84.29 mg/L) in *TNM* wines and by GABA (8.83 - 37.74 mg/L) in *Malvasia* wine. Similar results were usually obtained by others researchers in white wines (Csomós & Simon-Sarkadi, 2002; Herbert, Cabrita, Ratola, Laureano, & Alves, 2006). Before the thermal procedure, *TNM* sweet wine had the highest content in amino compounds, about 666 mg/L, while the corresponding dry wine had at least 3 times less. This result confirms that extensive fermentations lower the amino acid content. The *Malvasia* wine, also a sweet wine, did not show a similar profile to the other one, only presenting about 225 mg/L. This result may be related with the fermentation process, which was slightly more extensive (lower density, see Table 6.2) and consequently, a higher amino acid consumption was observed, or even, this grape variety already offered a smaller amount of this kind of nitrogen source, associated to the fact that the yeast strain, naturally present in these grapes, could be different. Moreover, this result can justify the versatility of *TNM* variety that is being broadly used for the production of wines with different sweetness levels, whilst *Malvasia* is almost exclusively used for sweet wines.

TABLE 6.3.A – Content of primary amino acids and amines found in ***TNM* sweet** wines at different stages: initial (0 m), after 1, 2 and 3 months of baking at 45 °C and after 1 month at 70 °C. The percentage of each compound in samples relative to the total value is also presented (%).

Amino	**0 m**			**1 m, 45 °C**			**2 m, 45 °C**			**3 m, 45 °C**			**1 m, 70 °C**		
compound	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
Tinta Negra Mole sweet															
Asp	22.09	0.08	3.4	16.71	0.08	3.6	23.38	0.02	4.1	25.40	0.13	4.4	24.38	0.29	6.4
Glu	31.99	0.06	4.9	17.10	0.11	3.7	17.98	0.03	3.1	14.24	0.11	2.5	2.66	0.02	0.7
Cys	n.d			n.d			n.d			n.d			n.d		
Asn	7.12	0.10	1.1	4.78	0.05	1.0	5.64	0.02	1.0	5.19	0.08	0.9	1.90	0.01	0.5
Ser	19.15	0.09	2.9	13.62	0.12	3.0	18.44	0.09	3.2	19.06	0.14	3.3	13.70	0.04	3.6
Gln	n.d.			n.d.			n.d.			n.d.			3.80	0.09	1.0
His	4.04	0.17	0.6	1.65	0.08	0.4	2.50	0.12	0.4	2.06	0.12	0.4	5.76	0.03	1.5
Gly	4.03	0.09	0.6	3.41	0.04	0.7	4.85	0.10	0.8	5.40	0.10	0.9	5.40	0.11	1.4
Thr	26.78	0.10	4.1	18.48	0.09	4.0	23.89	0.28	4.1	24.92	0.26	4.4	14.24	0.03	3.8
Arg	355.89	1.15	54.4	254.40	1.05	55.3	306.48	1.47	53.2	301.79	3.81	52.8	172.41	0.32	45.4
Ala	84.29	0.30	12.9	61.48	0.06	13.4	82.32	0.41	14.3	84.22	0.37	14.7	74.46	0.25	19.6
GABA	37.74	0.55	5.8	25.22	0.18	5.5	33.32	0.80	5.8	32.92	0.79	5.8	16.52	0.23	4.4
Tyr	14.40	0.02	2.2	10.30	0.06	2.2	13.80	0.05	2.4	13.96	0.17	2.4	11.44	0.04	3.0
Unk*	9.77	0.04	1.5	6.61	0.04	1.4	7.73	0.04	1.3	7.19	0.02	1.3	3.32	0.05	0.9
Trp	7.72	0.02	1.2	5.43	0.02	1.2	7.52	0.00	1.3	7.70	0.07	1.3	6.90	0.06	1.8
Phe	7.96	0.07	1.2	5.59	0.03	1.2	7.63	0.04	1.3	7.75	0.08	1.4	5.96	0.04	1.6
Ile	4.52	0.02	0.7	3.17	0.01	0.7	4.45	0.01	0.8	4.55	0.04	0.8	3.87	0.02	1.0
Leu	8.19	0.04	1.3	5.76	0.02	1.3	7.91	0.02	1.4	8.02	0.09	1.4	6.06	0.02	1.6
Lys	8.00	0.11	1.2	6.01	0.19	1.3	7.88	0.22	1.4	7.63	0.13	1.3	6.63	0.16	1.7
TOTAL AA	**653.68**			**459.72**			**575.71**			**572.02**			**379.42**		
Him	0.82	0.00	6.9	n.d.			0.79	0.00	6.7	0.82	0.01	6.8	0.73	0.01	6.2
Phm	1.22	0.00	10.2	1.16	0.01	10.7	1.19	0.02	10.0	1.22	0.01	10.3	1.22	0.00	10.4
Ism	2.15	0.01	18.0	1.92	0.01	17.8	2.08	0.04	17.6	2.05	0.02	17.2	2.00	0.01	17.0
Cad	7.74	0.01	64.9	7.73	0.01	71.5	7.75	0.03	65.6	7.83	0.01	65.7	7.81	0.01	66.4
TOTAL BA	**11.93**			**10.82**			**11.82**			**11.92**			**11.76**		

The results are expressed in mg/L. *- The unknown compound was expressed in Gly equivalents; n.d. - under LOD

TABLE 6.3.B – Content of primary amino acids and amines found in ***TNM* dry** wines at different stages: initial (0 m), after 1, 2 and 3 months of baking at 45 °C and after 1 month at 70 °C. The percentage of each compound in samples relative to the total value is also presented (%).

Amino compound	0 m			1 m, 45 °C			2 m, 45 °C			3 m, 45 °C			1 m, 70 °C		
	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
Tinta Negra Mole dry															
Asp	11.21	0.10	5.9	13.14	0.12	7.5	15.63	0.07	8.9	17.31	0.00	10.5	30.34	0.13	19.8
Glu	21.84	0.03	11.6	16.78	0.16	9.6	14.28	0.02	8.1	11.68	0.00	7.1	2.70	0.01	1.8
Cys	5.74	0.15	3.0	1.44	0.04	0.8	1.38	0.02	0.8	1.19	0.03	0.7	0.77	0.00	0.5
Asn	5.95	0.17	3.2	5.66	0.07	3.3	5.44	0.03	3.1	4.11	0.02	2.5	1.95	0.02	1.3
Ser	4.49	0.03	2.4	4.54	0.06	2.6	5.14	0.11	2.9	5.02	0.03	3.0	5.89	0.05	3.8
Gln	n.d.			n.d.			n.d.			n.d.			n.d.		
His	1.76	0.05	0.9	0.84	0.02	0.5	1.00	0.04	0.6	1.00	0.02	0.6	0.59	0.02	0.4
Gly	7.01	0.03	3.7	6.97	0.09	4.0	7.39	0.08	4.2	7.58	0.08	4.6	7.72	0.01	5.0
Thr	4.93	0.19	2.6	4.82	0.21	2.8	4.94	0.10	2.8	4.86	0.07	2.9	3.22	0.04	2.1
Arg	31.93	0.06	16.9	30.24	0.47	17.4	30.01	0.05	17.1	27.12	0.11	16.4	23.83	0.35	15.6
Ala	28.66	0.05	15.2	27.68	0.34	15.9	28.36	0.13	16.2	27.77	0.08	16.8	28.42	0.13	18.6
GABA	15.81	0.08	8.4	14.53	0.12	8.3	14.38	0.20	8.2	13.60	0.12	8.2	8.97	0.08	5.9
Tyr	6.06	0.06	3.2	5.89	0.06	3.4	6.09	0.08	3.5	5.99	0.02	3.6	6.13	0.01	4.0
Unk*	11.04	0.02	5.9	9.76	0.06	5.6	9.30	0.12	5.3	8.06	0.07	4.9	3.54	0.04	2.3
Trp	4.02	0.01	2.1	3.92	0.04	2.3	4.16	0.03	2.4	3.90	0.01	2.4	4.41	0.02	2.9
Phe	5.75	0.00	3.0	5.68	0.06	3.3	5.88	0.06	3.4	5.59	0.02	3.4	5.65	0.03	3.7
Ile	2.66	0.01	1.4	2.65	0.03	1.5	2.84	0.03	1.6	2.81	0.01	1.7	3.17	0.02	2.1
Leu	8.72	0.02	4.6	8.49	0.08	4.9	8.83	0.06	5.0	8.43	0.05	5.1	8.65	0.04	5.6
Lys	11.09	0.48	5.9	11.05	0.43	6.3	10.53	0.57	6.0	9.52	0.03	5.8	7.19	0.25	4.7
TOTAL AA	**188.66**			**174.08**			**175.58**			**165.55**			**153.13**		
Him	n.d.			n.d.			n.d.			n.d.			n.d.		
Phm	1.21	0.01	11.2	1.18	0.01	11.0	1.16	0.00	10.9	1.12	0.01	10.5	1.08	0.01	10.3
Ism	1.78	0.01	16.5	1.76	0.00	16.4	1.73	0.01	16.2	1.72	0.00	16.2	1.71	0.01	16.2
Cad	7.81	0.01	72.3	7.79	0.01	72.6	7.79	0.01	72.9	7.78	0.01	73.3	7.76	0.01	73.5
TOTAL BA	**10.81**			**10.73**			**10.68**			**10.62**			**10.56**		

The results are expressed in mg/L. *- The unknown compound was expressed in Gly equivalents; n.d. - under LOD

TABLE 6.3.C – Content of primary amino acids and amines found in ***Malvasia*** wines at different stages: initial (0 m), after 1, 2 and 3 months of baking at 45 °C and after 1 month at 70 °C. The percentage of each compound in samples relative to the total value is also presented (%).

Amino compound	0 m			1 m, 45 °C			2 m, 45 °C			3 m, 45 °C			1 m, 70 °C		
	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
Malvasia															
Asp	6.13	0.26	2.7	5.63	0.05	3.0	5.94	0.07	3.4	6.21	0.07	3.9	6.53	0.32	7.3
Glu	12.73	0.07	5.6	9.32	0.06	4.9	7.82	0.08	4.5	6.37	0.08	4.0	2.03	0.02	2.3
Cys	1.33	0.06	0.6	n.d.			n.d.			n.d.			n.d.		
Asn	2.40	0.06	1.1	1.87	0.08	1.0	1.48	0.03	0.8	1.05	0.02	0.7	n.d.		
Ser	5.48	0.13	2.4	5.94	0.08	3.2	5.32	0.07	3.0	5.07	0.06	3.2	3.49	0.03	3.9
Gln	n.d.			n.d.			n.d.			n.d.			n.d.		
His	3.45	0.18	1.5	2.27	0.11	1.2	1.57	0.05	0.9	0.83	0.06	0.5	1.66	0.03	1.9
Gly	3.48	0.09	1.5	3.97	0.02	2.1	4.10	0.08	2.3	4.47	0.05	2.8	3.65	0.08	4.1
Thr	5.91	0.07	2.6	5.31	0.05	2.8	4.85	0.04	2.8	4.22	0.04	2.7	1.46	0.01	1.6
Arg	111.99	3.75	49.7	91.88	2.22	48.7	82.94	1.26	47.5	73.56	0.63	46.5	37.10	0.31	41.6
Ala	17.00	0.20	7.5	16.02	0.09	8.5	16.37	0.22	9.4	15.98	0.12	10.1	12.84	0.15	14.4
GABA	30.84	0.35	13.7	25.41	0.35	13.5	24.74	0.40	14.2	22.55	0.12	14.3	8.83	0.02	9.9
Tyr	1.96	0.05	0.9	1.82	0.03	1.0	1.79	0.03	1.0	1.68	0.02	1.1	1.28	0.02	1.4
Unk*	9.60	0.41	4.3	7.01	0.21	3.7	5.81	0.08	3.3	4.67	0.03	2.9	1.09	0.01	1.2
Trp	2.86	0.04	1.3	2.73	0.01	1.4	2.75	0.04	1.6	2.69	0.04	1.7	2.11	0.11	2.4
Phe	2.69	0.08	1.2	2.44	0.03	1.3	2.43	0.05	1.4	2.28	0.03	1.4	1.65	0.07	1.8
Ile	1.20	0.01	0.5	1.21	0.04	0.6	1.26	0.05	0.7	1.25	0.02	0.8	0.99	0.01	1.1
Leu	3.27	0.06	1.4	2.99	0.07	1.6	2.99	0.11	1.7	2.77	0.04	1.8	1.92	0.07	2.2
Lys	3.15	0.09	1.4	2.74	0.10	1.5	2.51	0.04	1.4	2.57	0.12	1.6	2.59	0.02	2.9
TOTAL AA	**225.48**			**188.57**			**174.66**			**158.22**			**89.20**		
Him	n.d.			n.d.			n.d.			n.d.			n.d.		
Phm	1.05	0.01	10.0	1.05	0.00	10.0	1.05	0.00	9.9	1.05	0.01	10.0	1.03	0.00	9.9
Ism	1.78	0.01	16.9	1.76	0.01	16.6	1.74	0.01	16.5	1.73	0.00	16.5	1.70	0.01	16.2
Cad	7.72	0.02	73.1	7.75	0.00	73.4	7.75	0.00	73.5	7.73	0.00	73.6	7.72	0.00	73.9
TOTAL BA	**10.55**			**10.56**			**10.54**			**10.50**			**10.45**		

The results are expressed in mg/L. *- The unknown compound was expressed in Gly equivalents; n.d. - under LOD

Notice that Arg seems to be the amino acid which consumption, during the alcoholic fermentation, was more pronounced, given that the content in the *TNM* dry was about 11 times smaller than the correspondent sweet wine. The Arg consumption seems to be followed

by Thr and Ser. Amino acid consumption during fermentation is usually associated with formation of aromas, namely alcohols, but others compounds can also be formed. Particularly, Arg consumption may be related with the production of proline, citrulline (precursor of ethyl carbamate) and others compounds like amines. Ser depletion may be associated with Cys formation through condensation with homocysteine formed by yeasts, through sulphide sequestration, during fermentation (Ugliano & Henschke, 2009). Indeed, Cys levels were higher in *TNM* dry wine than in sweet wines (less fermented), indicating that its occurrence may be essentially from Ser decomposition.

Analysing Table 6.3 (A-C) it is also observed that the total amino acid content decreased after 3 months at 45 °C, about 12% for the *TNM* wines and 30% for the *Malvasia* wine. The diminishment is more pronounced when wines were submitted to stress conditions (1 month at 70 °C), falling down up to 60% in the case of the wine made of *Malvasia* grapes. This result indicates that temperature seems to favour the consumption of some amino acids during this stage. Probably their consumption is related with the flavour formation through Strecker degradation, as it was suggested by Escudero et al. (2000) and Marchand et al. (2000). Cys (in average 90%, when present), Gln (51%) and His (56%) revealed an evident decrease, when wines were heated at standard conditions, as well as, when baking was set to 1 month at 70 °C. In this case, the decrease was more expressive and at least 6 amino acids reduce more than 50%. Other amino acids slightly increased during the heating period, especially Asp, Gly and Ile, mostly at stress conditions, wherein Asp raised 3 times more. During the traditional elaboration of sparkling wines the amino acid increase was also observed (Martínez-Rodríguez & Puey, 2009) and this increase was related with the yeast autolysis which occurs during its ageing. Peptides and amino acids are considered the major compounds released into wine during autolysis. In the case of Madeira wine production, the yeast autolysis probably occur before the heating step, but eventually the temperature (especially high temperatures) can promote the hydrolysis of peptides present in wine, and consequently enhance some amino acids. It was also verified that the amino acid evolution with time was not so linear, probably due to the balance between release and consumption.

Regarding amines, from the 6 analysed only 4 (Him, Phm, Ism and Cad) were found in the studied wines. Considering that OIV (*Organisation Internationale de la Vigne et du Vin*) or any other European legislation, including Portugal, did not set any maximum limits for biogenic amines in wines, we chose to follow the suggestion of Leitão et al. (2005), that considered the contents of biogenic amines lower than 8 mg/L, a reasonable starting value for discussion. The concentrations found in wines of His and Phm did not represent any concern, since 2 mg/L were never exceeded. Tym was not found in these wines and Cad amount, usually associated with deficient sanitary conditions, was higher than other amines, but never exceeding the 8 mg/L. The total concentration of biogenic amines of all samples never exceeded the 12 mg/L, which is quite low. This result was expected, since malolactic fermentation is not encouraged in Madeira wine production, as biogenic amine development is usually associated with this type of fermentation. It was observed that amine amounts practically remained constant during the heating period, so that, it seems that temperature and also time did not promote their formation or development. Moreno and Azpilicueta (2004) also showed that natural oak ageing of red wines did not have any influence on the accumulation of biogenic amines.

The obtained results lead us to conclude that these wines appear to be safe from a healthy point of view, since the levels of histamine were very low and tyramine was not found. Additionally, these wines seem to be in adequate sanitary conditions as cadaverine never surpassed the 8 mg/L.

6.3.3 *Principal Components Analysis (PCA)*

The analysis performed on previous section allows us to understand the evolution of a particular wine according to a specific amino acid composition. However, when one looking for comparing all analysed samples to identify evolution trends, understand and explain their differences and similarities, it makes sense use all the measured information instead of using just one of the 22 variables quantified. In this regard, PCA becomes a natural framework for analysing such data, since allows compressing data, retaining essential information, in order to facilitate its visualization and interpretation.

Mathematical description of PCA can be found elsewhere (Jackson, 2004; Jolliffe, 2002; Westerhuis, Gurden, & Smilde, 2000). Briefly, PCA determines underlying information from multivariate raw data, by constructing new variables, known as principal components (PC), which are determined by their ability to account variability. The first component (t_1) is computed as the linear combination of the original *x*-variables (amino acids and biogenic amines composition) with the highest possible variance. The vector defining the linear combination is denoted by (p_1). The second component is defined in the same way, but under the constraint that is uncorrelated with (t_1), and the second direction vector is denoted by (p_2), being orthogonal to (p_1). The process continues until the desired number of components has been determined, in such a way that matrix can be approximated by a product of the first scores and corresponding loadings ($X \approx T \times P$), such data with few variables it is possible to describe a very large proportion of the variability in multivariate data, highlighting the differences among the various samples and determining at the same time which variables are principally involved in the patterns identified.

To carry out PCA, the data matrix of samples (45) by variables (22) was scalded, that is, each variable (relative to a given compound) was centred in its mean and divided by its sample standard deviation. In Figure 6.3, it is represented the biplot of the first two dimensions of PCA model which best approximate the original data, explaining altogether 77% of the total variability present in the original data set. Through biplot representation it is possible to simultaneously analyse how samples cluster together in the reduced PCA subspace, and which variables contribute more significantly for the observed separation patterns. From Figure 6.3, it is possible to identify three different clusters, each one representing a different type of wine (*TNM* dry wines, *TNM* sweet wines and *Malvasia* wines). Across the first PC, the clusters formed appear to be in agreement with wine type, while the second PC contributes to make the distinction among sweet and dry wines. In order to analyse which variables contribute to differentiate these clusters and also, to analyse how variables are related to each other, the loading information was analysed. This analysis could also be carried out from biplot, namely according to the coordinates of the variables, which reveal the weights that each variable has in each principal component. With exception of Cys and Gly, we can conclude that remaining

amino acid and biogenic amines are closely related to the distinction found across the first PC, and correlated positively among them. On the other hand, Cys and Gly correlate positively on the second PC, indicating their importance in sweet and dry wines distinction. Also, GABA, Arg and His are prominent compounds in that differentiation.

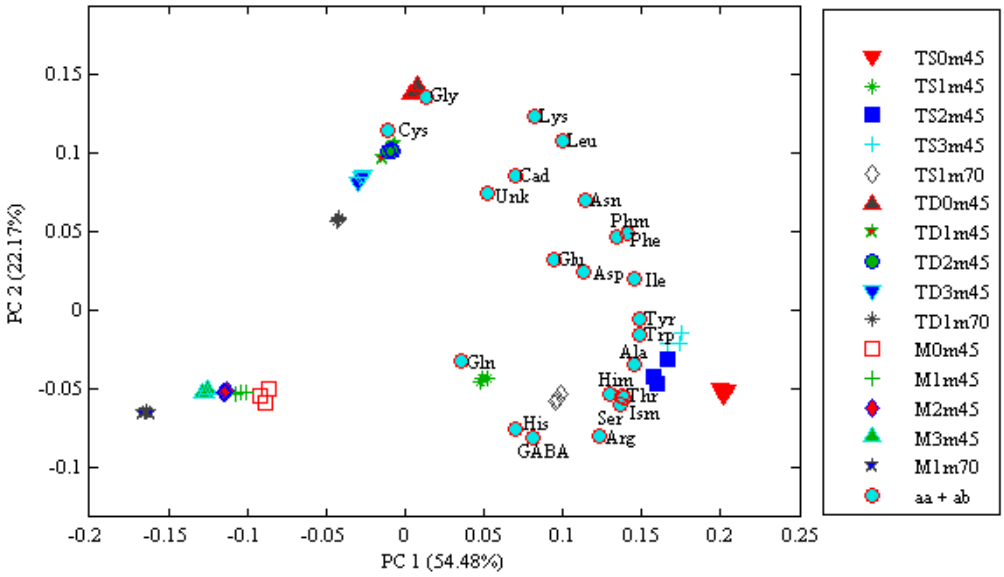

FIGURE 6.3 – Biplot of the first two PC relative to the amino acids profile of the studied Madeira wines submitted to *estufagem.*

The amino acids Arg, GABA, His, Ser, Thr, Ala, Trp, Tyr and Gln and the amines Him and Isn are associated with *TNM* sweet wines group. According to PCA subspace analysis, it can be seen a differentiation according to the fermentation extent, since the same variety produced under different conditions was well differentiated, essentially due to the content of the amino acids Gly, Lys, Cys, Leu, GABA, Arg and His not be the same. Moreover, PCA also enabled distinguish the two varieties despite of being both sweet wines (closer fermentation times). In previous sections, two hypothesis were supposed to explain the amino acid contents of *Malvasia*, namely the higher extent of fermentation process or/and as a consequence of an intrinsic characteristic of this grape variety. The PCA analysis leads us to consider that the latter hypothesis is more likely than the first one, since a similar behaviour regarding sweet *TNM* wines on principal component subspace was expected if the fermentation extent determined the amino acid content in these wines.

Analysing each type of wine separately, it can be recognized a small evolution trend relative to the heating process, developed along PC1. In fact, all clusters identified follow a negative direction in the first PC, with exception of *TS*1m45 which does not seem to fit into this pattern. *TNM* dry wines have also exhibit a clear evolution trend along PC2, once again to the negative side.

6.4 Conclusions

The current study showed some interesting results related with the heating step followed in Madeira wine production, specially related with the decrease of specific amino acids. The *TNM* sweet wine had the higher content of total amino acids, clearly showing the decrease of amino acids during fermentation (initially, *TNM* dry wine had contents about 3 times lower than *TNM* sweet), but surprisingly had a higher content and different profile than sweet *Malvasia*, even if this variety is usually considered having a more complex bouquet. This result can also explain the versatility of *TNM* that is being extensively used for the production of wines with different sweetness, while *Malvasia* is almost exclusively used for sweet wines. The decrease of total amino acids was more pronounced in sweet wines (about 109 mg/L of amino acids were transformed), which can be related with the development of the wine *bouquet* with ageing. This can explain the lower evolution of dry wines during the heating process, showing that the conditions for heating sweet and dry wines are not necessarily the same, as is currently done. This also increases the evidence that most changes during heating are related with Maillard reactions and sugar degradation, involving specific amino acids and controlled by the residual sugars after fermentation time.

Cysteine was not detected in sweet wines, probably as a consequence of the short fermentation period. Dry *TNM* showed a significant lower amount of serine than sweet *TNM*, which can explain the important amount of cysteine in the dry wine. This amino acid decreases during heating processes probably due to its involvement in Maillard and sugar degradation reactions. Arginine was the most abundant amino acid and presented a significant reduction (up to about 35% in *Malvasia*, after 3 months at 45 °C) clearly showing its involvement in the formation of ageing aromas.

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CHAPTER 7

Evolution of the polyphenolic composition, antioxidant potential and colour of Madeira wines during *estufagem*

This chapter is based on the following publication:

Evolution of the polyphenolic composition, antioxidant potential and colour of Madeira wines during its traditional thermal processing

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(Submitted)

Abstract

This chapter is focused on the evolution of the polyphenolic composition, antioxidant potential and colour changes in Madeira wines during the baking step, at 45 °C for 3 months. Three different types of Madeira were studied: a traditional sweet *Malvasia* (white), a sweet and a dry *Tinta Negra Mole* (red). Results obtained at overheating conditions (1 month at 70 °C) were also included. The polyphenolic composition was assessed by determining the total polyphenols (TP), total monomeric anthocyanins (TMA) and total flavonoids (TF), as well as by the non-anthocyanin polyphenols, determined individually by HPLC-DAD. Furfural and 5-hydroxymethylfurfural were assessed due to their sensitivity to heating conditions. Antioxidant potential (AP) was estimated by ABTS, DPPH and FRAP assays, while colour was evaluated using chromatic parameters generated by Glories and CIELab systems.

The results showed that the TP content of the wines slightly decreased during the heating process, varying between 434.42 and 617.10 mg (GAE)/L, which is comparable with most white wines, whilst TMA levels decreased progressively in the wines produced from red grapes. Several polyphenols were found in these wines: 6 hydroxybenzoic acids, 3 hydroxycinnamic acids, 1 stilbene, 3 flavonols and 3 flavan-3-ols. The most abundant phenolics were hydroxycinnamates and hydroxybenzoates, even after baking. Most polyphenols decreased during the heating step, with the exception of caffeic, ferulic, *p*-coumaric, gallic and syringic acids. Finally, both chromatic systems revealed that all wines tend to the same chromatic characteristics when the heating procedure was applied, with white wine turning to brownish colour and red wines becoming clearer, with yellow tones becoming predominant.

7.1 Introduction

Together with aroma and taste, colour is an essential feature in the sensory evaluation criteria of wine quality, influencing wine consumer selection. Polyphenols are main contributors for certain organoleptic characteristics of wines, as astringency and bitterness but in particular colour. In addition, the interest for wine phenolics is still increasing due to their antioxidant and free radical-scavenging proprieties, supported by the health benefits resulting from the moderate wine consumption with respect to cardiovascular diseases, cancer, diabetes, and others (López-Vélez, Martínez-Martínez, & Valle-Ribes, 2003).

Phenolics structure and occurrence in wine is strongly affected by grape variety and vineyard location (soil, climate and sun exposure), vine cultivation practices, ripening stage at harvesting time and vinification techniques, like the fermentation with the grape solids, pressing, sulphite addition, maturation, fining and ageing techniques (Lachman, Šulc, Faitová, & Pivec, 2009). The most important fraction of wine phenolics is firstly removed from grapes during wine vinification, mainly from skins but also from seeds, stems and pulp. Additionally, yeasts and wood-ageing can improve the wine polyphenolic content (López-Vélez, et al., 2003).

In general, white wines possess less polyphenols than red wines, with hydroxycinnamates as the major ones. These compounds, namely the esters of tartaric acid

(caftaric and coutaric acids) together with some flavanols, like *(+)*-catechin and *(-)*-epicatechin, are considered the major oxidation substrates and browning precursors of white wines, to form yellow-brown products due to the polymerisation of *ortho*-quinones (Betes-Saura, Andres-Lacueva, & Lamuela-Raventos, 1996; Kallithraka, Salacha, & Tzourou, 2009). Flavanols react with other flavanols through direct or acetaldehyde- and glyoxylic acid-mediated condensations (Monagas, Gómez-Cordovés, & Bartolomé, 2006). In the case of red wines, free anthocyanins are progressively transformed into more stable oligomeric and polymeric pigments, since the beginning of the vinification and ageing. Indeed, anthocyanins condense with flavanoids (catechins), directly or mediated by acetaldehyde, and with yeast metabolites (essentially pyruvic acid) to form pyranoanthocyanins (Sun, Leandro, de Freitas, & Spranger, 2006). The reactivity of polyphenols increases the complexity due to the variety of new resulting compounds and has an important effect on the sensorial properties of wines, especially on colour due to wine browning, but also in taste and colloidal stability during storage and ageing (Es-Safi, Fulcrand, Cheynier, & Moutounet, 1999).

Besides sensory attributes, antioxidant potential can presumably be affected by the oxidation of polyphenolic compounds in wines that developed non-enzymatic browning. It would be expected that oxidation of phenolics could lead to a lower antioxidant capacity, but this is not necessarily true as novel polyphenolic compounds may be produced.

In recent years, several studies were published concerning the effect of the ageing on colour and phenolic content especially in red wines, including the wood effect (Cadahía, Fernández de Simón, Sanz, Poveda, & Colio, 2009; del Álamo, Nevares, & Cárcel, 2006; Fernández de Simón, Hernández, Cadahía, Dueñas, & Estrella, 2003; Gutiérrez, Lorenzo, & Espinosa, 2005; Revilla & González-SanJosé, 2002) and ageing in bottle (Monagas, Martín-Álvarez, Bartolomé, & Gómez-Cordovés, 2006). In the case of white wines, the attention has been centred in browning due to polyphenolic oxidation, considered as an undesirable occurrence in table wines (Fernandez-Zurbano, Ferreira, Escudero, & Cacho, 1998; Kallithraka, et al., 2009; A. Lopez-Toledano, Mayen, Merida, & Medina, 2004; Lopez-Toledano, Mayen, Merida, & Medina, 2006; Maria-Ioanna, Samatina, & Irini, 2008; Mayén, Barón, Mérida, & Medina, 1997; Peinado, Lopez de Lerma, Moreno, & Peinado, 2009; Sioumis, Kallithraka, Makris, & Kefalas, 2006). In contrast, browning of Madeira wines, made from white and red grapes, is seen as a pleasant phenomenon, associated to quality and typical characteristics. The characteristic taste and complex bouquet together with the browning colour, can be associated to polyphenolic oxidation and eventual sugar degradation through Maillard type reactions, acidic degradation or even caramelization, especially in sweet wines (Li, Guo, & Wang, 2008).

Considering the limited information available, the main objective of the present study was to determine the influence of baking step in the development of some organoleptic characteristics, specifically colour, focused on the polyphenolic composition and the antioxidant potential. The study involved the estimate of colour, total phenolics, antioxidant potential and polyphenolic composition on sweet and dry wines produced from *Tinta Negra Mole (TNM)* grapes (red variety), compared with a traditional sweet wine produced from *Malvasia* grapes (white variety). The wines were fortified and heated at 45 °C for a 3 month

period. Additionally, in order to evaluate the temperature effect, the wines were also submitted to overheating conditions, at 70 °C during 1 month.

7.2 Experimental

7.2.1 *Wine samples*

Two *Vitis Vinifera L.* grapes varieties recommended for the production of Madeira wine, *Tinta Negra Mole* (*TNM*, red variety) and *Malvasia* (white variety), were used in the study. The grapes were collected during the 2007 harvest and the corresponding wines were elaborated following the procedures of a local Madeira wine-producing cellar. The alcoholic fermentation was conducted under controlled temperature without maceration or adding any commercial yeast.

Two types of wines were produced from *TNM* grapes in adequate stainless steel tanks, stopping the fermentation by the addition of natural grape spirit when the density of grape must reached 1025 g/cm^3 (115 g/L of reducing sugars) or 986 g/cm^3 (about 4g/L of reducing sugars) for sweet and dry *TNM* wine, respectively. The sweet *Malvasia* was prepared in a similar way and the fermentation was stopped when density reached 1019 g/cm^3 (about 96 g/L of reducing sugars). After fortification, each wine was forced-aged in a special pilot scale system equipped with 200 L stainless steel tanks fitted with heating coils, allowing the circulation of pre-heated tap water, and maintained at 45 °C during 3 months. For comparison purposes, about 250 mL of each wine were submitted to overheating conditions, 70 °C during 1 month, carried out in a lab oven. The wines were monthly sampled and kept at -20 °C before analysis.

7.2.2 *Standards and reagents*

Folin-Ciocalteu reagent and gallic acid (≥ 98.0%) were supplied by Fluka Biochemika AG (Buchs, Switzerland) while sodium carbonate (99.8%), potassium chloride and sodium acetate (≥ 99%) were from Panreac Química S.A. (Barcelona, Spain). Aluminium chloride-6-hydrate and quercetin (≥ 99%) were from Riedel de Haën (Seelze, Germany) and methanol HPLC gradient grade was supplied by Fisher Scientific (Loughborough, United Kingdom).

For the preparation of the PBS buffer solution the following chemicals were used: sodium chloride, potassium chloride, sodium hydroxide, di-sodium hydrogen phosphate 12-hydrate and potassium di-hydrogen phosphate, supplied by Panreac Química S.A. (≥ 98%). 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt (98.0%) and potassium persulfate (≥ 98.0 %) were supplied by Fluka Biochemika AG.

For the DPPH assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$, 90.0%) and Trolox (6-hydroxy-2,5,7,8-tetramethylchloromane-2-carboxylic acid, ≥ 98%) were obtained from Fluka Biochemika AG. For the FRAP assay 2,2'- dipyridyl (99%), trichloroacetic acid (TCA, ≥ 99.0%), sodium tartrate (≥ 99.0%) and citric acid monohydrate (≥ 99.5%) were purchased from Fluka Biochemika AG while iron chloride (≥ 99%) was supplied by Riedel de Haën.

The following polyphenolic standards (purity higher than 95%) were used: gallic acid, gentisic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, ellagic acid, cinnamic acid, *p*-hydroxybenzoic acid, *(+)*-catechin, *(-)*-epicatechin, *(-)*-epigallocatechin, myricetin, sinapic acid, rutin and kaempferol from Fluka Biochemika AG, protocatechuic acid, vanillin, syringic acid and *trans*-resveratrol from Sigma-Aldrich (St. Louis, MO, USA), syringaldehyde, HMF and furfural were from Acros Organics (Geel, Belgium) and quercetin from Riedel-de-Haën.

7.2.3 *Polyphenolic composition*

Wines were assayed for total polyphenols (TP, gallic acid equivalents, mg/L), total monomeric anthocyanins (TMA, cyanidin-3-glucoside equivalents, mg/L) and total flavonoids (TF, quercetin equivalents, mg/L).

TP were analysed by the Folin-Ciocalteu's method adopted from OIV Compendium (MA-E-AS2-10-INDFOL), using gallic acid as standard. The method is based on the reduction of phosphomolybdic and phosphotungstic acids, present in Folin Ciocalteau reagent, by phenolics in the presence of sodium carbonate, forming blue products with maximum absorption close to 750 nm. Briefly, 100 µL of wine/calibration standards were diluted 100 times adding the following reagents strictly in this sequence: 5 mL of distillate water, 0.5 mL of Folin–Ciocalteu reagent and 2 mL of 20% (w/v) sodium carbonate aqueous solution, bringing the volume to 10 mL with distilled water and mixed. The mixture was then incubated at room temperature for 30 min, followed by absorbance measurement at 750 nm. The concentration of phenolic compounds was calculated according to the following gallic acid calibration curve: $A_{750} = 0.0008\ GAE(mg/L) + 0.0058\ (R^2 = 0.999)$, set for the range of 100 to 800 mg/L.

The TMA of the wines was determined using the pH-differential method proposed by AOAC, Official Method 2005.02 (Lee, Durst, & Wrolstad, 2005). This determination was only performed for the red wines. Wine samples were tentatively diluted in 0.025 M potassium chloride buffer pH 1.0 until the absorbance at 510 nm was lower than unity. The same samples were then diluted using the determined factor (5) in 0.4 M sodium acetate buffer pH 4.5. Absorbance readings at 510 and 700 nm in each buffer were performed, using distilled water as blank. The concentration of TMA was calculated with the following formula and expressed as cyanidin-3-glycoside (cyd-3-glu) equivalents (mg/L): $TMA = (A \times MW \times DF \times 10^3) / (\varepsilon \times l)$, with $A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}$ and where *MW* is the cyd-3-glu molecular weight (449.2 g/mol), *DF* is the dilution factor, *l* is the path length cell (1 cm), ε is cyd-3-glu molar absorptivity coefficient (26,900 L/mol cm) and 10^3, a conversion factor (g to mg).

The TF were determined according to the aluminium chloride colorimetric method proposed by Meda et al. (2005), with small adjustements: 5 mL of 2% (w/v) aluminium chloride ($AlCl_3$) solution in methanol was mixed with the same volume of wine/standard. Absorbance readings at 415 nm were carried out after 10 min of incubation at room temperature, using methanol as blank. The concentrations of TF were calculated according to the obtained equation of the standard quercetin calibration graph (10 – 50 mg/L): $A_{415} = 0.0240\ QE(mg/L) - 0.0100\ (R^2 = 0.999)$.

All determinations were performed in a Perkin-Elmer Lambda 2 spectrophotometer (Waltham, MA, USA) using 1 cm quartz cells. The average of the relative standard deviation (%RSD) among replicates was 14%.

Individual polyphenols were determined by direct injection of the samples in a high performance liquid chromatography (HPLC) system, following the methodology previously described (Pereira, Câmara, Cacho, & Marques, 2010), with slight modifications. Briefly, chromatographic system (Waters Alliance, Milford, MA, USA) was equipped with an auto-injector (Waters 2695, separations module), a photodiode array detector (Waters 2996) and the Empower Pro software, for data handling. The polyphenolic compounds were separated in an Atlantis T3 column (250 × 4.6 mm, i.d.; 5 µm; Milford, MA, USA) using the gradient described in Table 7.1, based on 3 solvents: A (10 mM of phosphate buffered at pH 2.70), B (acetonitrile) and C (methanol), and setting the column temperature to 30 °C. 20 µL of each sample/standard were injected after filtration using 0.45 µm Acrodisc® GHP filters (Pall Gelman Sciences, Ann Arbor, MI, USA). All standards and wine samples were injected in triplicate.

TABLE 7.1 – Polyphenols gradient program. Mobile phase solvents: A – 10 mM of phosphate buffered at pH 2.70; B – acetonitrile; C – methanol.

Time (min)	Flow (mL/min)	%A	%B	%C	Curve
---	1.00	100.0	0.0	0.0	---
30.00	1.00	79.0	10.0	11.0	6
42.00	1.00	73.0	10.0	17.0	6
55.00	1.00	40.0	60.0	0.0	6
58.00	1.00	40.0	60.0	0.0	6
65.00	1.00	100.0	0.0	0.0	1

Detection was performed at specific wavelengths after scanning from 200 to 780 nm. The identification of the analytes was carried out by comparing retention times and spectra with those of original standards, when available. All others were tentatively identified based on spectra obtained from the literature and assayed by assuming similar molar absorptivities to compounds with structural similarities. Quantitative determinations were attempted using standard external calibration method. Wavelengths used for quantification were 210 nm for flavan-3-ols and benzoic acids, 280 nm for furans, 315 nm for hydroxycinnamic acids and *trans*-resveratrol, and 360 nm for flavonoids and ellagic acid.

7.2.4 *Determination of antioxidant potential*

The antioxidant potential was estimated by different methods, namely by the interaction of antioxidants compounds with highly reactive free radicals or reactive oxygen species: ABTS, DPPH and FRAP assays. This interaction can be based on one or both of the following transference mechanisms, single electron transfer and hydrogen atom transfer. All photometric measurements were carried out in triplicate, with RSD values below 9%.

7.2.4.1 *ABTS assay*

The antioxidant potential was firstly measured according to the ABTS assay based on Re et al. (1999) with some modifications, using gallic acid as antioxidant standard. This method is based on the ability of antioxidants compounds to interact with the radical cation ABTS ($ABTS^{\bullet+}$, blue chromofore), decreasing its absorbance at 734 nm. Firstly, a phosphate buffered saline (PBS) solution was prepared as follows: 8.18 g NaCl, 0.27 g KH_2PO_4, 3.94 g $Na_2HPO_4.12H_2O$ and 0.15 g KCl in 1 L of distilled water. Then, a 2 mM $ABTS^{\bullet+}$ stock solution was prepared by reacting the ABTS salt with 200 µL of 70 mM potassium persulfate in 50 mL of PBS and allowing the mixture to stand in the dark at room temperature for 16 h before use. The $ABTS^{\bullet+}$ stock solution was then diluted with PBS to obtain an absorbance value of 0.800±0.030 at 734 nm. Finally, 12 µL of sample were mixed with 3 mL of the $ABTS^{\bullet+}$ working solution, and absorbance measurements were performed at room temperature during 20 min, at every 60 to 60 seconds, using PBS as blank sample. The antioxidant power was calculated as the percentage of inhibition $(\%I = [(A_{734(0\ min)} - A_{734(20\ min)})/A_{734(0\ min)}] \times 100$, with $A_{734(0\ min)}$ as the absorbance of the $ABTS^{\bullet+}$ at 734 nm at t = 0 min, $A_{734(20\ min)}$ as the absorbance of the remaining radical at the end of the reaction (t = 20 min)) and converted into gallic acid equivalents (GAE) by means of the following calibration curve (50 – 240 mg/L), submitted to the same procedure described above: $\%I = 0.328\ GAE(mg/L) + 11.638\ (R^2=0.996)$.

7.2.4.2 *DPPH assay*

The antioxidant potential was also evaluated by the ability of wines to scavenge DPPH free radicals, adapting the DPPH assay proposed by Paixão and co-workers (2007), and expressing the results as efficient concentration EC_{50} and as Trolox® equivalent antioxidant capacity (TEAC). The DPPH is known to be stable in the radical form due to the delocalisation of the unpaired electron over whole molecule, also responsible to its purple colour (Molyneux, 2004). When wine antioxidant substances interact with the DPPH radical solution, they can donate a hydrogen atom reducing its radical form. This interaction is traduced by the loss of the purple to a residual yellow colour (derived from the picryl group belonging to DPPH molecule) and can be measured at 515 nm. Aliquots of 6, 12, 18, 24, 36 and 60 µL of wine sample were individually mixed with 2.5 mL of $DPPH^{\bullet}$ (60 µM in methanol, daily prepared) and analysed immediately. The absorbance of the remaining $DPPH^{\bullet}$ was determined after 20 min, in 30 sec periods, at 515 nm, using methanol as blank. The EC_{50} determination was achieved by firstly plotting the $DPPH^{\bullet}$ linear regression in the range 6 to 60 µM: $A_{515}=0.0121[DPPH^{\bullet}]-0.0223$ ($R^2=0.992$), calculating the percentage of remaining $DDPH^{\bullet}$ as follow: $\%[DPPH^{\bullet}]_{REM} = [DPPH^{\bullet}]_{(20\ min)} / [DPPH^{\bullet}]_{(0\ min)} \times 100$ and fitting the best curve to the graph sample concentration (µL) against $\%[DPPH^{\bullet}]_{REM}$. Finally, The EC_{50} parameter was calculated for each sample as the substrate concentration to produce 50% reduction of the DPPH.

To express the TEAC, Trolox® concentration was plotted vs. %I: $\%I = 0.0752\ TE(mg/L)+1.9269\ (R^2=0.999)$, with $\%\ I = [(A_{515\ (0\ min)} - A_{515\ (20\ min)})/A_{515\ (0\ min)}] \times 100$, and $A_{515\ (0\ min)}$ as the absorbance value measured at the beginning of the reaction and $A_{515\ (20\ min)}$ the absorbance value after 20 min of reaction of 18 µL of the Trolox® standards (25 – 1250 mg/L), following the above procedure.

7.2.4.3 *FRAP assay*

The ferric reducing/antioxidant power (FRAP), a simple direct test, was also performed to estimate the antioxidant potential of the wines. FRAP assay is based on the formation of a coloured Fe (II) form (ferrous) from the reduction of a colourless Fe (III) compound (ferric) by the action of electron donating antioxidants, measured at 525 nm. The current determination was based on the protocol established by Makris et al. (2003), using ferric chloride (3 mM $FeCl_3$ in 5 mM citric acid) as oxidant and measuring the coloured ferrous product formed with 2,2'-dipyridyl at 525 nm. Briefly, 250 µL of working FRAP solution, daily prepared, was mixed with 250 µL of sample properly diluted, and then mixed with 4.5 mL of 0.5% 2,2'-dipyridyl in 1.2% TCA after a 20-min incubation at 50 °C in a water bath. After 5 min, the FRAP values were obtained from the absorbance recordings at 525 nm and expressed as quercetin equivalents determined from the linear regression set from 3 to 60 mg/L, $A_{525} = 0.0099\ QE(mg/L + 0.3893$ (R^2=0.999), and introducing the dilution factor.

7.2.5 *Colour study*

The colour of the wines submitted to *estufagem* was determined by means of the chromatic Glories and CIELab parameters. Both colour measurements were performed using 1 cm path length quartz cells. The wine samples were filtered by cellulose membranes prior to the spectrophotometric analysis.

The Glories parameters, yellow percentage (%Ye), red percentage (%Re), blue percentage (%Bl), colour intensity (CI) and tonality (To), were determined at 420, 520 and 620 nm. These chromatic indexes are currently used by oenologists, but as the CIELab space defines better the wine colour and differentiation (Heredia, Troncoso, & Guzmán-Chozas, 1997; Pérez-Magariño & González-San José, 2006), the CIELab parameters (*L**, *a**, *b**) were also determined measuring the transmittance from 380 to 770 nm at 5 nm intervals, following the recommendations of the International Organization of Vine and Wine (OIV, resolution OENO 1/2006) and considering the illuminant D65 (daylight source) and 10° standard observer (human perception). The colour-opponent coordinates, *a** and *b**, correspond to reddish / greenish and yellowish / bluish colours, respectively, and the colour lightness, *L**, is evaluated in a black and white scale (ranging from 0 to 100). The psychophysical parameters *C**, *H** and *S** were also estimated (Meléndez, Sánchez, Íñiguez, Sarabia, & Ortiz, 2001), where the chromaticity (*C**) was calculated as $C^* = \sqrt{(a^*)^2 + (b^*)^2}$ and determines the degree of distinction of each hue when compared with the same lightness grey, the hue as $H^* = \arctan(b^*/a^*)$, which is the attribute allowing the differentiation of a colour with reference to same lightness grey and S* as $S^* = C^*/L^*$, which represents the saturation.

7.2.6 *Data processing*

All analyses were performed in triplicate and the results were expressed as the mean value ± standard deviation. Regular statistical analyses were performed with Microsoft Office Excel 2007.

7.3 Results and discussion

The current experiments were focused on the transformations that take place in the polyphenolic composition, antioxidant potential, and colour of Madeira wines submitted to the heating step usually undertaken, with the purpose of setting up common patterns of change and the influence of temperature. These issues will be further discussed.

7.3.1 *Polyphenolic composition*

The effect of temperature on the polyphenolic composition was estimated by spectrophotometric measurements and correlated with individual determination by HPLC-DAD. Table 7.2 summarizes the attained results in terms of total polyphenols (TP), total flavonoids (TF) and total monomeric anthocyanins (TMA). The results showed that young wines produced from *TNM* grapes (red wines) presented similar levels of TP when compared with the white variety *Malvasia*. This may be related to the fact that the fermentation of Madeira wines is usually performed as in white table wines, in absence (or limited contact) of grape solids. As shorter fermentation times should lead to lower phenolic contents, sweet wines were expected to have similar phenolic contents.

TABLE 7.2 – Total polyphenols (TP), total flavonoids (TF) and total monomeric anthocyanins (TMA) of Madeira wines at the initial stage and at the end of each month of heating at 45 °C (3 months) and 70 °C (1 month).

Samples		TP GAE (mg/L)	± SD	TMA Cyd-3-glu (mg/L)	± SD	TF QE (mg/L)	± SD
TNM sweet	**0 m**	469.98	13.63	15.02	0.01	28.96	0.39
	1 m, 45 °C	332.17	9.58	4.43	0.03	23.47	1.55
	2 m, 45 °C	474.15	15.64	3.16	0.03	38.06	0.33
	3 m, 45 °C	434.42	9.03	0.93	0.02	30.70	0.54
	1 m, 70 °C	444.01	17.21	1.62	0.05	51.44	0.60
TNM dry	**0 m**	609.98	25.83	22.05	0.05	49.42	0.56
	1 m, 45 °C	576.68	23.60	2.99	0.10	45.97	0.31
	2 m, 45 °C	561.02	4.17	5.05	0.02	45.80	0.25
	3 m, 45 °C	493.09	3.04	2.50	0.02	45.39	0.16
	1 m, 70 °C	573.57	6.37	0.30	0.04	82.87	2.92
Malvasia	**0 m**	617.10	7.22	-		28.48	0.11
	1 m, 45 °C	565.11	47.84	-		33.60	0.32
	2 m, 45 °C	517.12	18.02	-		46.48	1.60
	3 m, 45 °C	492.16	40.92	-		47.76	0.48
	1 m, 70 °C	466.02	18.06	-		133.74	4.18

Ageing can also affect the content of phenolics, as they can suffer hydrolysis, oxidations and complexations, with temperature increasing their degradation (Recamales, Sayago, González-Miret, & Hernanz, 2006). The heating process carried out in current the Madeiras promoted some changes on the phenolic content, decreasing up to 25% of the initial amount. After the baking step at 45 °C, the TP was found to vary from 469.98 to 434.42 mg (GAE)/L in *TNM* sweet wine, and from 609.98 to 493.09 and 617.10 to 492.16 mg (GAE)/L in *TNM* dry and *Malvasia* wines, respectively (Table 7.2). Increasing the temperature, no

significant changes were detected, showing that temperature has a relatively small effect on TP decrease. TP final values range from 434.42 to 573.57 mg (GAE)/L, which were comparable with those presented in the literature for white wines (Li, Wang, Li, Li, & Wang, 2009; Minussi et al., 2003; Paixão, et al., 2007), or slightly higher (Mitić, Obradović, Grahovac, & Pavlović, 2010; Roussis, Lambropoulos, & Soulti, 2005; Sánchez-Moreno, Larrauri, & Saura-Calixto, 1999). Although only responsible for up to 4% of the phenolics, anthocyanins may also contribute for the diminishment. The loss of anthocyanins was proved by the progressive decrease of the total monomeric anthocyanins (TMA) obtained in *TNM* wines (Table 7.2). The initial TF values were in general very low, ranging from 28.48 to 49.42 mg/L, as result of the small skin contact in winemaking. These values were close to those obtained by Mitić et al. (2010) (between 45 to 81 mg/L as catechin equivalents) when they analysed 10 Serbian white wines using the same test. The flavonoids did not represent more than 8% of the total polyphenolic content of the wines at the initial circumstances and, surprisingly, the aluminum chloride assays revealed that TF values increased with the heating period, especially when overheating temperature was applied. This was not expected since it is frequently refered that flavonoids participate in several reactions, namely anthocyanin and flavanols degradation to form new polymeric complexes (Monagas, Gómez-Cordovés, & Bartolomé, 2006), which could induce the decrease in the TF values. Eventually, other substances with similar structure of flavonoids were formed during this period and respond positively to this test.

Regarding the HPLC-DAD analysis, 16 polyphenols (from the 22 standards assayed) were found in the current sample set of Madeira wines (Table 7.3 (A-C)), including non-flavonoids: 6 hydroxybenzoic acids, 3 hydroxycinnamic acids and 1 stilbene; and flavonoids: 3 flavonols and 3 flavan-3-ols. Additionally, 2 furans were also found: 5-hydroxymethylfurfural (HMF) and furfural, while some compounds were never found, as gentisic, sinapic and cinnamic acids and the flavonol rutin. Moreover, the most important unknown peaks were tentatively identified, by the elution order and UV spectrum when compared with those found in literature. The first 6 unknown peaks exhibit cinnamic-type UV spectra, and is believed that they correspond to hydroxycinnamates, currently found in wines. In fact, evidences indicate that some of them are hydroxycinnamoyltartaric acids. These compounds were recently investigated by Buiarelli and colleagues (2010) that identified them in wine by HPLC-tandem mass spectrometry. Using a similar chromatographic column, they established the following elution order: caftaric, coutaric, fertaric, caffeic, *p*-coumaric and ferulic. Darias-Martín et al. (2008) also reported that the *cis* forms elute first than *trans*. Consequently, comparing the UV spectra with those obtained by Guerrero et al. (2009), Mozetič et al. (2006) and Gutiérrez et al. (2005), Unk 1 (maximum at 326 nm with a shoulder at 300 nm) was identified as *trans*-caftaric acid, Unk 2 and Unk 3 as *cis*- and *trans*-coutaric acids, with maximums at 310 and 313 nm, respectively, and Unk 4 as fertaric acid (maximum at 327 nm with a shoulder at 287 nm), probably the *trans* form, the most common in wines. Unk 5 and 6 should correspond to the hydroxycinnamate family, but the identification was not established. Unk 7, has a UV spectrum similar to rutin (standard available) and may have structural similarities.

TABLE 7.3.A – Individual polyphenols (mg/L) of ***TNM* sweet** wines during the heating at 45 °C (3 months) and 70 °C (1 month).

		***TNM* sweet**									
		0 m	± SD	1 m, 45 °C	± SD	2 m, 45 °C	± SD	3 m, 45 °C	± SD	1 m, 70 °C	± SD
Non-flavonoids											
Hydroxybenzoic acids											
	Gallic acid	3.70	0.01	4.91	0.07	5.29	0.03	6.23	0.12	9.16	0.09
	Protocatechuic acid	2.57	0.11	2.35	0.06	1.97	0.02	1.55	0.08	1.64	0.05
	p-Hydroxybenzoic acid	0.94	0.05	0.57	0.02	0.77	0.03	0.51	0.02	1.00	0.06
	Vanillic acid	2.52	0.09	2.57	0.06	2.23	0.06	2.24	0.11	3.08	0.08
	Syringic acid	4.89	0.06	5.29	0.08	5.03	0.18	5.03	0.06	5.93	0.04
	Ellagic acid	n.d.		n.d.		n.d.		n.d.		n.d.	
	Total	14.62		15.69		15.29		15.56		20.81	
Hydroxycinnamates											
	Unk 1*	14.53	0.01	13.37	0.01	11.31	0.02	10.82	0.05	3.24	0.04
	Unk 2*	4.45	0.04	3.71	0.02	2.91	0.01	2.73	0.11	0.62	0.02
	Unk 3*	7.87	0.02	7.66	0.01	7.07	0.03	6.67	0.15	2.99	0.06
	Unk 4*	0.91	0.02	0.82	0.02	0.71	0.02	0.78	0.01	1.80	0.05
	Unk 5*	2.20	0.04	2.57	0.10	2.34	0.03	2.40	0.03	2.70	0.02
	Caffeic acid	1.72	0.02	1.82	0.07	2.06	0.02	2.53	0.10	3.34	0.15
	Unk 6*	1.96	0.05	2.07	0.02	1.91	0.01	2.05	0.02	2.09	0.01
	p-Coumaric acid	0.58	0.01	0.85	0.02	1.02	0.01	1.27	0.01	4.31	0.09
	Ferulic acid	n.q.		0.45	0.02	0.47	0.00	0.68	0.03	0.45	0.02
	Total	34.22		33.32		29.81		29.93		21.52	
Stilbene											
	trans-Resveratrol	n.d.		n.d.		n.d.		n.d.		n.d.	
Flavonoids											
Flavonols											
	Unk 7**	8.03	0.07	7.13	0.06	5.71	0.09	5.30	0.04	0.56	0.03
	Myricetin	n.d.		n.d.		n.d.		n.d.		n.d.	
	Quercetin	n.q.		n.q.		n.q.		0.53	0.01	0.67	0.01
	Kaempferol	n.q.		n.q.		n.q.		n.q.		n.q.	
	Total	8.03		7.13		5.71		5.83		1.23	
Flavan-3-ols											
	(-)-Epigallocatechin	n.d.		n.d.		n.d.		n.d.		n.d.	
	(+)-Catechin	3.94	0.02	2.49	0.05	1.48	0.02	0.93	0.03	0.47	0.00
	(-)-Epicatechin	0.92	0.01	0.65	0.03	0.47	0.03	0.40	0.01	0.36	0.01
	Total	4.86		3.13		1.95		1.32		0.84	
Furans											
	HMF	1.56	0.02	17.90	0.00	49.32	0.03	97.50	0.12	1728.07	2.80
	Furfural	n.d.		n.q.		2.02	0.05	3.08	0.05	20.26	0.18
	Total	1.56		17.90		51.34		100.58		1748.32	

* Quantification relative to caffeic acid; ** Quantification relative to rutin
n.d. - not detected, bellow LOD; n.q. - not quantified, bellow LOQ

Before heating, hydroxycinnamates represent in average 59% of the non-anthocyanin polyphenols, followed by hydroxybenzoic acids (about 20 %). Caftaric acid was the most abundant compound found in all wines, varying from 14.53 to 37.34 mg/L (caffeic acid equivalents). Similar amounts were found by Fernández-Pachón and colleagues (2006) in sherry wines (6.29 to 42.90 mg/L). During accelerated ageing, a noticeable decrease of caftaric, coutaric and fertaric acids was registered, especially when heating conditions were more severe. Conversely, caffeic, *p*-coumaric and ferulic contents increased during the same period, suggesting the hydrolysis of the correspondent hydroxycinnamoyltartaric acids, increasing with temperature (Table 7.3). Flavan-3-ols, initially ranging from 0.55 mg/L for *(-)*-epigallocatechin to 16.19 mg/L for *(+)*-catechin, also progressively declined during the baking process. Similar findings have also been pointed out by others researchers during wine ageing (Fernandez-Zurbano, et al., 1998; Kallithraka, et al., 2009; Recamales, et al., 2006).

TABLE 7.3.B – Individual polyphenols (mg/L) of ***TNM* dry** wines during the heating at 45 °C (3 months) and 70 °C (1 month).

		***TNM* dry**									
		0 m	± SD	1 m, 45 °C	± SD	2 m, 45 °C	± SD	3 m, 45 °C	± SD	1 m, 70 °C	± SD
Non-flavonoids											
Hydroxybenzoic acids											
	Gallic acid	9.47	0.37	9.81	0.01	9.91	0.14	9.50	0.05	10.44	0.08
	Protocatechuic acid	6.84	0.39	3.53	0.15	2.47	0.14	2.19	0.09	4.74	0.06
	p-Hydroxybenzoic acid	1.15	0.02	1.31	0.05	1.22	0.06	1.28	0.04	1.67	0.06
	Vanillic acid	4.92	0.17	3.81	0.11	3.42	0.03	3.23	0.06	3.27	0.08
	Syringic acid	3.39	0.16	3.72	0.05	4.27	0.09	4.11	0.08	4.90	0.04
	Ellagic acid	n.q.		n.q.		n.q.		n.q.		n.q.	
	Total	25.77		22.18		21.29		20.31		25.02	
Hydroxycinnamates											
	Unk 1*	37.34	0.14	32.78	0.02	27.63	0.01	27.64	0.04	5.71	0.02
	Unk 2*	5.14	0.02	4.37	0.05	3.69	0.04	3.67	0.00	0.64	0.01
	Unk 3*	20.55	0.15	19.60	0.04	17.37	0.08	17.32	0.01	5.03	0.12
	Unk 4*	n.d.		n.d.		n.d.		n.d.		n.d.	
	Unk 5*	2.20	0.06	2.66	0.01	2.68	0.03	2.66	0.03	2.93	0.01
	Caffeic acid	2.75	0.09	4.29	0.03	5.18	0.04	5.21	0.04	6.00	0.02
	Unk 6*	1.63	0.03	2.19	0.03	2.19	0.08	2.19	0.06	2.62	0.03
	p-Coumaric acid	1.61	0.03	2.31	0.05	2.78	0.04	2.77	0.00	8.32	0.02
	Ferulic acid	0.45	0.01	0.66	0.02	0.66	0.01	0.65	0.02	0.51	0.02
	Total	71.69		68.85		62.19		62.12		31.76	
Stilbene											
	trans-Resveratrol	0.63	0.02	0.51	0.01	n.q.		n.q.		n.q.	
Flavonoids											
Flavonols											
	Unk 7**	6.13	0.03	4.93	0.07	4.04	0.06	3.98	0.10	0.46	0.01
	Myricetin	0.71	0.03	0.75	0.01	0.72	0.01	n.q.		1.15	0.03
	Quercetin	0.82	0.01	0.89	0.01	0.81	0.01	0.81	0.01	1.53	0.01
	Kaempferol	n.q.		n.q.		n.q.		n.q.		n.q.	
	Total	7.66		6.57		5.56		4.79		3.15	
Flavan-3-ols											
	(-)-Epigallocatechin	3.54	0.12	3.18	0.06	1.47	0.04	1.41	0.05	n.d.	
	(+)-Catechin	16.19	0.02	13.06	0.06	6.16	0.06	6.20	0.04	4.27	0.11
	(-)-Epicatechin	4.78	0.18	3.21	0.06	1.46	0.07	1.30	0.06	1.07	0.03
	Total	24.52		19.45		9.08		8.91		5.33	
Furans											
	HMF	n.d.		1.61	0.01	2.70	0.03	2.68	0.03	41.28	0.05
	Furfural	n.d.		n.q.		1.66	0.01	1.65	0.00	12.20	0.02
	Total	0.00		1.61		4.36		4.33		53.47	

* Quantification relative to caffeic acid; ** Quantification relative to rutin
n.d. - not detected, bellow LOD; n.q. - not quantified, bellow LOQ

The decrease of hydroxycinnamic acid esters and flavanols - yellow pigments (Jackson, 2000) - due to oxidation is referenced to contribute to the development of the brownish shades in white wines, although flavanols have been considered more effective in browning, especially *(-)*-epicatechin (Es-Safi, et al., 1999).

Some hydroxybenzoic acids increased during the heating period (gallic and syringic acid) and others declined (protocatechuic, *p*-hydroxybenzoic and vanillic acids). Gallic acid was the major hydroxybenzoate and its growth during ageing is usually explained by the hydrolysis of gallic tannins (García Parrilla, Heredia, & Troncoso, 1999; Moreno-Arribas & Polo, 2009). The values found (3.70 to 13.85 mg/L) are slightly above of those found by Darias-Marin and co-workers (2008) in white wines from Canary Islands (0.97 to 1.64 mg/L), but similar to those reported by Fernández-Pachón et al. (2006) in sherry wines (4.42 to 10.70 mg/L). Syringic acid

increase during ageing is usually related with the anthocyanins cleavage or the breakdown of lignin during wine wood-ageing (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). The former could explain the result obtained for *TNM* wines. The degradation of the others hydroxybenzoates may be related with the formation of ethyl esters of vanillic and *p*-hydroxybenzoic acids, and methyl esters of vanillic and protocatechuic acids already found in wines (Moreno-Arribas & Polo, 2009). Regarding ellagic acid, only trace amounts were detected.

TABLE 7.3.C – Individual polyphenols (mg/L) of ***Malvasia*** wines during the heating at 45 °C and 70 °C.

		Malvasia									
		0 m	± SD	1 m, 45 °C	± SD	2 m, 45 °C	± SD	3 m, 45 °C	± SD	1 m, 70 °C	± SD
Non-flavonoids											
Hydroxybenzoic acids											
	Gallic acid	11.62	0.42	13.73	0.07	13.85	0.02	13.27	0.08	12.93	0.12
	Protocatechuic acid	3.40	0.01	2.67	0.15	2.66	0.03	2.95	0.05	3.48	0.15
	p-Hydroxybenzoic acid	0.92	0.03	0.88	0.04	0.68	0.03	0.61	0.01	0.90	0.01
	Vanillic acid	1.71	0.03	0.93	0.04	0.66	0.01	0.57	0.02	0.55	0.01
	Syringic acid	n.d.		n.d.		n.d.		0.93	0.05	0.75	0.03
	Ellagic acid	n.d.		n.d.		n.d.		n.d.		n.d.	
	Total	17.66		18.21		17.85		18.33		18.61	
Hydroxycinnamates											
	Unk 1*	37.33	0.07	30.47	0.02	23.31	0.02	16.87	0.02	7.25	0.04
	Unk 2*	7.40	0.03	6.32	0.02	5.28	0.31	3.91	0.13	0.93	0.00
	Unk 3*	15.51	0.08	13.27	0.06	10.76	0.01	8.20	0.04	4.88	0.00
	Unk 4*	3.08	0.05	2.71	0.16	2.14	0.03	1.60	0.02	0.86	0.02
	Unk 5*	0.62	0.01	0.48	0.01	n.q.		n.q.		n.d.	
	Caffeic acid	1.71	0.03	3.10	0.02	3.60	0.02	3.55	0.05	6.72	0.02
	Unk 6*	n.d.		n.d.		n.d.		n.d.		n.d.	
	p-Coumaric acid	0.76	0.01	1.35	0.00	1.63	0.02	1.65	0.05	7.72	0.09
	Ferulic acid	0.37	0.02	0.66	0.01	0.72	0.04	0.62	0.01	1.00	0.02
	Total	66.77		58.36		47.42		36.39		29.36	
Stilbene											
	trans-Resveratrol	n.q.		n.q.		n.q.		n.d.		n.d.	
Flavonoids											
Flavonols											
	Unk 7**	7.25	0.07	6.80	0.11	5.31	0.18	3.79	0.06	0.48	0.03
	Myricetin	n.d.		n.d.		n.d.		n.d.		n.d.	
	Quercetin	0.64	0.01	0.96	0.01	0.85	0.00	0.65	0.01	0.75	0.01
	Kaempferol	n.q.		n.q.		n.q.		n.q.		n.q.	
	Total	7.89		7.76		6.17		4.44		1.24	
Flavan-3-ols											
	(-)-Epigallocatechin	0.55	0.01	n.q.		n.q.		n.q.		0.60	0.02
	(+)-Catechin	6.98	0.05	4.23	0.06	1.25	0.03	0.49	0.02	n.q.	
	(-)-Epicatechin	1.47	0.04	0.90	0.02	0.49	0.03	0.39	0.01	0.37	0.02
	Total	9.00		5.12		1.74		0.88		0.97	
Furans											
	HMF	1.48	0.00	12.17	0.01	30.42	0.01	52.10	0.15	1651.50	2.78
	Furfural	n.d.		n.q.		1.80	0.02	2.33	0.05	19.48	0.01
	Total	1.48		12.17		32.22		54.43		1670.98	

* Quantification relative to caffeic acid; ** Quantification relative to rutin

n.d. - not detected, bellow LOD; n.q. - not quantified, bellow LOQ

Flavonols, common in the skins of both red and white grapes in glycoside form, with the aglycone form prevailing in wines, were found but represent a small fraction (less than 13%). The results were consistent with the absence or limited contact with grape solids during fermentation, and the highest values were found in *TNM* dry wines due to a more extensive fermentation. Unk 7 is important and decreased with baking. Quercetin was found in small

amounts (ranging from 0.53 to 1.53 mg/L) and did not present a regular trend with the baking process, as myricetin, only found in the *TNM* dry wine (about 0.71 mg/L). Traces of kaempferol were found but below the quantification limit and *trans*-resveratrol (not detected in sweet wines and up to 0.63 mg/L in *TNM* dry wine) is clearly affected by temperature (Table 7.3). This value is within the range of 0.1–0.8 mg/L found in white wines (Moreno-Arribas & Polo, 2009). In the opposite side, HMF and furfural, formed essentially from sugar degradation, were clearly improved with temperature, as pointed out in a previous work (Pereira, Albuquerque, Ferreira, Cacho, & Marques, 2011), especially HMF in the sweet wines. Low levels obtained for the dry wine (less than 3 mg/L), except in overheating conditions, suggest the need of accurate control of the temperature used in the *estufagem*, with eventual differentiation according to wine sweetness.

7.3.2 *Antioxidant potential*

TABLE 7.4 – Antioxidant potential of Madeira wines during the heating at 45 °C (3 months) and 70 °C (1 month) expressed in terms of ABTS, DPPH and FRAP assays.

Samples		ABTS assay		DPPH assay				FRAP assay	
		GAE (mg/L)	± SD	TEAC (mg/L)	± SD	EC_{50} (µL)	± SD	QE (mg/L)	± SD
TNM sweet	0 m	150.76	1.16	313.99	12.34	49.99	2.56	40.65	2.18
	1 m, 45 °C	103.07	2.52	276.66	13.27	65.51	0.86	19.88	0.69
	2 m, 45 °C	174.07	3.16	308.73	16.45	50.93	1.51	51.50	2.37
	3 m, 45 °C	153.93	3.50	305.52	2.36	53.85	2.39	53.49	0.92
	1 m, 70 °C	149.82	1.23	234.84	5.37	68.90	3.49	42.98	0.29
TNM dry	0 m	268.61	0.13	502.93	19.46	25.68	0.49	36.83	3.22
	1 m, 45 °C	249.74	9.13	496.66	13.50	25.84	0.78	28.67	2.42
	2 m, 45 °C	235.36	8.50	504.98	9.22	27.06	0.28	33.67	1.90
	3 m, 45 °C	198.45	7.25	409.66	9.42	36.79	0.31	102.46	4.31
	1 m, 70 °C	218.21	6.32	403.53	5.13	34.30	0.68	120.27	1.86
Malvasia	0 m	183.93	4.95	445.92	7.00	32.56	0.73	70.21	3.39
	1 m, 45 °C	214.76	9.34	426.01	3.55	34.67	0.55	75.30	1.25
	2 m, 45 °C	219.29	8.08	389.33	22.98	39.07	0.65	60.76	2.55
	3 m, 45 °C	177.06	2.85	362.99	6.36	43.10	0.45	55.33	3.89
	1 m, 70 °C	144.65	0.39	351.60	6.39	60.54	2.79	38.81	1.85

The antioxidant potential (AP) was determined by three different tests: ABTS, DPPH and FRAP, and the results are depicted in Table 7.4. DPPH (R^2 = 0.725) and ABTS (R^2 = 0.7411) assays likely reflected better the AP of the studied wines rather than FRAP assay (R^2=0.1158), because of the higher correlation with the TP. DPPH assay presented the highest values (in average 382.22 mg (Trolox)/L) and the FRAP the lowest (about 55.39 mg (QE)/L). FRAP assay measures the reducing capacity of antioxidants and depends totally on the electron transference mechanisms while DPPH and ABTS assays determine the radical scavenging activity by electron and hydrogen transfer (Prior, Wu, & Schaich, 2005). The results showed that with the baking step no drastic changes took place in the AP (DPPH and ABTS); only a slightly decrease was observed even when the heating process was carried out at higher temperatures. At the end of the heating procedure the AP values were in the range 234.84 - 409.66 mg/L in terms of TEAC (or 0.94 – 1.64 mM) slightly above of the results obtained by

Fernández-Pachón et al. (2006) in sherry wines (0.49 – 0.98 mM), and comparable of those found by de Quirós et al. (2009) in Spanish white wines (0.77 – 2.01 mM).

7.3.3 *Colour study*

As colour is one of the principal attributes of a wine and is considered decisive for the choice of consumers, colour studies can be a helpful tool in the recognition of the typical characteristics of a wine or on the influence of the vinification procedures. As Madeira wines can present pronounced colour changes during *estufagem*, Glories and CIELab systems were applied in this study. Glories parameters, %Ye, %Re and %Bl, are presented in Figure 7.1 while colour intensity (CI), tonality (To) and the absorbance readings at 420, 520 and 620 nm are reported in Table 7.5. The results showed that before heating, red colour (about 47%) predominates in *TNM* dry wine (*TD0m*), while the yellow tones characterized sweet wines, especially *Malvasia* (*M0m*) with 65% (Figure 7.1) and limited contribution of blue hue (up to 16%). The heating process clearly affected colour, expressed in the increase of yellow tones (%Ye increase reaches about 35% and 15% in the dry and sweet wine submitted to heating at 45 °C for 3 months, *TD3m45* and *TS3m45*, respectively) and in the decrease of red tones. The change was intensified at overheating temperatures, up to 81% in *TD1m70* and 59% in *TS1m70*. The decrease of the reddish and the increase of the yellowish shades are in agreement with the observations reported by other authors (Cadahía, et al., 2009; Monagas, Martín-Álvarez, et al., 2006) and can be associated to the anthocyanins degradation, to form new polymeric complexes. Indeed, the anthocyanin degradation was confirmed by the TMA analysis (see Table 7.2).

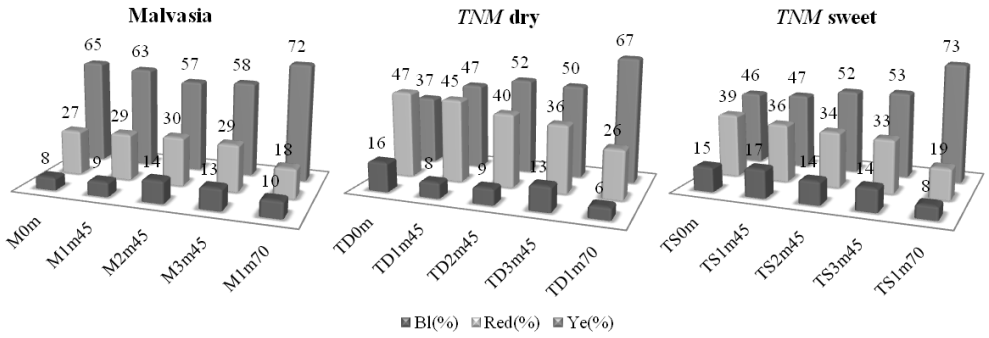

FIGURE 7.1 – Glories parameters, %Ye, %Re and %Bl of Madeira wines during the heating at 45 °C (3 months) and 70 °C (1 month).

In *Malvasia* wine, the yellow pigments were always preponderant (at least 57%), as expected for a white variety. *TNM* wines presented high CI values before baking (Table 7.5) which decreased with *estufagem,* following the decrease of red hues, while To slightly increased. Almost the opposite is observed for *Malvasia.*

TABLE 7.5 – Glories chromatic parameters: intensity (CI), tonality (To) and absorbance readings at 420, 520 and 620 nm of the Madeira wines submitted to heating at 45 °C (3 months) and 70 °C (1 month).

Samples		$A_{420\ nm}$	± SD	$A_{520\ nm}$	± SD	$A_{620\ nm}$	± SD	CI	± SD	To	± SD
TNM sweet	**0 m**	1.435	0.033	1.224	0.031	0.479	0.024	3.14	0.09	1.17	0.00
	1 m, 45 °C	1.840	0.117	1.403	0.104	0.687	0.077	3.93	0.30	1.31	0.01
	2 m, 45 °C	1.174	0.120	0.772	0.094	0.323	0.059	2.27	0.27	1.52	0.03
	3 m, 45 °C	1.167	0.052	0.716	0.043	0.308	0.034	2.19	0.13	1.63	0.03
	1 m, 70 °C	2.149	0.019	0.556	0.016	0.238	0.014	2.94	0.05	3.87	0.08
TNM dry	**0 m**	1.580	0.019	1.970	0.018	0.671	0.026	4.22	0.06	0.80	0.00
	1 m, 45 °C	1.064	0.004	1.013	0.003	0.189	0.001	2.27	0.01	1.05	0.00
	2 m, 45 °C	1.311	0.007	1.000	0.006	0.220	0.002	2.53	0.02	1.31	0.00
	3 m, 45 °C	1.743	0.043	1.255	0.034	0.457	0.021	3.46	0.10	1.39	0.00
	1 m, 70 °C	0.948	0.003	0.372	0.001	0.089	0.002	1.41	0.00	2.55	0.00
Malvasia	**0 m**	0.692	0.001	0.293	0.001	0.082	0.000	1.07	0.00	2.36	0.00
	1 m, 45 °C	1.070	0.004	0.488	0.002	0.151	0.001	1.71	0.01	2.19	0.00
	2 m, 45 °C	1.253	0.030	0.654	0.019	0.298	0.012	2.21	0.06	1.92	0.01
	3 m, 45 °C	0.859	0.011	0.420	0.010	0.192	0.008	1.47	0.03	2.05	0.02
	1 m, 70 °C	2.135	0.016	0.530	0.018	0.301	0.019	2.97	0.05	4.03	0.11

Usually used as a browning index in white wines, the absorbance at 420 nm did not revealed a consistent trend during the heating period, but the absorbance increased significantly in sweet wines under overheating conditions (70 °C), in good agreement with Mayén and co-workers (1997), which found that A_{420nm} did not increase during the browning of white wines from *Pedro Ximenez* and *Baladi* grapes at accelerated ageing (50 °C), in corked bottles. However, an increase was registered when the bottles were opened and exposed to air. Recently, Kallithraka and colleagues (2009) reported that $A_{420\ nm}$ of white wines significantly increased only after accelerated ageing at 55 °C over a period of 10 days.

In addition, Fernandez-Zurbano et al. (1998) established three categories for the browning of white dry wines, considering intense when absorbance (AU) was higher than 0.5, moderate between 0.2 and 0.5 AU and light when less than 0.2 AU. Considering these categories, the current Madeira wines presented an intense browning at the end of the baking process, especially when sweet wines were baked at 70 °C, for 1 month (Table 7.5). At the initial stage, *TNM* wines already presented very high $A_{420\ nm}$ values, possibly due to the presence of anthocyanins and others phenolics. At overheating conditions, reactions between polyphenolic compounds and sugars degradation, namely caramelization, were certainly favoured.

The CIELab chromatic coordinates *a**, *b** and *L** were also obtained and are represented in a 3D plot (Figure 7.2). Colour differences were more noticeable than with the Glories procedure, confirming the conclusions. Major changes were observed again in *TNM* dry wine, reflecting the decrease on the *a** positive coordinate (red hue) associated with an increase of the *b** positive values (yellow hue), even more pronounced at overheating conditions. The variations with the heat presented by *TNM* sweet wine and *Malvasia* were significantly smaller, except for overheating conditions.

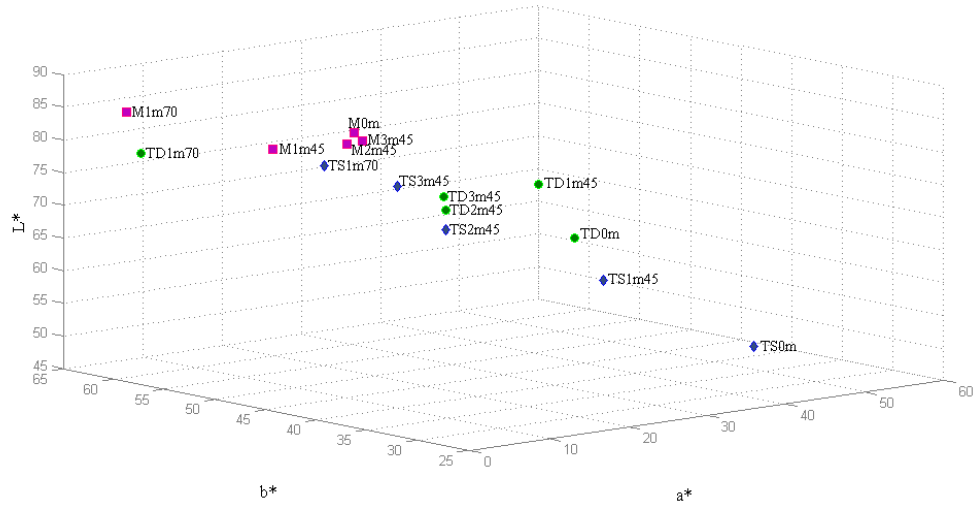

FIGURE 7.2 – 3D representation of the CIELab chromatic coordinates *a**, *b** and *L** of the Madeira wines submitted to heating at 45 °C (3 months) and 70 °C (1 month).

TABLE 7.6 – CIELab chromatic parameters: chromaticity (C*), hue (H*) and saturation (S*) of the Madeira wines submitted to heating at 45 °C (3 months) and 70 °C (1 month).

Samples		*C**	± SD	*H**	± SD	*S**	± SD	ΔE*
***TNM* sweet**	**0 m**	47.05	0.00	0.91	0.00	0.69	0.00	-
	1 m, 45 °C	32.58	0.00	1.12	0.00	0.40	0.00	-
	2 m, 45 °C	49.85	0.00	1.11	0.00	0.70	0.00	-
	3 m, 45 °C	41.80	0.00	1.21	0.00	0.54	0.00	16.45
	1 m, 70 °C	63.33	0.00	1.46	0.00	0.82	0.00	34.90
***TNM* dry**	**0 m**	63.46	0.00	0.62	0.00	1.32	0.00	-
	1 m, 45 °C	58.18	0.00	0.82	0.00	0.99	0.00	-
	2 m, 45 °C	62.56	0.00	1.01	0.00	0.98	0.01	-
	3 m, 45 °C	55.32	0.00	1.14	0.00	0.75	0.00	40.21
	1 m, 70 °C	51.36	0.00	1.31	0.00	0.65	0.00	50.55
Malvasia	**0 m**	41.11	0.00	1.43	0.00	0.47	0.00	-
	1 m, 45 °C	54.88	0.00	1.35	0.00	0.68	0.00	-
	2 m, 45 °C	43.16	0.00	1.41	0.00	0.51	0.00	-
	3 m, 45 °C	40.64	0.00	1.42	0.00	0.47	0.00	1.34
	1 m, 70 °C	61.46	0.00	1.52	0.00	0.72	0.00	20.96

Figure 7.2 clearly shows that all wines tend to the same chromatic characteristics when the heating procedure is applied: red wines become clearer (*L** increases) due to anthocyanin decrease, while yellow tones (*b** increases) predominate rather than red (*a** decreases). This can also be visualised in Figure 7.3. No defined trend was observed for chromaticity (*C*,* ranging from 32.53 to 63.46 units), hue (*H*,* ranging from 0.62 and 1.52 units) and saturation (*S*,* ranging from 0.40 to 1.32 units) during the heating period (Table 7.6). Differences detectable by the human eye were estimated by the measurement of colorimetric differences

(*ΔE**) for every pair of wines after the heating period, as follows: $\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$, assuming that *ΔE** higher than 3 units means that the colour of the samples is different enough to be easily distinguished by human observers (Gómez-Míguez, Gómez-Míguez, Vicario, & Heredia, 2007). Results (Table 7.6) revealed that the colour of the wines after being baked was clearly distinguishable from the initial stage (16.45 < *ΔE**< 50.55), with the exception of *Malvasia,* at standard conditions.

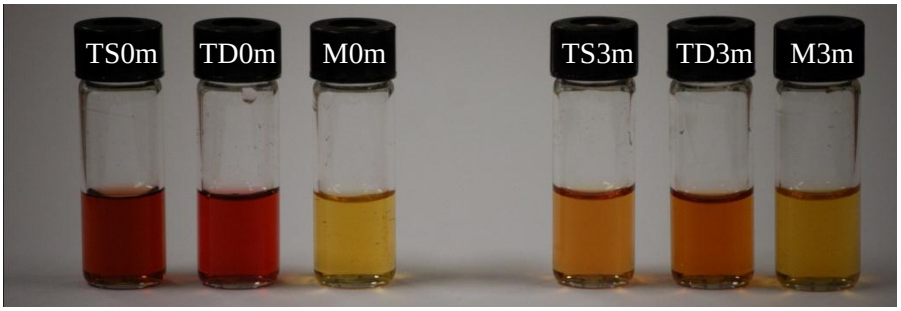

FIGURE 7.3 – Colour evolution of Madeira wines submitted to heating at 45 °C. TS0m – initial *TNM* sweet; TD0m – initial *TNM* dry; M0m – initial *Malvasia*; TS3m – *TNM* sweet after 3 months of heating; TD3m – *TNM* dry after 3 months of heating; M3m – *Malvasia* after 3 months of heating.

7.4 Conclusions

The work showed that the *estufagem* did not greatly affected the total content of polyphenols of the Madeira wines submitted to this procedure and moderately decreased the total polyphenolic composition, up to 25%, with at least 434.42 mg (GAE)/L of total polyphenols present after *estufagem*, comparable with most white wines. The antioxidant potential (0.94 – 1.64 mM) was also similar to white wines. In terms of individual polyphenols, 6 hydroxybenzoic acids, 3 hydroxycinnamic acids, 1 stilbene, 3 flavonols and 3 flavan-3-ols were found in these wines, with hydroxycinnamates and hydroxybenzoates being the most abundant phenolics. Most polyphenols decreased during the *estufagem*, except caffeic, ferulic, *p*-coumaric, gallic and syringic acids.

Finally, the colour of wines tends to the same chromatic characteristics when the heating procedure was applied and even red wines became clearer, with yellow tones becoming predominant, as monomeric anthocyanins gradually declined. Browning index values (absorbance at 420 nm) did not reveal a consistent trend during the heating period, but increased significantly at overheating conditions, especially for sweet wine, indicating the probable relation between Madeira wine browning and sugar degradation.

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CHAPTER 8

The effect of *estufagem* on the volatile profile and organic acids of Madeira wines

This chapter is based on the following publication:

Evolution of the volatile profile of Madeira wines submitted to the traditional thermal processing

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(Submitted)

Abstract

The evolution of monovarietal Madeira wines from *Tinta Negra Mole* (*TNM*) and *Malvasia* grape varieties, aged by the traditional thermal processing, was studied in terms of volatile compounds. Additionally, the organic acid profile was also evaluated. The wines were monthly analysed after heating at 45 °C for 3 months (standard conditions) and at 70 °C for 1 month (overheating conditions). The characterization of the volatile profile was performed analysing the dichloromethane extracts (SPE) by GC–MS, while the organic acid profile was examined by the simple injection of diluted samples (1:3) into the HPLC-DAD equipment.

The results showed that *estufagem* introduced significant changes in the volatile composition of Madeira wines, especially promoting the increase of the volatile fraction of both Madeira wine types (dry and sweet). At least 190 volatile compounds were identified, 53 of which were only encountered in wines after baking. Most chemical families increased after heating, especially furans and esters. On the contrary, alcohols, acetates and fatty acids presented a slight decrease after heating. Additionally, several varietal aromas, such as monoterpenic alcohols, especially encountered in *Malvasia* wine, disappear after baking. The obtained results also showed that estufagem favoured the development of some volatiles previously reported as typical aromas of Madeira wines, particularly phenylacetaldeyde, β-damascenone and 5-ethoxymethylfurfural. Additionally, ethyl butyrate, ethyl 2-methylbutyrate, ethyl hexanoate, ethyl isovalerate, guaiacol, 5-hydroxymethylfurfural and γ-decalatone were also found as potential contributors to the global aroma of baked wines. The obtained data also showed that Madeira wines are especially rich in malic acid (about 55%). Most acids declined during the thermal processing, especially succinic acid (up to 75%). However, lactic, acetic and formic acids did not show the same trend, increasing especially when overheating conditions were performed.

8.1 Introduction

Flavour is one of the most significant factors of wine quality, determining the consumer acceptance or rejection. Generally speaking, the aroma of wines is influenced by several different compounds, originated from grapes or resulting from winemaking and storage. In fact, these compounds act as a fingerprint for each wine type. Notwithstanding, in some cases, the occurrence of a particular compound is enough to give the characteristic aroma of a wine.

Madeira wine is characterized by marked and intense flavours. Its winemaking can include a peculiar maturation process, a baking phase so-called *estufagem*, wherein the fortified wines are heated up to about 45 °C, for at least 3 months. Then, the oxidative ageing goes further, since wine is placed in oak used casks (leaving some space at the top), ageing for a minimum period of 3 years. With baking a premature ageing takes place, being acquired some characteristics typical of the finest Madeiras (older wines only maturated in oak casks during several years), namely some aromas.

It is known that heating promotes important changes in the aroma of foods. Until date, there are some studies dealing with the accelerated oxidative ageing, promoted by thermal processing, on the volatile profile of wines. According to Cutzach et al. (1999), Deibner and Bernard, in 1956, studied the effect of the heat treatment on wine, pointing out the important role of Maillard reactions in the formation of the aroma of thermal processed sweet fortified wines, but were unable to identify the respective compounds. Cutzach et al. (1999) investigated the ageing of red and white sweet fortified wines (*Vins doux Naturels*) following an experimental laboratory study, in which, wines were forced-aged through heating at 37 °C for 12 months. Among the developed molecules during the accelerated ageing, they found that sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone), 5-ethoxymethylfurfural, 5-hydroxymethylfurfural (HMF), furfural acetylformoin and hydroxymaltol were involved in the aroma of sweet fortified wines. Latter, Escudero et al. (2000) also performed studies dealing with wine oxidative ageing, through laboratory oxidized samples at 20 °C for several weeks. According to olfactometric studies (GC-O analysis), they have found that the impact odorants of oxidized white wines were essentially 2, 4, 5-trimethyldioxolane, methional, sotolon and eugenol. Changes in the volatile content of *Fino* Sherry wines exposed to high temperature (45 °C) and UV–Vis radiation has also been reported (Benítez, Castro, Natera, & Barroso, 2006). These experiments revealed the decrease of most esters, acids and alcohols, and the increase of furfural and benzaldehyde. Recently, López de Lerma (2010) thermally processed sweet Pedro Ximénez wines at 65 °C up to 30 days and reported the increase of volatile Maillard products, specifically of HMF, 5-ethoxymethylfurfural, dihydromaltol, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), 2-methyltetrahydrofuran-3-one, furaneol, dihydro-2-methyl-3(2H)-furanone and cyclotene (corylon). Finally, Loscos et al. (2010) reported that the accelerated ageing, at 50 °C for 9 weeks, of wines supplemented with grape flavour precursors also introduced important changes in the volatile composition. This study showed that the main differences were observed in the first week of accelerated ageing. Most compounds first showed a significant increase and later a steady reduction, including Riesling acetal, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and *(E)*-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB). However, vanillin derivatives, furan linalool oxides, 3-oxo-*ϐ*-ionone, actinidols, 4-ethylphenol, and guaiacol showed a continuous increase during the ageing process.

In the case of Madeira wines, which are traditionally forced-aged, few studies were done regarding the effect of the baking step on their volatile profile. The first study, carried out by Oliveira e Silva (2008) based on GC-O analysis, highlight the occurrence of those volatiles imparting notes considered typical of finest Madeiras. Those volatiles were the following volatile Maillard products: sotolon, furfural, 5-methylfurfural, 5-ethoxymethylfurfural, methional, and phenylacetaldehyde. In this sense, the aim of this study was to evaluate the effect of *estufagem* on the volatile profile of Madeira wines. For this purpose, three Madeira wines were prepared: dry and sweet *Tinta Negra Mole* (*TNM*), and the traditional sweet *Malvasia*, and heated at 45 °C during 3 months, after fortification. Additionally, overheating conditions, 70 °C for 1 month, were also accomplished to force the development of volatiles specific from heating. The organic acid profile was also evaluated during this period, to understand their involvement in the development of the volatile profile.

8.2 Experimental

8.2.1 *Wines*

Three Madeira wines were prepared from two *Vitis Vinifera L.* grapes varieties from the 2007 harvest: dry *TNM* (red variety), sweet *TNM* and sweet *Malvasia* (white variety). The wines were produced using the winemaking practices of a local Madeira wine-producing cellar. The elaboration of these wines was conducted in separate stainless steel tanks. The alcoholic fermentation was conducted under controlled temperature and without adding any commercial yeast. The fermentation of the sweet *TNM* was stopped by the addition of natural grape spirit when grape must density attained 1025 g/cm^3, remaining 115 g/L of reducing sugars, while for the dry *TNM* it was allowed the density reaches 986 g/cm^3 before fortification, maintaining a low level of residual sugars (about 4 g/L). In the case of sweet *Malvasia,* the must was fermented until 1019 g/cm^3 before fortification, maintaining 96 g/L of reducing sugars. After the final adjustments, each wine was heated at 45 °C during 3 months in a special pilot scale system equipped with 200 L stainless steel tanks, fitted with heating coils for the circulation of hot tap water. To force the development of volatiles specific from heating, about 250 mL of each wine were overheated in a lab oven at 70 °C during 1 month (overheating conditions). All wines were monthly sampled and kept at -20 °C before being analysed.

8.2.2 *Standards and reagents*

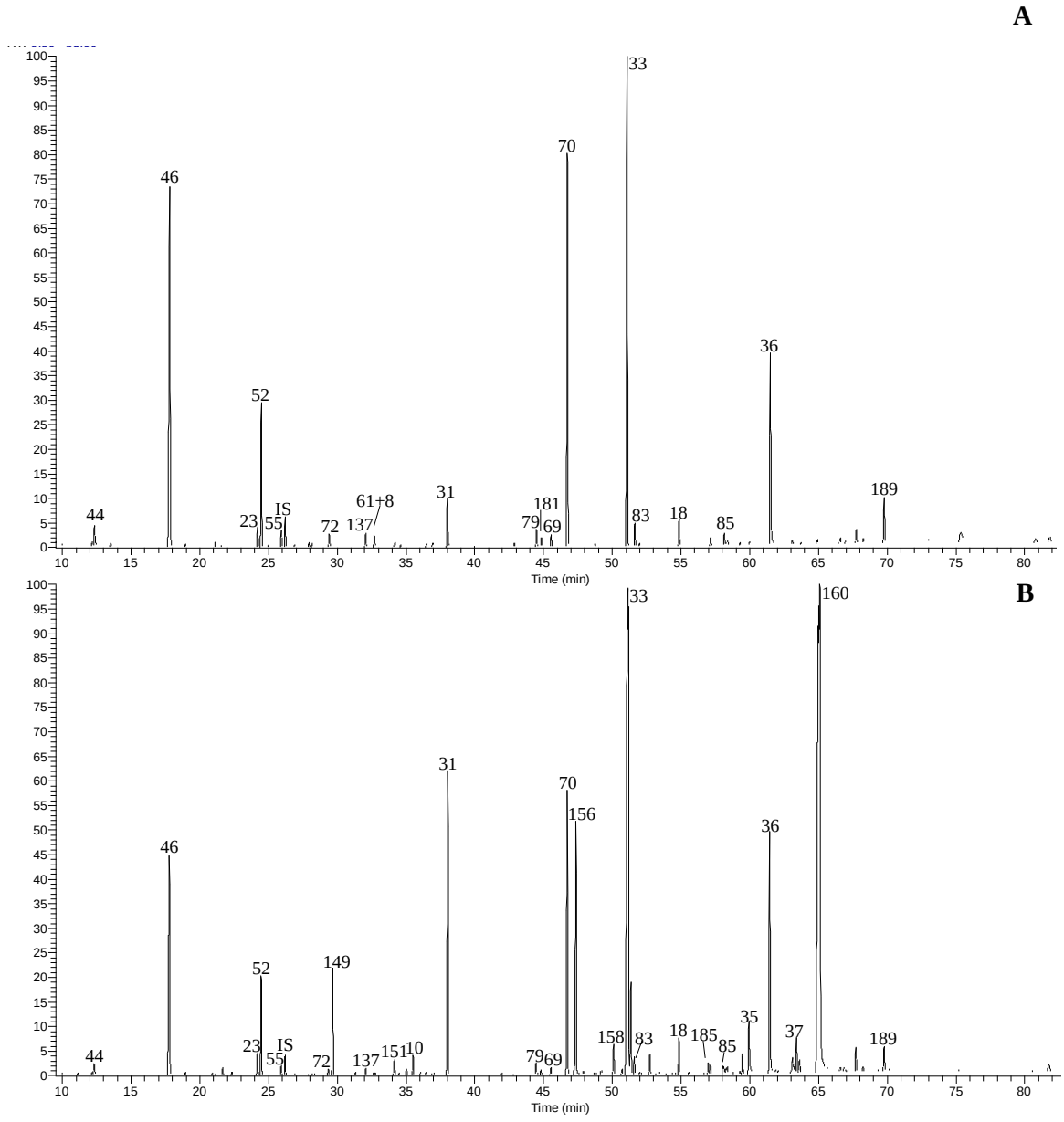
All reagents were of analytical quality. HPLC-grade dichloromethane, acetonitrile and methanol were from Fisher Scientific (Loughborough, UK) while absolute ethanol was supplied by Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). Disodium hydrogen phosphate dihydrate was supplied by Panreac Química S.A. (Barcelona, Spain) while sulphuric acid was supplied by Riedel-de-Haën (Seelze, Germany). Solid anhydrous sodium sulphate was from JMGS (Lisbon, Portugal) while the 3-octanol standard was from Sigma–Aldrich. LiChrolut EN resin was supplied by Merck Co. (Darmstadt, Germany). 6 mL polypropylene cartridges and respective frits were obtained from Supelco (Bellefonte, PA, USA). Solid phase extraction was carried out in a 12-port Visiprep™ SPE vacuum manifold from Supelco. For the HPLC analyses, eluents were previously filtered with 0.45 µm membrane filters obtained from Pall (Ann Arbor, MI, USA). The organic acids standards were those previously reported in Chapter 4 (section 4.2.1).

8.2.3 *Analysis of volatile compounds*

The screening of volatile compounds was accomplished based on the solid phase extraction (SPE) method proposed by López et al. (2002). Briefly, 120 mg of LiChrolut EN resin, a poly-(styrene-divinyl benzene) polymer used as reverse phase sorbent, was packed in a 6 mL cartridge. After conditioning the resin, in the SPE station, with 4 mL of dichloromethane, 4 mL of methanol and 4 mL of ethanolic solution (18%, v/v), 50 mL of wine spiked with 25 µL of 3-octanol (491 mg/L) were passed through the resin at about 2 mL/min. Then, the sorbent was dried by letting pass a small flow of air for 15 min. Finally, wine volatiles were eluted with 1.3

mL of dichloromethane and the extract dried with sodium sulphate, being kept at −20 °C until analysis. All samples were extracted in duplicate.

FIGURE 8.1 – Typical chromatograms of the dichloromethane extracts of *TNM* sweet wine before (A) and after (B) the heating process at 70 °C for 1 month. For peak identification see Table 8.2. The highlighted peaks correspond to the major peaks identified.



The extracts were analysed by GC-MS using the TRACE GC Ultra gas chromatograph equipped with the ISQ single quadrupole (electron impact mode) and the TriPlus autosampler (liquid mode) from Thermo Scientific (Hudson, NH, USA). Before injection the extracts were diluted in dichloromethane (1:5). Then, 1 µL of extract was vaporized in the injector port set to 230 °C, in splitless mode. All extracts were injected twice. The column was a DB-WAXetr 30 m × 0.250 mm with 0.50 µm film thickness from Agilent J&W (Folsom, CA, USA). The carrier gas was He at 1 mL/min. The ionization voltage was 70 eV with transfer line and ion source temperatures kept at 230 and 240 °C, respectively. The oven temperature program started at

40 °C for 5 min then increased up to 230 °C at 3 °C/min and finally was kept at 230 °C for 15 min. A 30–400 m/z mass range was recorded and the extracted ion peaks (m/z) described in Table 8.2 were considered to estimate the volatile concentration, overcoming some coelution problems. Figure 8.1 depicts typical chromatograms of the dichloromethane extracts of wines before and after the heating process at overheating conditions. The identification of compounds was made by comparison of the mass spectra obtained with those present in NIST08 and Wiley 6.0 MS library database, and comparing the obtained Kovats indexes with those stated on NIST Chemistry WebBook (Stein, 2008). Sixteen compounds (highlighted with * in Table 8.2) were only identified by comparison of the mass spectra obtained with those present in NIST08 and Wiley 6.0 MS library database, but all with at least a fair match (> 70%). A C_7–C_{30} n-alkanes mixture (Supelco) was used to calculate the Kovats indexes. 3-octanol was chosen as internal standard being added to each sample. The amount of each volatile compound was estimated, semi-quantitatively, regarding the added amount of internal standard (246 μg/L) and the relative concentrations of the investigated compounds, which were calculated by dividing the compounds area by the 3-octanol area. The coefficient of variation (% CV) was in average 7%. The concentration became underestimated when the single ion monitoring (SIM) was used, since the accounted area was smaller. To minimize this effect, the sum of the characteristics ion peaks was considered.

8.2.4 *Analysis of organic acids*

Organic acids were analysed based on the RP-HPLC-DAD method previously proposed (V. Pereira, et al., 2010). The analyses were carried out using a Waters Alliance liquid chromatograph (Milford, MA, USA) equipped with an auto-injector (Waters 2695, separations module), a photodiode array detector (Waters 2996) and the Empower Pro software, for data handling. Briefly, the samples were simply diluted (1:3) with Milli-Q water and 20 μL were directly injected in an Atlantis T3 column (250 × 4.6 mm, i.d.; 5 μm; Waters), after being filtered through membrane filters Acrodisc® CR PTFE (0.45 μm, Pall). The analysis was performed in 25 min using the gradient described in Table 8.1. All organic acids elute in the first 10 min and the next 15 min correspond to the regeneration and equilibrium period. The eluent flow was set to 1.0 mL/min and the column thermostated at 30 °C. The analytes were detected at 210 nm. All standards and wine samples were analysed in triplicate.

TABLE 8.1 – Organic acids gradient. Mobile phase solvents: A – 10 mM of phosphate solution buffered at pH 2.70; B – acetonitrile; C – methanol.

Time (min)	Flow (mL/min)	%A	%B	%C	Curve
---	1.00	100.0	0.0	0.0	---
10.00	1.00	90.0	10.0	0.0	6
12.00	1.00	50.0	25.0	25.0	6
16.00	1.00	50.0	50.0	0.0	1
25.00	1.00	100.0	0.0	0.0	1

Chromatographic peaks were identified comparing their retention times with those of standards and spiking samples with pure compounds. The quantification was carried out using the external standard method.

8.3 Results and discussion

8.3.1 *Volatile compounds*

Table 8.2 resumes the data of volatiles obtained for the current Madeira wines, before and after the heating at standard conditions (45 °C for 3 months) and at overheating conditions (70 °C for 1 month). The concentrations given in Table 8.2 should be interpreted with caution since they are rough estimates, but still, give an orientation to which order of magnitude the compounds are sensorially active and elucidate about the evolution of each compound with heating.

The GC-MS analyses of the current sample set allowed the identification of 190 volatile compounds including 42 esters, 29 alcohols, 18 carbonyl compounds, 19 volatile phenols, 17 fatty acids, 15 furan compounds, 15 monoterpenes, 8 acetals, 7 lactones, 4 sulphur compounds, 6 norisoprenoids and also 10 miscellaneous compounds. At least 171 compounds could not be identified by the regular strategies and should require specific methods of isolation and characterization. Acetaldehyde and ethyl acetate, both extremely polar and small odorants, usually referred as present in Madeira wines (Câmara, Alves, & Marques, 2006), could not be measured since in this conditions they elute with the solvent peak, which was not recorded.

The current study also revealed that a large number of compounds were developed during the traditional heating of Madeira wines, at least 53 compounds were only found in wines after baking. The results showed that *TNM* dry wine presented the highest fraction of volatiles, about 52 mg/L before heating, which is in agreement with the fermentation extension, essentially due to the higher levels of esters and higher alcohols. This study also showed that the volatile fraction of these Madeira wines increased after the heating process has been performed: up to 88% in sweet wines and up to 28% in the dry wine.

8.3.1.1 *Esters*

Esters were one of the most abundant groups. Before the heating step they represented in average 35% of the volatiles of these wines. This chemical family was represented by 19 fatty acid ethyl esters, 18 esters of organic acids and 5 acetates, but it was the esters of organic acids that represented the highest fraction (Table 8.2). Indeed, esters are generally ubiquitous in wines since they are secondary aromas, usually considered important to the sensory properties of wines, contributing with positive aromas, essentially with fruity notes. As expected, they appeared at higher amounts in the dry wine namely because it was more fermented. The most abundant fatty acid ethyl ester was diethyl 2-hydroxypentanedioate followed by ethyl hexanoate. The first is not so commonly found in wines but it was also found by Lee & Noble (2003) when they characterize the odour-active compounds of Californian Chardonnay wines. They reported that this ester has a cotton candy aroma.

TABLE 8.2 - Concentrations of volatiles in *Malvasia, TNM* sweet and dry wines before and after the heating at 45 °C (3 months) and 70 °C (1 month)

#	Compounds	KI	m/z	*TNM* sweet (µg/L)						*TNM* dry (µg/L)						*Malvasia* (µg/L)						Oth	Common descriptors
				0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	(µg/L)	
	Esters (42)																						
	Fatty acid ethyl esters (19)																						
1	ethyl butyrate	1053	43+71+88+101	6.2	0.2	9.5	0.5	11.5	0.5	19.2	1.3	**27.5**	**0.6**	**52.7**	**20.8**	4.8	0.3	7.8	0.1	9.5	0.3	20 [a]	fruity [a]
2	ethyl 2-methylbutyrate	1069	30-400	n.d.		n.d.		n.d.		n.d.		8.4	0.4	**25.3**	**8.5**	n.d.		n.d.		6.7	1.0	18 [a]	fruity [a]
3	ethyl hexanoate	1251	30-400	**25.7**	**0.5**	**30.0**	**1.7**	**37.4**	**2.6**	**96.6**	**5.4**	**96.4**	**2.4**	**150.8**	**3.5**	**33.0**	**3.5**	**43.8**	**0.6**	**48.9**	**1.7**	14 [a]	fruity [a]
4	ethyl 3-hexenoate	1320	30-400	n.d.		n.d.		n.d.		n.d.		2.8	0.5	n.d.		3.1	0.3	3.7	0.4	3.7	0.3	---	---
5	ethyl 3-ethoxypropionate	1354	30-400	n.d.		n.d.		7.9	0.3	n.d.		n.d.		n.d.		n.d.		1.8	0.3	8.1	0.3	---	---
6	ethyl octanoate	1452	41+57+60+73+ 88+101+127	14.3	0.4	12.0	0.7	14.0	1.3	95.9	3.4	57.8	1.3	92.0	2.2	20.1	2.5	30.5	0.6	20.6	0.6	580 [a]	fruity, apple [c]
7	ethyl 3-hydroxybutyrate	1544	30-400	n.d.		3.3	0.2	4.1	0.2	13.0	0.4	14.0	0.8	14.3	1.0	n.d.		n.d.		n.d.		20,000 [a]	---
8	ethyl 2-hydroxy-4-methylpentanoate	1568	43+69+87+104	9.1	0.1	15.0	0.1	18.3	0.4	46.5	0.8	83.6	1.6	95.8	1.4	24.0	0.2	32.6	0.8	42.5	0.3	---	---
9	ethyl 4-oxobutyrate	1576	85+102	5.5	0.1	7.1	0.0	9.4	0.5	3.3	0.3	5.2	0.3	4.6	0.1	2.5	0.2	5.3	0.2	5.8	0.8	---	---
10	ethyl levulinate	1636	43+74+99+129	n.d.		6.4	0.0	131.7	0.8	n.d.		7.6	0.1	24.8	1.4	1.1	0.1	9.4	0.1	145.3	2.2	---	---
11	ethyl decanoate	1655	41+43+55+70+ 73+88+101+115 +155+157	5.9	0.2	4.8	0.2	3.6	0.4	92.8	2.2	36.3	3.0	30.1	0.6	6.5	1.2	7.0	0.5	3.5	0.3	200 [a]	grape [e]
12	ethyl 9-decenoate	1708	30-400	n.d.		n.d.		n.d.		6.1	0.6	n.d.		n.d.		n.d.		n.d.		n.d.		---	---
13	ethyl 4-hydroxybutyrate	1834	30-400	11.5	1.1	23.0	2.7	26.2	1.9	159.5	6.8	74.2	2.4	51.8	3.2	38.4	1.4	3.6	0.4	26.2	0.3	---	---
14	ethyl dodecanoate	1858	30-400	5.6	0.8	n.d.		n.d.		12.2	1.1	n.d.		n.d.		4.6	0.9	n.d.		n.d.		>800 [d]	sweet, floral, fruity, cream [d]
15	diethyl adipate	1921	30-400	n.d.		n.d.		n.d.		4.7	0.7	4.0	0.5	n.d.		n.d.		3.9	0.4	6.3	0.4	---	---
16	ethyl 3-methylbutyl butanedioate	1925	30-400	3.3	0.4	7.7	0.3	7.4	0.7	28.2	1.6	46.6	1.8	75.5	4.5	n.d.		9.0	0.7	15.9	1.4	---	---
17	propyl ethyl hydroxybutanedioate*	2159	43+71+89+117+ 131+145	n.d.		n.d.		n.d.		n.d.		n.d.		12.5	0.9	n.d.		n.d.		n.d.		---	---
18	diethyl 2-hydroxypentanedioate	2196	30-400	232.7	4.0	524.1	12.1	486.8	18.5	1,037.1	45.1	2,208.5	89.4	2,127.1	74.6	420.9	4.2	900.5	14.6	849.6	13.5	---	cotton candy [o]
19	ethyl palmitate	2268	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		11.7	2.1	n.d.		n.d.		---	---
	Subtotal			319.9		643.0		758.2		1,615.1		2,672.9		2,757.5		570.8		1,058.9		1,192.7			
	%			1.6		1.8		1.2		3.1		4.0		4.1		1.8		2.3		1.5			
	Esters of organic acids (18)																						
20	monomethyl succinate	1062	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		28.6	8.7	n.d.		n.d.		n.d.		---	---
21	ethyl isovalerate	1085	30-400	n.d.		n.d.		**6.9**	**1.0**	n.d.		**14.1**	**0.8**	**47.5**	**18.4**	n.d.		**4.3**	**0.4**	**9.2**	**1.3**	3 [b]	fruity, apple [l]
22	ethyl pyruvate	1293	30-400	10.9	0.2	10.8	0.6	28.3	0.9	26.4	0.9	27.9	0.8	20.7	1.4	34.4	0.1	26.6	1.0	39.0	1.1	---	herbaceous, oil painting [c]
23	ethyl lactate	1368	30-400	168.2	3.2	251.3	3.0	298.5	3.8	1,033.7	15.2	1,349.8	29.3	1,228.0	34.5	290.1	5.8	381.8	4.7	447.4	9.6	154,636 [a]	strawberry, raspberry [c]
24	ethyl glycolate	1445	30-400	n.d.		5.6	0.4	9.2	0.3	n.d.		4.5	0.4	4.6	0.4	46.8	4.2	12.2	0.8	11.2	0.7	---	---
25	ethyl 2-hydroxy isovalerate	1450	43+55+73+76+ 104	2.2	0.1	6.2	0.1	9.7	0.2	11.3	0.7	34.4	1.8	52.1	0.2	n.d.		21.1	0.3	30.3	0.8	---	---
26	isobutyl lactate	1483	30-400	n.d.		n.d.		n.d.		12.9	0.7	32.3	2.3	23.3	2.9	n.d.		n.d.		n.d.		---	---
27	isoamyl lactate	1593	30-400	n.d.		n.d.		n.d.		37.7	0.6	49.6	1.1	60.3	2.9	n.d.		4.3	0.5	4.7	0.3	---	---
28	diethyl malonate	1602	88+114+133	4.6	0.1	5.4	0.1	2.5	0.1	n.d.		n.d.		n.d.		2.0	0.1	6.9	0.1	n.d.		---	---
29	ethyl methylsuccinate	1662	55+59+101+115 +129	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		2.6	0.1	9.1	0.2	13.4	0.3	---	---
30	diethyl methylsuccinate	1665	30-400	n.d.		3.4	0.3	12.1	1.2	n.d.		8.7	1.0	26.0	0.4	n.d.		n.d.		n.d.		---	---
31	diethyl succinate	1699	101+128+129	253.3	4.6	1,627.4	13.3	2,643.4	74.3	1,277.8	14.9	4,677.0	49.0	6,240.2	988.7	691.2	6.9	3,170.3	33.9	4,742.7	20.1	200,000 [a]	fruity [d, e]; wine [e]
32	ethyl glutarate	1803	87+114+115+ 143	1.3	0.0	5.2	0.0	7.6	0.3	4.1	0.1	17.7	0.2	23.4	0.8	2.4	0.0	9.2	0.0	12.5	0.3	---	---
33	diethyl malate	2077	30-400	4,824.1	50.1	13,657.0	815.7	16,017.9	1,186.2	3,836.4	150.2	11,597.3	210.8	13,007.9	1,967.0	4,935.0	264.9	13,125.3	291.4	15,906.6	51.8	760,000 [n]	over-ripe, peach, cut grass [n]

TABLE 8.2 (continued)

#	Compounds	KI	m/z	*TNM* sweet (µg/L)						*TNM* dry (µg/L)						*Malvasia* (µg/L)						Oth	Common descriptors
				0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	(µg/L)	
34	ethyl phenyl lactate	2314	30-400	52.5	1.7	90.2	3.2	99.3	6.5	408.3	17.7	534.6	16.2	529.7	23.9	137.7	3.3	242.4	7.1	313.4	5.1	---	---
35	diethyl tartrate	2371	30-400	49.5	3.7	406.5	7.5	947.9	30.8	70.7	7.4	330.5	24.2	759.9	17.4	38.6	7.3	409.1	27.2	1,139.4	30.8	---	earth, must [e]
36	ethyl hydrogen succinate	2426	30-400	1,928.7	25.8	3,997.2	241.7	3,620.3	229.2	6,723.4	880.5	8,836.9	236.3	8,001.3	1,172.6	4,354.1	239.0	6,487.2	106.9	6,435.1	83.7	---	sweet, sour, fruity [c]
37	ethyl citrate	2499	30-400	n.d.		46.3	0.3	239.8	12.0	n.d.	0.0	59.0	3.0	239.7	12.0	n.d.		92.2	16.1	219.6	9.9	---	---
	Subtotal			7,295.3		20,112.4		23,943.5		13,442.7		27,574.2		30,293.0		10,546.5		24,002.0		29,324.5			
	%			37.4		54.9		37.2		25.9		41.7		44.6		34.0		51.8		38.0			
	Acetates (5)																						
38	isoamyl acetate	1141	30-400	10.6	0.3	3.1	0.5	n.d.		**91.7**	**5.0**	19.8	1.9	22.2	0.2	16.1	1.5	4.3	0.6	n.d.		30 [a]	banana [a]
39	hexyl acetate	1289	30-400	2.9	0.2	n.d.		n.d.		6.7	0.6	n.d.		n.d.		3.0	0.4	0.0	0.0	n.d.		1,500 [a]	fruity, pear [d]
40	ethyl phenylacetate	1812	30-400	4.4	0.5	8.0	0.5	9.5	0.3	11.9	0.5	22.7	1.0	15.7	1.0	13.9	0.6	19.2	1.0	30.1	2.3	73 [p]	fruity, sweet [e]; honey-like [p] roses [a, c];
41	phenylethyl acetate	1844	43+91+104	7.8	0.1	n.d.		n.d.		39.0	0.7	11.7	0.5	10.4	0.3	14.9	0.3	3.7	0.2	n.d.		250 [a]	apple, honey, sweet [c]
42	ethyl 4-hydroxyphenylacetate	2958	30-400	n.d.		n.d.		n.d.		24.6	1.9	39.4	5.6	42.9	1.5	31.3	2.0	42.9	7.4	43.7	3.7	---	---
	Subtotal			15.1		8.0		9.5		82.3		73.8		69.0		63.0		65.8		73.8			
	%			0.1		0.0		0.0		0.2		0.1		0.1		0.2		0.1		0.1			
	Total			7,630.3		20,763.5		24,711.2		15,140.1		30,320.9		33,119.5		11,180.3		25,126.7		30,591.1			
	%			39.1		56.7		38.4		29.2		45.8		48.8		36.0		54.2		39.6			
	Higher alcohols (29)																						
43	1-propanol	1056	31+42+59+60	7.1	0.6	7.2	0.8	7.7	0.6	49.1	6.7	81.0	28.2	52.6	17.9	19.1	0.6	16.7	1.1	22.5	1.2	50,000 [d]	fresh, alcohol [d]
44	isobutyl alcohol	1108	30-400	234.7	4.7	224.8	3.2	196.0	12.5	1,227.6	36.6	1,337.7	187.8	998.5	44.8	441.3	18.9	403.3	5.9	412.1	11.1	40,000 [d]	fusel, alcohol [d]
45	1-butanol	1163	30-400	5.8	0.4	6.9	0.5	4.6	0.6	21.3	1.6	22.0	2.0	15.9	1.3	5.6	0.6	12.8	0.7	7.1	0.5	150,000 [d]	medicinal, alcohol [d]
46	isoamyl alcohol	1228	30-400	4,181.4	93.0	4,335.6	198.2	3,679.7	168.7	16,556.2	1,580.6	15,336.1	353.7	13,465.9	1,512.1	7,448.5	578.4	6,937.7	170.9	6,988.2	251.6	30,000 [a]	fusel [a]
47	1-pentanol	1270	30-400	4.8	0.4	4.1	0.5	2.7	0.1	13.2	0.7	9.6	0.5	6.6	0.2	6.9	0.9	6.1	0.4	5.2	0.3	80,000 [f]	fruity, balsamic [f]
48	4-methyl-1-pentanol	1334	30-400	3.0	0.2	3.3	0.1	3.9	0.5	13.5	0.9	13.7	0.5	15.9	0.5	5.7	0.4	4.6	0.3	6.1	0.4	50,000 [f]	almond, toasted [n]
49	2-heptanol	1339	45+55+70+83	1.8	0.1	1.7	0.0	1.7	0.1	2.3	0.1	2.4	0.1	2.5	0.1	n.d.		n.d.		n.d.		70 [f]	fruity, mouldy, musty [f]
50	*(Z)*-2-penten-1-ol	1343	30-400	2.1	0.3	1.8	0.3	2.3	0.3	2.7	0.4	2.9	0.5	2.9	0.4	3.6	0.6	3.3	0.6	3.6	0.5	---	---
51	3-methyl-1-pentanol	1348	30-400	4.0	0.3	3.6	0.3	4.5	0.5	19.3	0.3	19.1	0.7	19.5	0.3	7.7	0.6	6.8	0.7	7.9	0.6	---	---
52	1-hexanol	1374	30-400	1,247.5	13.9	1,268.4	23.6	1,255.3	27.6	1,088.4	8.1	1,054.1	14.7	1,090.7	10.0	1,117.6	36.6	992.8	11.1	1,058.9	10.5	8,000 [a, d]	green, grass [d]
53	*(E)*-3-hexen-1-ol	1385	30-400	22.6	0.9	21.7	0.6	22.2	1.2	17.6	0.2	19.8	0.7	17.3	0.4	21.6	0.7	25.2	0.5	22.3	0.3	400 [f]	green, floral [f]
54	3-ethoxy-1-propanol	1396	30-400	2.1	0.3	1.7	0.1	n.d.		14.7	0.4	13.5	0.8	11.7	0.3	6.0	0.3	5.3	0.2	6.2	0.4	---	fruity [e]
55	*(Z)*-3-hexen-1-ol	1405	30-400	150.9	2.6	142.6	1.9	132.0	0.7	164.0	1.3	147.0	4.0	142.4	1.5	291.6	3.7	253.1	1.5	251.0	4.8	400 [a, f]	grass [a], green [f]
56	*(E)*-2-hexen-1-ol	1429	30-400	18.4	0.4	18.1	0.1	20.5	0.7	3.2	0.1	3.8	0.5	4.7	0.5	17.7	0.2	17.0	0.3	17.1	0.4	400 [f]	grass, herb [f]
57	*(Z)*-2-hexen-1-ol	1439	30-400	7.3	0.7	7.3	0.5	8.0	0.3	4.1	0.3	4.9	0.7	5.1	0.4	5.0	0.7	4.9	0.6	4.2	0.5	400 [f]	grass, herb [f]
58	1-heptanol	1476	30-400	4.8	0.3	4.3	0.1	4.5	0.3	9.9	0.1	9.7	0.5	10.6	0.3	5.9	0.5	4.9	0.2	5.9	0.2	1,000 [f]	grape, sweet [f]
59	2-ethyl-1-hexanol	1510	30-400	5.0	0.2	8.7	0.2	4.1	0.1	5.8	0.2	13.7	0.3	8.4	0.6	5.4	0.6	10.5	0.6	4.6	0.3	8,000 [f]	mushroom, sweet fruit [f]
60	3,5,5-trimethylhexanol*	1527	30-400	2.9	0.1	3.4	0.4	4.3	0.2	6.7	0.4	6.7	0.5	9.1	0.5	n.d.		n.d.		4.1	0.2	---	---
61	2,3-butanediol, isomer 1	1566	43+45+57	81.0	1.4	69.7	16.1	28.5	6.5	173.7	72.2	267.2	111.4	255.2	4.3	n.d.		n.d.		n.d.		120,000 [f]	butter, cream [f]
62	1-octanol	1578	30-400	8.2	0.9	8.3	0.9	9.3	0.6	14.4	1.4	12.8	0.9	11.6	1.9	16.4	2.1	9.3	0.7	7.7	1.3	120 [f]	citrus, roses [f]
63	2,3-butanediol, isomer 2	1602	45+57	n.d.		n.d.		n.d.		28.7	14.3	50.1	25.6	43.8	1.9	n.d.		n.d.		n.d.		---	---
64	1,2-propanediol	1619	30-400	3.9	0.4	n.d.		n.d.		4.1	1.4	10.9	5.4	8.3	1.4	n.d.		n.d.		n.d.		---	---
65	1-nonanol	1680	55+56+69+70+83+84+97+98	1.6	0.1	1.7	0.1	2.0	0.1	4.0	0.1	4.1	0.2	4.4	0.1	2.9	0.0	2.7	0.1	n.d.		---	fat, green [e]
66	1-methoxy-2-butanol*	1683	31+45+59+75	n.d.		n.d.		n.d.		8.5	2.5	10.1	2.5	10.3	0.3	n.d.		n.d.		n.d.		---	---
67	1-decanol	1783	30-400	n.d.		n.d.		n.d.		2.6	0.2	2.7	0.6	5.4	0.9	2.7	0.2	n.d.		n.d.		400 [d, f]	orange flowery, fatty [d, f]

TABLE 8.2 - (continued)

#	Compounds	KI	m/z	*TNM* sweet (µg/L)						*TNM* dry (µg/L)						*Malvasia* (µg/L)						Oth	Common descriptors
				0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	(µg/L)	
68	buthoxyethoxyethanol*	1819	30-400	35.1	0.9	7.0	0.5	8.7	0.8	6.9	0.6	n.d.		n.d.		12.9	1.6	12.3	1.1	13.3	1.8	---	---
69	benzyl alcohol	1909	30-400	120.7	2.5	111.5	0.7	114.5	5.6	219.8	6.9	198.2	9.8	198.8	4.5	117.0	1.7	115.3	2.3	128.4	2.5	200,000 [b, f]	Citrusy, sweet [f]; almonds [c]
70	phenylethyl alcohol	1945	30-400	3,782.2	41.9	4,148.3	88.5	4,062.6	130.6	13,672.5	2,289.4	12,982.1	360.7	12,244.3	2,053.4	8,027.4	648.7	7,832.3	149.9	8,263.5	114.1	14,000 [a, f]	flowery, pollen, perfumed [f]; roses [a]
71	2-ethoxybenzyl alcohol*	1978	30-400	n.d.		25.3	0.3	62.0	0.2	n.d.		30.0	1.7	49.9	1.4	n.d.		32.0	2.4	108.9	5.1	---	---
	Total			9,939.0		10,442.3		9,694.4		33,354.6		31,666.0		28,712.4		17,588.3		16,708.9		17,404.0			
	%			50.9		28.5		15.1		64.4		47.8		42.3		56.7		36.1		22.6			
	Fatty acids (17)																						
72	acetic acid	1488	43+45+60	106.9	5.0	108.5	16.8	67.6	13.5	88.4	30.9	156.1	76.9	138.3	7.1	55.0	4.6	65.8	0.6	125.5	1.2	200,000 [f]	vinegar [a], acid, fatty [f]
73	propanoic acid	1573	45+57+73+74	n.d.		n.d.		2.5	0.2	5.5	0.8	3.8	0.4	n.d.		n.d.		n.d.		n.d.		8,100 [a, f]	vinegarish [f]
74	isobutyric acid	1601	41+43+73	14.8	0.2	11.5	0.5	9.3	0.5	46.4	2.0	45.2	4.0	41.9	1.1	19.0	3.5	19.4	0.6	19.9	0.4	2,300 [a]	rancid, butter, cheese [e]
75	butyric acid	1664	60+73	10.2	0.2	9.0	0.1	8.4	0.2	22.0	0.5	21.1	0.5	19.6	0.7	8.8	0.1	8.2	0.2	9.1	0.1	173 [a]	cheese [a, e]; rancid, sweat [e]
76	isovaleric acid	1704	60	n.d.		n.d.		n.d.		32.2	1.0	n.d.		n.d.		15.6	0.3	13.8	0.5	12.2	0.0	33.4 [b, f]	fatty, rancid [f]
77	2-methyl butyric acid	1704	41+57+74+87	13.1	0.3	n.d.		n.d.		**69.7**	**1.6**	47.9	6.3	n.d.		31.8	0.5	30.9	1.4	24.8	0.4	50 [a]	cheese, sweat [e]
78	pentanoic acid	1773	60+73	0.9	0.1	0.8	0.1	1.1	0.0	1.9	0.1	1.5	0.1	1.6	0.1	1.3	0.0	1.5	0.1	1.5	0.0	---	---
79	hexanoic acid	1880	41+60+73+87	92.9	3.4	88.2	1.0	103.5	4.1	308.61	6.65	301.32	12.31	294.95	3.78	152.3	2.3	140.7	3.2	148.2	2.1	420 [a]	cheese, rancid, fatty [f]
80	2-ethyl hexanoic acid	1981	30-400	13.0	1.3	n.d.		n.d.		n.d.		n.d.		17.8	2.4	19.5	1.8	11.5	1.6	0.0	0.0	---	---
81	(*E*)-4-hexenoic acid	1996	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		13.0	1.1	8.5	1.4	12.4	1.7	---	---
82	(*E*)-2-hexenoic acid	2003	30-400	28.8	1.7	33.6	0.2	36.6	1.2	16.2	1.4	18.5	1.1	17.8	0.7	15.0	0.8	10.0	1.5	21.6	0.8	---	musty, fatty [e]
83	octanoic acid	2094	30-400	200.7	6.6	199.7	7.4	240.3	17.4	**953.7**	**47.6**	**946.0**	**76.5**	**856.6**	**12.4**	448.9	2.2	371.3	10.7	317.5	9.2	500 [b, f]	rancid, harsh, cheese, fatty acid [f]
84	nonanoic acid	2202	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		13.9	1.8	n.d.		n.d.		---	green, fatty [e]
85	decanoic acid	2307	30-400	109.4	5.1	107.6	3.6	94.1	9.4	304.4	21.0	355.5	20.8	284.9	13.5	216.6	4.1	186.0	5.6	155.1	1.0	1,000 [a]	rancid, fatty [e]
86	benzoic acid	2482	51+77+105+122	30.8	3.7	26.7	5.0	36.2	2.4	25.2	1.2	33.0	4.7	40.6	2.8	45.3	1.1	37.3	7.0	29.5	3.2	1,000 [a]	urine [e]
87	dodecanoic acid	2519	30-400	n.d.		26.4	2.5	n.d.		53.1	9.4	40.8	3.5	60.4	9.0	44.9	2.2	n.d.		58.8	4.4	---	metal [e]
88	phenylacetic acid	2608	65+91+92+136	12.1	0.5	12.8	0.1	13.9	0.6	31.5	2.5	32.0	2.0	28.5	2.0	32.3	0.9	33.2	1.9	32.3	1.1	1,000 [a]	honey, flower [e]
	Total			621.6		612.1		599.7		1,927.3		1,970.7		1,774.4		1,101.0		905.1		936.1		---	---
	%			3.2		1.7		0.9		3.7		3.0		2.6		3.5		2.0		1.2			
	Volatile phenols (19)																						
89	ethyl salicylate	1839	30-400	17.4	0.3	10.0	0.2	9.0	0.8	6.7	0.4	6.2	0.6	7.8	0.6	n.d.		29.9	1.0	5.1	0.4	---	wintergreen, mint [e]
90	guaiacol	1892	30-400	2.2	0.2	4.3	0.3	**27.8**	**1.2**	n.d.		n.d.		**22.4**	**0.4**	n.d.		n.d.		n.d.		9.5 [b]	smoky, sweet, medicine [e]
91	4-ethylguaiacol	2065	77+91+122+137 +153	n.d.		n.d.		n.d.		1.9	0.1	1.8	0.1	2.2	0.1	5.7	0.2	5.3	0.2	6.8	0.2	33 [b]	spice, clove [b]
92	*m*-cresol	2120	30-400	n.d.		n.d.		7.3	1.3	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		68 [a]	leather [a]
93	4-ethylphenol	2215	30-400	n.d.		n.d.		6.0	0.9	8.6	0.4	6.7	0.8	9.0	0.9	8.5	1.4	10.1	0.6	11.1	1.0	440 [a]	phenolic [g]
94	4-vinylguaiacol	2234	77+107+135+ 150	n.d.		1.7	0.0	4.5	0.6	n.d.		1.6	0.3	13.9	0.3	n.d.		n.d.		40.6	2.1	1,100 [a]	clove-like, smoky [c]
95	syringol	2301	30-400	n.d.		16.1	2.1	107.2	7.6	n.d.		n.d.		118.6	17.4	n.d.		n.d.		n.d.		570 [a]	---
96	2,4-di-tert-butylphenol	2346	30-400	25.9	1.4	31.1	1.8	35.8	2.1	43.9	6.3	38.8	2.8	40.0	5.2	50.7	3.9	36.4	1.7	49.9	1.4	---	---
97	4-vinylphenol	2440	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		144.8	10.8	n.d.		n.d.		166.7	10.2	180 [a]	almond shell [e]
98	4-allyl-syringol	2579	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		18.8	0.2	n.d.		n.d.		n.d.		1,200 [a]	---
99	4-ethoxymethylphenol	2586	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		14.0	2.4	n.d.		---	---
100	vanillin	2613	81+109+123+ 151+152	38.4	1.0	42.5	0.8	39.5	1.4	13.7	0.9	27.1	0.7	17.0	0.9	22.2	0.5	54.4	2.2	28.2	1.3	60 [b]	vanilla-like, sweet [c]

TABLE 8.2 (continued)

#	Compounds	KI	m/z	*TNM* sweet (µg/L)						*TNM* dry (µg/L)						*Malvasia* (µg/L)						Oth	Common descriptors
				0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	(µg/L)	
101	methyl vanillate	2650	30-400	9.9	1.4	11.2	1.3	16.2	1.8	20.0	2.6	16.6	1.6	20.1	2.6	12.3	1.5	12.8	2.5	23.3	3.6	3,000 [b]	caramel, butterscotch, vanilla [e]
102	ethyl vanillate	2676	30-400	47.7	2.8	n.d.		n.d.		51.7	4.3	96.0	6.5	86.7	5.0	n.d.		n.d.		n.d.		990 [b]	flowery, fruity, sweet, vanilla [e]
103	acetovanillone	2687	30-400	17.7	1.8	24.3	0.6	18.3	1.4	26.9	4.2	31.1	6.2	33.7	4.0	26.7	2.0	66.7	6.4	49.4	4.4	1000 [a]	vanilla [e]
104	ethyl vanillyl ether	2780	30-400	n.d.		n.d.		n.d.		30.7	3.7	32.8	4.4	65.7	12.9	n.d.		n.d.		n.d.		---	---
105	3,4-dimethoxyphenol	2816	30-400	n.d.		n.d.		n.d.		14.8	2.3	n.d.		n.d.		n.d.		n.d.		n.d.		---	---
106	syringaldehyde	2989	30-400	54.6	4.8	94.3	29.2	60.1	3.2	n.d.		63.5	14.3	42.2	11.8	n.d.		27.6	5.3	n.d.		50,000 [a]	chocolate, grape, woody, smoky, wine-like [h]
107	4-hydroxybenzaldehyde	3015	30-400	101.8	11.1	105.0	7.8	104.2	10.0	51.3	7.6	177.1	15.9	107.9	12.0	139.5	2.0	268.8	21.7	46.5	5.2	---	---
	Total			315.7		340.6		436.0		270.2		499.3		750.8		265.5		526.1		427.7			
	%			1.6		0.9		0.7		0.5		0.8		1.1		0.9		1.1		0.6			
	Monoterpenes (15)																						
	Monoterpenic alcohols (12)																						
108	eucalyptol	1218	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		2.4	0.2	3.2 [i]	eucalyptus, fresh, cool, camphoraceous, medicinal [i]
109	linalool	1569	55+71+80+93+121	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		19.4	0.3	n.d.		n.d.		25 [a]	flowery [a]; citrus-like, bergamot [c]
110	3-terpinen-1-ol	1597	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		2.2	0.3	---	musty [e]
111	hotrienol	1634	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		3.3	0.3	n.d.		n.d.		---	hyacinth [e]
112	α-terpineol	1720	30-400	3.7	0.4	8.0	0.7	8.0	1.3	5.9	0.4	8.0	0.3	8.0	0.7	42.4	0.5	39.9	1.2	18.1	1.8	250 [a]	pine [c]; oil, anise, mint [e]
113	3,7-dimethyl-1,5-octadien-3,7-diol	1723	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		7.3	0.1	n.d.		n.d.		---	---
114	citronellol	1788	30-400	n.d.		n.d.		n.d.		3.9	0.3	n.d.		n.d.		n.d.		n.d.		n.d.		100 [a]	citronella [c]; rose [e]; green, lemon [f]
115	nerol	1825	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		4.1	0.5	n.d.		n.d.		---	sweet [e]
116	geraniol	1872	39+41+68+69+93+123	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		6.3	0.2	n.d.		n.d.		20 [a]	rose-like, citrus-like [c]; geranium [e]
117	2,6-dimethyl-3,7-octadien-2,6-diol	1974	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		49.5	0.9	7.2	1.0	n.d.		---	---
118	*p*-menthane-3,8-diol	2127	30-400	n.d.		n.d.		n.d.		n.d.		13.2	0.7	22.4	1.8	n.d.		n.d.		n.d.		---	---
119	3,7-dimethyl-1,7-octanediol	2235	30-400	n.d.		n.d.		n.d.		8.2	1.4	n.d.		n.d.		11.5	0.7	n.d.		n.d.		---	---
	Subtotal			3.7		8.0		8.0		18.0		21.2		30.4		143.9		47.1		22.7			
	%			0.0		0.0		0.0		0.0		0.0		0.0		0.5		0.1		0.0			
	Monoterpenic oxides (3)																						
120	*(E)*-linalool oxide	1462	30-400	n.d.		6.5	0.5	19.7	0.6	4.0	0.2	10.7	0.7	26.8	0.2	4.4	0.6	13.1	0.4	31.7	1.2	---	---
121	*(Z)*-linalool oxide	1490	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		10.0	0.3	n.d.		n.d.		n.d.		---	flowery [e]
122	linalool hydrate	2004	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		18.8	0.8	24.1	0.6	n.d.		---	---
	Subtotal			0.0		6.5		19.7		4.0		10.7		36.8		23.2		37.2		31.7			
	%			0.0		0.0		0.0		0.0		0.0		0.1		0.1		0.1		0.0			
	Total			3.7		14.6		27.7		22.0		31.9		67.2		167.1		84.3		54.4			
	%			0.0		0.0		0.0		0.0		0.0		0.1		0.5		0.2		0.1			
	Norisoprenoids (6)																						
123	2,6,6-trimethyl-2-cyclohexen-1-one	1422	30-400	n.d.		n.d.		2.8	0.2	n.d.		n.d.		3.7	0.6	n.d.		n.d.		2.6	0.4	---	---
124	vitispirane	1546	30-400	n.d.		2.5	0.3	2.9	0.3	n.d.		n.d.		4.7	0.5	n.d.		13.8	0.7	11.5	0.4	800 [j]	camphor, eucalyptus [j]
125	1-(2,4,6-trimethylphenyl)buta-1,3-diene*	1768	115+128+141+142+157+172	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		1.4	0.1	1.8	0.1	---	---

TABLE 8.2 - (continued)

#	Compounds	KI	m/z	*TNM* sweet (µg/L)						*TNM* dry (µg/L)						*Malvasia* (µg/L)						Oth	Common descriptors
				0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	(µg/L)	
126	1,1,6-trimethyl-1,2-dihydronaphthalene (TDN)	1770	142+157+172	n.d.		0.8	0.1	1.2	0.1	n.d.		n.d.		1.9	0.1	n.d.		n.d.		n.d.		20 [k]	petrol, kerosene-like [k]
127	*β*-damascenone	1846	30-400	n.d.		**3.5**	**0.3**	**12.0**	**1.7**	n.d.		n.d.		n.d.		n.d.		n.d.		**14.9**	**1.8**	0.05 [a]	baked apple [a]; rose, honey [e]
128	1-(2,3,6-trimethylphenyl)-3-buten-2-one	2146	30-400	n.d.		n.d.		36.9	4.9	n.d.		n.d.		53.7	4.4	n.d.		n.d.		51.5	1.2	---	---
	Total			0.0		6.8		55.7		0.0		0.0		64.1		0.0		15.2		82.4			
	%			0.0		0.0		0.1		0.0		0.0		0.1		0.0		0.0		0.1			
	Carbonyl compounds (18)																						
129	3-hexanone	1079	30-400	6.2	0.6	5.3	0.7	17.4	0.7	n.d.		n.d.		n.d.		n.d.		5.0	1.0	19.2	1.3	---	ether, grape [e]
130	hexanal	1098	30-400	6.2	0.4	5.3	0.6	4.8	0.5	n.d.		n.d.		n.d.		5.6	0.5	4.7	0.8	5.8	0.9	10 [g]	grass [a,g]; tallow, fatty [e]
131	*(E)*-3-penten-2-one	1145	30-400	4.9	0.3	3.7	0.5	4.7	0.8	n.d.		8.7	0.8	n.d.		5.5	0.7	5.4	0.2	5.3	0.7	---	---
132	4-ethoxy-2-pentanone*	1258	45+86+101	2.2	0.1	3.1	0.1	2.1	0.1	n.d.		4.8	0.2	0.9	0.0	3.1	0.0	7.7	0.1	3.3	0.1	---	---
133	4-ethoxy-2-butanone*	1280	30-400	n.d.		n.d.		4.5	0.3	n.d.		n.d.		n.d.		n.d.		n.d.		3.6	0.1	---	---
134	acetoin	1309	43+45+88	20.0	0.2	20.1	0.4	19.9	0.4	11.9	0.2	12.0	0.6	10.1	0.0	n.d.		n.d.		38.8	1.0	150,000 [a]	butter, cream [c]; flowery, wet [f]
135	acetol	1326	30-400	n.d.		5.0	0.2	52.8	1.8	n.d.		n.d.		n.d.		n.d.		n.d.		55.2	2.2	---	---
136	cyclopenten-3-one	1379	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		3.6	0.6	---	---
137	benzaldehyde	1551	30-400	127.6	1.5	102.9	1.3	103.0	2.6	10.2	0.2	50.6	2.9	22.0	2.2	71.8	0.9	77.2	1.1	62.5	1.6	2,000 [f]	almond [f]; burnt sugar [e]
138	2-cyclopentene-1,4-dione	1616	30-400	n.d.		n.d.		7.1	0.5	n.d.		n.d.		n.d.		n.d.		n.d.		9.8	0.7	---	---
139	phenylacetaldehyde	1673	30-400	**79.2**	**0.5**	**54.6**	**1.3**	**98.2**	**2.1**	**12.5**	**1.2**	**28.3**	**0.8**	**46.6**	**2.0**	**28.7**	**1.6**	**38.8**	**2.6**	**31.4**	**2.0**	1 [a]	green, honey [a]
140	phenylacetone	1756	43+65+91+92+134	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		6.0	0.2	3.7	0.1	---	---
141	ethyl nicotinate	1843	51+78+106+123+151	n.d.		3.5	0.1	7.6	0.3	n.d.		n.d.		10.0	0.2	n.d.		n.d.		6.9	0.1	---	---
142	cyclotene	1859	30-400	n.d.		n.d.		9.6	1.2	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		---	sweet, burnt [l]
143	ethyl picolinate*	2028	30-400	n.d.		n.d.		13.8	0.5	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		---	---
144	2H-pyran-2,6(3H)-dione	2033	30-400	n.d.		n.d.		n.d.		11.1	0.7	5.8	0.8	18.1	1.5	n.d.		n.d.		n.d.			
145	3-hydroxy-4-phenyl-2-butanone	2302	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		20.6	1.7	27.0	5.7	25.7	1.2	---	---
146	hydroxymaltol	2332	30-400	n.d.		49.8	4.8	81.9	10.7	n.d.		n.d.		n.d.		n.d.		119.4	8.8	31.7	3.3	---	---
	Total			246.1		253.4		427.3		45.8		110.2		107.7		135.4		291.1		306.7		---	---
	%			1.3		0.7		0.7		0.2		0.3		0.2		0.7		0.8		0.5		---	---
	Furan compounds (15)																						
147	2,2-dimethyl-5-(1-methyl-1-propenyl)-tetrahydrofuran	1258	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		3.0	0.1	---	---
148	dihydro-2-methyl-3(2H)-furanone	1286	30-400	n.d.		n.d.		8.5	0.3	n.d.		n.d.		n.d.		n.d.		n.d.		9.7	0.2	---	---
149	furfural	1492	95+96	8.0	0.1	159.4	3.3	987.5	16.1	5.6	0.0	98.8	1.6	563.1	18.0	9.2	0.2	143.9	2.2	1,038.2	14.4	14,100 [a,f]	almonds [c,e]; pungent [f]; bread, sweet [e]
150	2-acetylfuran	1533	95+110	n.d.		3.3	0.0	29.1	0.9	n.d.		2.3	0.1	15.8	0.4	n.d.		3.5	0.0	26.3	0.4	---	balsamic [e]
151	5-methylfurfural	1600	109+110	n.d.		6.9	0.1	116.7	3.3	n.d.		4.2	0.1	50.1	1.7	n.d.		5.2	0.1	98.1	2.8	20,000 [a]	almond, caramel, burnt sugar [e]
152	2-furyl ethyl ketone	1602	95+124	n.d.		n.d.		16.3	0.4	n.d.		n.d.		n.d.		n.d.		n.d.		25.9	0.3	---	---
153	2-acetyl-5-methylfuran	1642	30-400	n.d.		n.d.		6.7	0.4	n.d.		n.d.		6.8	1.6	n.d.		n.d.		10.8	0.6	---	---
154	ethyl 2-furoate	1650	30-400	5.8	0.8	12.7	0.4	44.1	0.8	5.5	1.0	12.0	0.5	57.9	1.4	12.3	0.6	21.6	1.5	76.9	1.6	---	vanilla, scorched [r]
155	furfuryl alcohol	1690	30-400	n.d.		2.6	0.4	9.7	0.6	4.9	0.9	n.d.		n.d.		n.d.		n.d.		13.5	2.0	2,000 [b]	burnt [e]
156	5-ethoxymethylfurfural	1962	30-400	n.d.		52.3	1.5	**3,616.4**	**114.8**	n.d.		n.d.		**117.7**	**9.8**	n.d.		26.2	1.7	**3,551.3**	**33.1**	90 [m]	---
157	2,5-furandicarboxaldehyde	2018	30-400	n.d.		6.6	0.4	101.5	7.4	n.d.		n.d.		n.d.		n.d.		n.d.		128.8	9.7	---	---
158	furyl hydroxymethyl ketone	2046	39+67+95+126	1.3	0.0	29.0	0.3	355.4	13.4	n.d.		n.d.		9.4	0.3	1.3	0.0	18.6	0.4	458.5	9.2	---	---
159	2,3-dihydrobenzofuran	2437	30-400	n.d.		21.2	1.4	44.2	4.2	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		---	---

TABLE 8.2 (continued)

#	Compounds	KI	m/z	*TNM* sweet (µg/L)						*TNM* dry (µg/L)						*Malvasia* (µg/L)						Oth	Common descriptors
				0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	(µg/L)	
160	5-hydroxymethylfurfural (HMF)	2551	30-400	41.3	0.8	3,124.7	141.3	**20,738.1**	**1,533.3**	35.7	14.8	82.0	5.4	868.7	49.7	32.0	2.3	1,518.1	97.7	**19,571.7**	**284.4**	12,000 [q]	caramel [r]
161	1-(5-hydroxymethyl-2-furanyl)-1-propanone*	2623	41+69+97+125+154	n.d.		n.d.		37.0	1.9	n.d.		n.d.		n.d.		n.d.		n.d.		131.7	6.7	---	---
	Total			56.4		3,418.8		26,111.2		51.6		199.2		1,689.5		54.8		1,737.1		25,144.4			
	%			0.3		9.3		40.6		0.1		0.3		2.5		0.2		3.7		32.6			
	Lactones (7)																						
162	γ-butyrolactone	1661	41+42+56+86	16.1	0.2	17.4	0.6	17.0	0.4	40.6	0.8	44.1	0.6	44.3	1.6	13.0	0.3	13.1	0.1	14.3	0.3	35,000 [a]	caramel, sweet [e]
163	α-angelica lactone	1460	30-400	n.d.		n.d.		3.9	0.4	n.d.		n.d.		n.d.		n.d.		n.d.		7.9	1.2	---	---
164	β-angelica lactone	1709	43+55+83+98+126	n.d.		n.d.		6.1	0.1	n.d.		n.d.		n.d.		n.d.		n.d.		6.9	0.2	---	---
165	γ-ethoxybutyrolactone	1756	47+56+57+58+85+86	14.4	0.2	13.2	0.2	17.5	0.2	9.7	0.5	14.9	0.4	13.2	0.6	5.2	0.1	9.4	0.7	11.4	0.3	---	---
166	γ-nonalactone	2060	30-400	n.d.		9.8	1.3	n.d.		8.7	2.2	10.1	0.5	20.0	2.0	n.d.		7.1	0.7	9.0	1.7	27 [g]	coconut, peach [e]
167	γ-decalactone	2112	43+57+85	n.d.		n.d.		**2.6**	**0.1**	**2.9**	**0.1**	**3.4**	**0.3**	**8.4**	**0.5**	n.d.		n.d.		n.d.		2.6 [g]	peach, fatty [e]; coconut [g]
168	γ-carboethoxy-γ-butyrolactone	2273	30-400	79.9	1.0	118.4	1.8	125.0	3.8	364.9	16.7	467.9	9.3	539.3	23.5	140.4	3.9	190.5	2.8	235.3	10.4	---	roast, smoke [e]
	Total			110.4		158.8		172.1		426.7		540.4		625.2		158.7		220.1		284.7			
	%			0.6		0.4		0.3		0.8		0.8		0.9		0.5		0.5		0.4			
	Sulphur compounds (4)																						
169	2-methyl-3-thiolanone	1556	30-400	n.d.		n.d.		n.d.		8.4	0.2	6.3	0.5	12.5	1.1	n.d.		n.d.		4.9	0.6	---	---
170	ethyl 3-(methylthio)propionate	1591	30-400	n.d.		n.d.		n.d.		11.3	0.7	14.2	2.0	17.4	2.2	n.d.		3.1	0.4	3.4	0.3	---	---
171	methionol	1747	30-400	n.d.		n.d.		n.d.		161.9	4.0	135.4	4.9	137.9	3.3	10.5	0.4	9.4	1.3	10.1	0.7	1,000 [b]	sweet, potato [e]
172	4-(methylthio)-1-butanol	1870	30-400	n.d.		n.d.		n.d.		9.3	0.4	7.2	0.8	8.5	0.9	n.d.		n.d.		n.d.		---	---
	Total			0.0		0.0		0.0		190.9		163.0		176.3		10.5		12.5		18.4			
	%			0.0		0.0		0.0		0.4		0.2		0.3		0.0		0.0		0.0			
	Acetals (8)																						
173	isovaleraldehyde diethyl acetal	1090	30-400	n.d.		n.d.		20.4	0.7	n.d.		n.d.		10.9	3.3	n.d.		n.d.		7.0	0.5	---	---
174	acetaldehyde ethyl amyl acetal	1121	30-400	n.d.		n.d.		n.d.		6.4	0.9	8.6	0.7	11.9	1.7	n.d.		3.2	0.4	n.d.		---	---
175	glycolaldehyde diethyl acetal*	1482	30-400	11.2	0.9	6.0	0.1	5.1	0.5	n.d.		n.d.		n.d.		6.5	0.5	13.3	1.1	5.7	0.6	---	---
176	furfural diethyl acetal	1484	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		20.3	0.7	---	---
177	*cis*-5-hydroxy-2-methyl-1,3-dioxane	1525	30-400	6.8	0.3	9.9	0.4	5.4	0.5	22.2	0.7	78.6	1.0	23.9	2.0	20.1	0.4	45.5	2.8	11.6	0.3	---	---
178	*cis*-4-hydroxymethyl-2-methyl-1,3-dioxolane	1639	43+45+57+59+87+103+117	5.2	0.2	7.0	0.2	61.7	1.3	31.2	0.5	39.6	0.6	19.3	0.4	11.1	0.2	20.2	1.0	5.2	0.4	---	---
179	phenylacetaldehyde diethyl acetal	1740	47+75+91+103+121+149	7.7	0.2	5.0	0.1	8.4	0.2	n.d.		n.d.		n.d.		6.3	1.0	2.0	0.1	2.9	0.4	---	---
180	*trans*-5-hydroxy-2-methyl-1,3-dioxane	1854	30-400	4.1	0.6	9.2	0.6	11.9	1.8	19.5	0.3	87.2	4.2	85.5	2.0	20.3	1.9	18.7	0.8	n.d.		---	---
	Total			35.0		37.1		112.9		79.4		214.0		151.6		64.2		102.8		52.5			
	%			0.2		0.1		0.2		0.2		0.3		0.2		0.2		0.2		0.1			
	Miscellaneous compounds (10)																						
181	N-(3-methylbutyl)acetamide	1889	30-400	n.d.		81.1	0.9	70.2	2.3	330.0	12.9	261.1	22.1	236.5	23.8	91.2	2.1	260.9	4.7	68.3	1.8	---	---
182	3,7-dimethyl-2,3-epoxy-6-octanyl-1-oxythiocarbonylimidazolide*	2083	30-400	n.d.		61.6	1.5	1,351.7	47.1	n.d.		n.d.		60.4	4.5	n.d.		44.2	4.7	1,264.4	8.6	---	---
183	ethyl pyrrole-2-carboxylate*	2117	30-400	n.d.		n.d.		7.5	0.7	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		---	---
184	2,3-dihydroxypyrazine*	2254	84+85+112	n.d.		n.d.		11.7	0.7	n.d.		14.9	1.6	26.9	2.3	n.d.		n.d.		9.5	0.5	---	---
185	curvulol*	2267	30-400	n.d.		24.1	1.6	150.4	5.8	n.d.		n.d.		76.3	1.2	n.d.		26.1	3.0	410.8	8.5	---	---
186	ethyl 2-formylpyrrole-1-acetate*	2308	30-400	n.d.		n.d.		n.d.		n.d.		n.d.		118.1	12.2	n.d.		n.d.		n.d.		---	---
187	diethyl phtalate	2404	30-400	16.3	1.5	32.4	1.1	n.d.		24.4	3.9	n.d.		n.d.		17.8	5.3	n.d.		n.d.		---	---
188	isobutyl phtalate	2574	30-400	14.1	2.0	11.9	1.0	n.d.		18.4	3.0	17.2	2.6	13.3	4.1	11.9	1.8	n.d.		n.d.		---	---

TABLE 8.2 - (continued)

#	Compounds	KI	m/z	*TNM* sweet (µg/L)						*TNM* dry (µg/L)						*Malvasia* (µg/L)						Oth (µg/L)	Common descriptors
				0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD	0 m	±SD	3 m, 45 °C	±SD	1 m, 70 °C	±SD		
189	dibuthyl phtalate	2729	30-400	457.3	8.0	325.8	3.4	403.2	48.6	113.8	9.7	139.8	6.4	91.7	12.8	165.6	2.8	150.2	7.5	127.5	9.0	---	---
190	phtalimide	2975	30-400	63.9	5.3	n.d.		n.d.		59.7	8.9	n.d.		n.d.		n.d.		n.d.		n.d.		---	---
	Total			551.7		536.9		1,994.8		546.2		433.0		623.1		286.5		481.4		1,880.6			
	%			2.8		1.5		3.1		1.0		0.7		0.9		0.9		1.0		2.4			
	TOTAL			19,506.1		36,579.8		64,253.3		52,054.9		66,148.8		67,808.1		31,012.4		46,211.3		77,076.1			

KI - Kovats index; m/z – masses selected to quantitation; SD – standard deviation; Oth - odour threshold; n.d. - not detected. In bold are highlighted the compounds above the odour threshold

a - (Campo, et al., 2006); **b** - (Gómez-Míguez, Cacho, Ferreira, Vicario, & Heredia, 2007); **c** - (Duarte et al., 2010); **d** - (Li, Tao, Wang, & Zhang, 2008); **e** - (Acree & Arn, 2004); **f** - (Jiang & Zhang, 2010); **g** - (Czerny et al., 2008); **h** - (Sigma-Aldrich, 2011); **i** - (Capone et al., 2011); **j** - (A. C. Silva Ferreira & Guedes de Pinho, 2004); **k** - (Mendes-Pinto, 2009); **l** - (El-Sayed, 2010); **m** - (Cutzach, Chatonnet, & Dubourdieu, 2000); **n** - (Sánchez-Palomo, Gómez García-Carpintero, Alonso-Villegas, & González-Viñas, 2010); **o** - (Lee & Noble, 2003); **p** - (Tat, et al., 2007); **q** - (Hauck, Landmann, Brühlmann, & Schwab, 2003); **r** - (Pisarnitskii, 2001)

Regarding to ethyl hexanoate, this ester is commonly reported in wines, namely was already reported as important odorant of young *TNM* wines (Perestrelo, Fernandes, Albuquerque, Marques, & Câmara, 2006), as well as of aged Madeira wines (Campo, Ferreira, Escudero, Marqués, & Cacho, 2006). In the current sample set, ethyl hexanoate levels (25.7 – 150.8 µg/L) appeared higher than its odour threshold (Table 8.2), so probably contributed to the aroma of these wines with fruity notes. Additionally, it was verified that both esters increased after the wines baking up to 125% for diethyl 2-hydroxypentanedioate and up to 56% for ethyl hexanoate. In fact, it was generally shown the increase of fatty acid ethyl esters after the heating step except for the following ethyl esters: octanoate, decanoate, dodecanoate and 4-hydroxybutyrate which, did not denoted a clear tendency. Temperature increase seems to accelerate the upward tendency. Contrary to these results, Câmara et al. (2006) observed the decrease of this esters during the oak-ageing of Madeira wines. Oliveira et al. (2008) found that ethyl esters of straight chain fatty acids related to yeast lipid metabolism (such as ethyl butyrate, hexanoate, octanoate and so on) decreased during bottle maturation of *Loureiro* wines but did not significantly decreased in *Alvarinho* wines. However, the same authors also reported the increased of ethyl esters of fatty acids related to yeast nitrogen metabolism of both varieties during the conservation period. Fatty acid ethyl esters increase during wine maturation or storage may be related with esterification while the decrease may be associated with hydrolysis reactions (Benítez, et al., 2006). Some fatty acid ethyl esters only appeared after baking, such as ethyl 2-methylbutyrate, ethyl 3-ethoxypropionate, ethyl levulinate and propyl ethyl hydroxypentanedioate. Ethyl levulinate was also encountered in sweet fortified wines by Cutzach et al. (1999) when they accelerated their ageing (heating at 37 °C for 12 months). According to them, levulinic acid, which can be formed by the heat breakdown of glucose, furfuryl alcohol or HMF in acidic medium and react with ethanol, being formed ethyl levulinate. Ethyl 3-ethoxypropionate, as far as we know, was only identified in brandies (Ledauphin et al., 2004; Schreier, Drawert, & Winkler, 1979) while propyl ethyl hydroxypentanedioate, apparently, was never identified in beverages.

Relative to esters of organic acids they represented the major fraction of esters (96%) and greatly increased (up to 72%) after baking, namely due to chemical esterification of the

corresponding organic acids. The most prevalent esters of organic acids were diethyl malate, ethyl hydrogen succinate, diethyl succinate and ethyl lactate, but none of them seemed to contribute to the flavour of these wines, because never surpassed their odour threshold, even after the heating. Some of them exhibited a large increase after *estufagem*, especially diethyl succinate, whose amount grew up to 6-fold after the standard baking and up to 10-fold at overheating conditions. Similar results were obtained by Câmara et al. (2006). Other ester that showed a marked development was diethyl tartrate, which came to grow up to almost 30-fold its initial value. This ester might be formed by esterification of tartaric acid with ethanol, contributing to the drop of the tartaric acid levels (Table 8.3). Once again, some esters were only found after wine's thermal processing: ethyl isovalerate, diethyl methylsuccinate and ethyl citrate. The concentration of ethyl isovalerate even surpassed its odour threshold (3 µg/L), especially in dry wine, and therefore accounted for a potential participation in the global aroma of these fortified wines. Diethyl methylsuccinate was previously identified in oak-aged Madeira wines by A. C. Pereira et al. (2010). Actually, these authors, following chemometric studies, found out that this ester together with ethyl lactate, ethyl methylsuccinate, diethyl succinate and ethyl hydrogen succinate were especially important in the ageing trends of older wines. Ethyl citrate was also found by Schneider et al. (1998) in sweet fortified wines from Grenache Noir. Its occurrence might be explained by the chemical esterification of citric acid with ethanol. In fact, this reaction may have contributed to the citric acid decline (Table 8.3).

Finally, the group of acetates was also present, although in much lower quantities (less than 0.2%). The major acetate found in these wines was isoamyl acetate, with higher levels in *TNM* dry. Actually, the young *TNM* dry wine presented higher levels of this acetate than its odour threshold, but the contribution of its banana-like scent diminished since its concentration decreased after the thermal processing. Similar to Câmara et al. (2006) results, most acetates declined after baking, except ethyl phenylacetate and ethyl 4-hydroxyphenylacetate, which indeed increased after this procedure. Both compounds are not commonly reported in wines, however it has been found in Aglianico del Vulture wines by Tat et al. (2007) and in Riesling wines by Güntert et al. (1986), respectively. Tat et al. (2007) suggested that ethyl phenylacetate occurrence might be related with shikimate pathway, such as phenylalanine and tyrosine, and also cinnamic acids, and therefore be produced during alcoholic fermentation by enzymatic esterification of phenylacetic acid. Likewise, Güntert et al. (1986) associated the occurrence of 4-hydroxyphenylacetate with the esterification of 4-hydroxyphenylacetic acid, a product of tyrosine metabolism. Probably the development of these esters with heating might be associated with the chemical esterification of phenylacetic acid with ethanol. In spite of its positive development, the concentration never attained the odour threshold (73 µg/L). The decrease shown by most of acetates after baking may have contributed for the loss of fruitiness of these wines.

8.3.1.2 *Higher alcohols*

Higher alcohols were quantitatively the largest group of volatiles, representing in average 57% of the total content of volatiles accumulated during the fermentation of these Madeira wines. However, taking into account their perception threshold, they did not seem to have influence on the aroma of these fortified wines.

These secondary aromas are essentially formed either from sugar catabolism or from amino acids decarboxylation and deamination (Ebeler, 2001). In this sense, unsurprisingly, sweet wines (short fermentation) presented the lowest content of alcohols (Table 8.2). From the 29 alcohols identified, isoamyl alcohol, phenylethyl alcohol and 1-hexanol were by far the most abundant higher alcohols, with concentrations between 1.0 and 16.5 mg/L. After the thermal processing of these wines, alcohols did not change very much, only small fluctuations were encountered, and so it was not possible identify a clear tendency. Câmara et al (2006) also obtained comparable results during Madeira wines oak-ageing. Only 2-ethoxybenzyl alcohol was formed during the heating step. As far as we know, this compound has never been reported in wines and may be derived from the reaction of ethanol with benzyl alcohol, which indeed slightly decreased after baking.

8.3.1.3 *Fatty acids*

Fatty acids in wines may have origin in grapes or be developed during the fermentative step by microbial organisms. Several compounds belonging to this chemical family were identified in these wines, including short chain (propanoic, butyric and pentanoic acids), medium chain (hexanoic, octanoic, nonanoic and decanoic acids), long chain (dodecanoic acid) and branched-chain fatty acids (isobutyric, isovaleric, 2-methylbutyric, 2-ethyl hexanoic, 4-hexenoic and *(E)*-2-hexenoic acids). Together, they did not represent more than 3.5% of the volatile fraction of these wines. Fatty acids were more abundant in *TNM* dry wine (Table 8.2), probably due to the extra fermentation time.

The most abundant was octanoic acid, with levels ranging from 199.7 to 953.7 µg/L. Actually, it was the only fatty acid exceeding its odour perception (500 µg/L), but only in *TNM* dry wine. Uncommonly, 4-hexenoic acid was encountered in the current *Malvasia* wine. According to deMan (1999), the presence of this acid in wines is usually related with the microbial degradation of sorbate, generating a geranium off-flavour note. Regarding to acetic acid, seems to be underestimated in the GC-MS analyses (values less than 156.1 µg/L) while its concentration was rigorously quantified by HLPC-DAD. The difference might be explained taking into consideration that the different signal responses of each compound in the GC-MS and the different capacity of LiChrolut EN extract each compound were not taken into account. Once again, acetic acid did not show a clear tendency with heating. In general, fatty acids decreased after the baking step (Table 8.2), probably due to their participation in esterification reactions with ethanol.

8.3.1.4 *Volatile phenols*

Nineteen volatile phenols were identified in this sample set of Madeira wines. Quantitatively, they represent a minority group (less than 1.1%) among the volatiles encountered. However, some of these compounds may negatively affect the overall aroma of a wine if present at concentrations above its low odour thresholds, imparting off-flavours described as animal, horse sweat, leather or medicinal. The most common examples are vinylphenols and ethylphenols which can be originated from the decarboxylation of *p*-coumaric and ferulic acids through the action of *Brettanomyces* yeasts or by pyrolysis

(Domínguez, Guillén, & Barroso, 2002). Actually, 4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol and 4-vinylguaiacol were identified in these wines but all bellow their odour threshold (Table 8.2). Guaiacol was found above its odour perception (up to 3-fold higher) only in the *TNM* wines heated at 70 °C. Guaiacol is frequently considered a cause of defects in wines, imparting smoky notes. This result suggests that temperature accelerates its development whereat high temperatures should be avoided in the heating process. In wine, guaiacol occurrence is normally associated with oak barrel maturation, being formed by the lignin breakdown during wood toasting and then transferred to wine (Arapitsas, Antonopoulos, Stefanou, & Dourtoglou, 2004). However, this route does not explain its development with the heating. Probably, its development may be related with hydroxycinnamates pyrolysis (Singleton, 1981). Vanillin and its esters, acetovanillone, syringaldehyde and 4-hydroxybenzadehyde were also detected, but all bellow their odour perception limit. The occurrence of vanillin in wines is also commonly related with the release during oak wood lignin breakdown (Pérez-Coello & Díaz-Maroto, 2009), but in this case this observation is not likely because these wines did not pass through oak-ageing. A reasonable explanation is that maybe vanillin and its esters were transferred from grapes to wines (Flamini & Traldi, 2010). The slight increase of acetovanillone after wines heating is also interesting. Similarly, Escudero et al. (2000) also found acetovanillone in laboratory oxidized white wines. According to them, wine oxidation may release the glycosylated acetovanillone extracted from grapes. Others volatile phenols were only detected after baking, namely 4-vinylguaiacol, 4-vinylphenol, 4-allyl-syringol and syringol. Actually, generally speaking, volatile phenols seem to be promoted by the thermal treatment of wines.

8.3.1.5 *Monoterpenes*

In the current sample set, 15 monoterpenes were found: 12 alcohols and 3 oxides. Monoterpenic compounds are usually considered as varietal compounds because they are present in grapes, especially on skins, or arise from grape precursors. This group represents a small fraction of the volatiles found in these wines, less than 0.5%. The highest levels were presented in young *Malvasia* wine (white variety), with at least 8 times more.

In general, monoterpenic alcohols such as linalool, hotrienol, citronellol, nerol and geraniol, when present, disappeared with the wines heating. It was also observed the same tendency for most monoterpenic diols, excepting *p*-menthane-3,8-diol, which was only detected in baked TNM dry wine. It is known that the profile and content of monoterpenes can be altered during ageing essentially due to acid-catalysed reactions. For example, linalool can be transformed into *α*-terpineol and successively in 1,8-terpines, and geraniol and nerol into linalool and *α*-terpineol (Versini, Dellacassa, Carlin, Fedrizzi, & Magno, 2008). Probably, this explains the arising of eucalyptol (1,8-cineole), 3-terpinen-1-ol, *p*-menthane-3,8-diol and the increasing *α*-terpineol in *TNM* wines. According to Marais (1983) review, eucalyptol was also found in wines heated at 70 °C (wine pH at 1.0). Relative to monoterpenic oxides, namely the isomeric forms of linalool oxides, they sharply increased their levels after the heating process, while most monoterpenic diols tend to disappear after baking like most monoterpenic alcohols. Linalool oxides may have been formed by linalool oxidation via epoxide (Marais, 1983).

8.3.1.6 *Norisoprenoids*

Norisoprenoids have been frequently found in wines and are usually associated with carotenoids degradation during ageing processes, but can also be released through glycoside hydrolysis (Vinholes, Coimbra, & Rocha, 2009). Six norisoprenoids were identified in these Madeira wines: 2,6,6-trimethyl-2-cyclohexen-1-one, vitispirane, 1-(2,4,6-trimethylphenyl)buta-1,3-diene, TDN, *β*-damascenone and 1-(2,3,6-trimethylphenyl)-3-buten-2-one. These compounds were only detected in wines after being heated, indicating that temperature accelerates their formation. Kanasawud and Crouzet (1990) demonstrated that 2,6,6-trimethyl-2-cyclohexen-1-one formation in aqueous medium is related with the thermal degradation of carotenoids, namely *β*-carotene. Apparently, this ketone was never been identified in wines.

In the current study, an isomer of TPB, the 1-(2,4,6-trimethylphenyl)buta-1,3-diene, was identified according to Wiley 6.0 MS library, with the following ion peaks (m/z), ordered according to their abundance: 157, 142, 141, 128, 172, 115, and eluting before *β*-damascenone with a KI of 1768. Janusz et al. (2003) were the first researchers to report the occurrence of TPB in wines as a potent grape-derived odorant, with a very low odour perception limit of 40 ng/L. According to them, this compound exhibits green and cut-grass notes at low concentrations and pungent or chemical scents when present at higher concentrations. They also reported that TPB elutes just after the elution of *β*-damascenone on a Carbowax column, with a Kovats GC retention index of 1830 and a mass spectrum with the following ions, ordered according to their abundance: m/z 157, 142, 141, 172, 128, 115. Additionally, in the current study was also found in the studied Madeiras another compound with structural similarities with TPB, the 1-(2,3,6-trimethylphenyl)-3-buten-2-one, which, as far as we know, was only identified by Nykanen (Nykanen, 1986) in wine and distilled alcoholic beverages.

Finally, vitispirane and TDN were never been encountered above their odour perception, however *β*-damascenone, only detected in baked sweet wines, reached 300-fold above its odour threshold (0.05 µg/L) in wines heated at overheating conditions. This result leads us to conclude that this compound might play an important role in the aroma of Madeira baked wines. Campo et al. (2006) found that *β*-damascenone has a great contribution to the aroma of oak-aged Madeira wines.

8.3.1.7 *Carbonyl compounds*

Carbonyls group includes aldehydes and ketones. In the current sample set 18 carbonyls were found, of which 3 were aldehydes.

It is known that the major wine aldehyde is acetaldehyde, typically reaching concentrations between 350 to 450 mg/L and occasionally as a high as 1000 mg/L (Peinado & Mauricio, 2009). However, Câmara et al. (2006) has reported lower values for oak-aged Madeira wines (less than 117 mg/L). On the other hand, Campo et al. (2006) has observed that acetaldehyde was quite important for the aroma of oak-aged Madeira wines, reaching values up to 18.5 mg/L. In the current samples it was not possible to quantify this aldehyde since, as

already mentioned, elutes to earlier. However, other aldehydes were identified, namely hexanal, benzaldehyde and phenylacetaldehyde. The latter compound reached values far above its odour perception (98-fold higher) and it seems that baking favours its development (Table 8.2). These results indicate that phenylacetaldehyde was apparently important to the aroma of these wines. Actually, Oliveira e Silva (2008) through GC-O analysis found out that phenylacetaldehyde impart a significant contribution for the definition of baked Madeira wines flavour. Similar results were obtained by Campo et al. (2006) in oak-aged Madeira wines. Phenylacetaldehyde has been related with wine oxidative processes, imparting honey-like scents (Silva Ferreira, Guedes de Pinho, Rodrigues, & Hogg, 2002). The occurrence of this aldehyde in beverages has been related with Maillard reactions, specifically with Strecker degradation, through the phenylalanine degradation (Soares da Costa et al., 2004). According to Campo et al. (2006) the presence of hexanal is most likely due to the direct oxidation of hexanol.

Several ketones were identified in these wines, specifically some usually found in sweet fortified wines, namely cyclotene, 3-hydroxy-4-phenyl-2-butanone and hydroxymaltol (Brock, Kepner, & Webb, 1984; Câmara, et al., 2006; Cutzach, et al., 1999; López de Lerma, et al., 2010). Cyclotene and hydroxymaltol were only detected in baked wines at levels ranging from 9.6 to 119.4 μg/L. The presence of these two carbonyls in wines is usually associated with Maillard reactions (Pérez-Coello & Díaz-Maroto, 2009). Were also identified several carbonyls which are rarely identified in wines, namely *(E)*-3-penten-2-one, 4-ethoxy-2-pentanone, 4-ethoxy-2-butanone, cyclopenten-3-one, 2-cyclopentene-1,4-dione, phenylacetone, ethyl nicotinate, ethyl picolinate, and 2H-pyran-2,6(3H)-dione. *(E)*-3-penten-2-one, already encountered in wines before heating, was also found by Perestrelo et al. (2006) in young *TNM* wines. Cyclopenten-3-one, only detected in *Malvasia* baked at 70 °C, has been detected in foods submitted to thermal processing, especially in coffees (Nebesny, Budryn, Kula, & Majda, 2007; C. Sanz, Maeztu, Zapelena, Bello, & Cid, 2002). The formation of 2-cyclopentene-1,4-dione in foods has been related with the sugar degradation promoted by heating, namely in Maillard reaction (Ames, Guy, & Kipping, 2001; Tai & Ho, 1998). In fact, this compound was only found in sweet wines submitted to heating. As far as we know, this carbonyl has never been detected in wines. It seems that phenylacetone formation is also associated with the heating process since was only detected in baked *Malvasia* wines. Actually, it was identified in roasted model reactions consisting of glucose and phenylalanine (Baltes & Mevissen, 1988). 2H-Pyran-2,6(3H)-dione was previously identified in oak aged Madeira wines (A. C. Pereira, et al., 2010). It seems that the ethyls nicotinate and picolinate were only detected in these baked wines.

8.3.1.8 *Furan compounds*

At least 15 furan compounds were developed by the thermal processing of Madeira wines. Generally speaking, the heating developed more furans in sweet wines than in dry wines as well as higher contents. In sweet wines baked at standard conditions furans characterized in average 6.5% of the volatile fraction, while in the dry wine they represent less than 0.3%. Nevertheless, after baking at 70 °C, furans representation raised up to 40.6% in sweet wines and less than 2.5% in *TNM* dry wine. These results can be explained by the fact

that furans in wines are especially generated by the thermal degradation of sugars due to acid-catalysed reactions, or even through Maillard reaction (M. L. Sanz & Martínez-Castro, 2009). Some furans provided remarkable increases with baking, especially 5-hydroxymethylfurfural (HMF), 5-ethoxymethylfurfural, furfural, furyl hydroxymethyl ketone, 2,5-furandicarboxaldehyde and 5-methylfurfural (Table 8.2). Two furans exceeded their odour threshold in wines baked at 70 °C, 5-ethoxymethylfurfural and HMF. The former has surpassed 40-fold the odour perception and the second only almost 2-fold. This result indicates that these furans may be potential odorants of baked Madeira wines. Indeed, Oliveira e Silva (2008) observed that 5-ethoxymethylfurfural had an important role in the definition of the global aroma of baked Madeira wines. Cutzach et al. (1999) observed the same in sweet fortified wines and suggested that 5-ethoxymethylfurfural was obtained by the reaction of 5-hydroxymethylfurfural with ethanol catalysed by the wine acidic medium and temperature.

Other minor furans, usually reported in aged wines or wine vinegars (Chinnici et al., 2009; Cutzach, et al., 1999; Lee & Noble, 2003; A. C. Pereira, et al., 2010) were also identified, such as 2-acetylfuran, 2-acetyl-5-methylfuran, ethyl 2-furoate and furfuryl alcohol. Additionally, were also detected in baked wines minor furans rarely detected in wines, namely 2,2-dimethyl-5-(1-methyl-1-propenyl)-tetrahydrofuran, dihydro-2-methyl-3(2H)-furanone, 2-furyl ethyl ketone and 1-(5-hydroxymethyl-2-furanyl)-1-propanone. 2,2-Dimethyl-5-(1-methyl-1-propenyl)-tetrahydrofuran was already detected in the volatile fraction of ice wine (Setkova, Risticevic, & Pawliszyn, 2007).

8.3.1.9 *Lactones*

Lactones are essentially formed by yeast during alcoholic fermentation (Jackson, 2000). However, significant odorants lactones are usually accumulated during wine ageing. The most common example is sotolon which imparts powerful nutty, sweet, burnt, curry notes on those wines that is present. Very small amounts, slightly above than 5 µg/L, are sufficient to characterize the flavour of some wines, especially of fortified wines such as Port, Sherry and Madeira (Câmara, Marques, Alves, & Silva Ferreira, 2004; Martin, Etievant, Le Quere, & Schlich, 1992; Silva Ferreira, Barbe, & Bertrand, 2003). Specifically, Oliveira e Silva (2008) confirmed the importance of sotolon in the typicity of Madeira wines and observed the progressively increase of sotolon in Madeira wines submitted to *estufagem*. However, in the current baked Madeiras, this lactone was not detected, probably because the used procedure was not appropriated to detect this lactone. Nonetheless, 7 lactones were currently detected, most of them never surpassing their odour perception. The exception was *γ*-decalactone, which slightly surpassed its low flavour threshold (2.6 µg/L) in *TNM* dry wine. The results also showed that this lactone, as well as most lactones, suffered a slight increased after baking. Quantitatively, the most abundant lactone was *γ*-carboethoxy-*γ*-butyrolactone, with values ranging between 79.9 and 539.3 µg/L. Its content was higher in *TNM* dry wine indicating that longer fermentations favour its formation. Additionally, the results also showed that *γ*-carboethoxy-*γ*-butyrolactone greatly increased after heating, indicating that temperature favours its formation. This lactone was already reported in Madeira wines aged in oak casks (A. C. Pereira, et al., 2010). *γ*-Butyrolactone, commonly considered the most abundant wine lactone since it is essentially derived from the fermentative process, was also detected with values (13.0 –

44.3 µg/L) well below from those often found in wines (about 1 mg/L) (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). *γ*-Ethoxybutyrolactone was also detected (5.2 – 17.5 µg/L), probably formed by the reaction between ethanol and γ-butyrolactone. This lactone was previously identified in the volatile fraction of toasted wood chips (Fernández de Simón, Esteruelas, Muñoz, Cadahía, & Sanz, 2009; Vichi et al., 2007). Other minor lactones were also detected, namely *γ*-nonalactone and the two isomers (*α* and *β*) of angelica lactone. Pisarnitskii (2001) has suggested that angelica lactones in wines are products of sugar decomposition. Actually, these lactones were only detected in sweet wines baked at overheating conditions.

8.3.1.10 *Sulphur compounds*

Volatile sulphur compounds in wines are usually responsible for offensive odours, even when present in trace amounts, since they possess low perception thresholds (Jackson, 2000). Usually, their presence in wines is associated with the enzymatic (fermentative process) or non-enzymatic (storage or maturation processes) degradation of sulphur-containing compounds, especially amino acids (Mestres, Busto, & Guasch, 2000). In the current wines only 4 sulphur compounds were encountered and all apparently below their odour perception limit. The volatile profile of *TNM* sweet wine did not show any sulphur compound, even after *estufagem*. Methionol was the most abundant sulphur compound, especially in *TNM* dry wine (135.4 - 161.9 µg/L). In fact, *TNM* dry wine accumulated higher contents, probably because it was more fermented. Besides methionol, ethyl 3-(methylthio)propionate was also detected (3.1 – 17.4 µg/L). Both compounds are usually considered products of methionine metabolism (Mestres, et al., 2000). 4-(Methylthio)-1-butanol, usually related with homomethionine metabolism (Mestres, et al., 2000), was also found in *TNM* dry wine (7.2 - 8.5 µg/L). 2-Methyl-3-thiolanone, already identified in other wines (Aznar, López, Cacho, & Ferreira, 2001; Ferreira, Aznar, López, & Cacho, 2001), was also encountered in the current Madeira wines, with values ranging from 4.9 to 12.5 µg/L. Generally speaking, it seems that the heating process did not promote significant changes in the levels of sulphur compounds.

8.3.1.11 *Acetals*

The origin of acetals in wines is usually associated with the reaction of aldehydes with hydroxyl groups of two alcohols (Jackson, 2000). Madeira wines present favourable conditions for their production, since the alcoholic content is high and several aldehydes are present. In fact, 8 acetals were detected in the current sample set.

Similar to Câmara et al. (2003) we also encountered heterocyclic acetals in the current Madeiras, specifically *cis*-5-hydroxy-2-methyl-1,3-dioxane (*cis*-dioxane), *cis*-4-hydroxymethyl-2-methyl-1,3-dioxolane (*cis*-dioxolane) and *trans*-5-hydroxy-2-methyl-1,3-dioxane (*trans*-dioxane). Trans-dioxolane was not detected in these current sample set. Interestingly, *cis*-dioxane always increased when the baking was conducted at standard conditions, but did not always increased when the heating took place at higher temperatures. Similar trends were usually observed for *cis*-dioxolane and *trans*-dioxane.

Four diethyl acetals were also identified, namely the diethyl acetals of isovaleraldehyde, glycolaldehyde, furfural and phenylacetaldehyde, with values ranging from 2.0 to 20.4 µg/L. Additionally, an acetal probably originated from the reaction of acetaldehyde with ethanol and amyl alcohol (1-pentanol), the acetaldehyde ethyl amyl acetal, was also encountered. The acyclic acetals did not present a clear trend with the heating process.

8.3.1.12 *Miscellaneous compounds*

Finally, 11 compounds which do not belong to the aforementioned chemical families were also found in the current Madeiras. Among them were 4 phtalates, usually transferred to wines during winemaking from the plastic materials degradation (Carrillo, Salazar, Moreta, & Tena, 2007). Specifically, diethyl phthalate, isobutyl phthalate, dibutyl phthalate and phtalimide were detected. The most abundant was dibutyl phthalate, with values between 91.7 and 457.3 µg/L. In general, phtalates concentration decreased after the thermal processing.

Another compound, N-(3-methylbutyl)acetamide, that is often reported in wines (Oliveira, et al., 2008; Perestrelo, et al., 2006) was also found in the current Madeiras, especially in *TNM* dry wine. Finally, some compounds were only developed after baking, which was the case of the following compounds, tentatively identified by mass spectra (comparison with NIST and Wiley libraries): 3,7-dimethyl-2,3-epoxy-6-octanyl-1-oxythiocarbonylimidazolide, ethyl pyrrole-2-carboxylate, 2,3-dihydroxypyrazine, curvulol and ethyl 2-formylpyrrole-1-acetate. Interestingly, the compound identified as 3,7-dimethyl-2,3-epoxy-6-octanyl-1-oxythiocarbonylimidazolide sharply increased after baking from not detected up to 1,351.7 µg/L. Its increase was especially high in sweet wines.

8.3.2 *Organic acids*

The determination of organic acids in wines is of high interest, since they play an important role on the final characteristics of wines. During ageing, they can participate in the development of volatiles, namely ethyl esters, and in this sense contribute to the wine aroma. 8 organic acids were determined along with the Madeira wine heating process. Table 8.3 present the organic acids levels found during the thermal processing of the current Madeira wines.

The most abundant organic acid found on these Madeira wines was malic acid, ranging from 3.519 to 4.541 g/L. These values were higher than those found for other wines, such as fortified wines (0.491 – 2.378 g/L (Cunha, Fernandes, Faria, Ferreira, & Ferreira, 2002; Esteves, Lima, Lima, & Duarte, 2004)), red wines (0.040 – 2.627 g/L (Casella & Gatta, 2001; Peres et al., 2009; Villiers, Lynen, Crouch, & Sandra, 2003)) and white wines (0.656 – 3.103 g/L (Casella & Gatta, 2001; Peres, et al., 2009; Villiers, et al., 2003)). These values suggest that possibly the grapes used for the preparation of these wines should hold at harvest time, a large amount of this acid. The concentration of malic acid represented in average 55% of the acids present in these Madeira wines and generally decreased 16% during their thermal processing at standard

conditions. The temperature increase (overheating conditions) did not enlarge this effect. Malic acid was followed by acetic and tartaric acids.

TABLE 8.3 – Concentrations of organic acids in *Malvasia*, *TNM* sweet and dry wines during the heating at 45 °C (3 months) and 70 °C (1 month).

Organic acids (g/L)	0 m	± SD	1 m, 45 °C	± SD	2 m, 45 °C	± SD	3 m, 45 °C	± SD	1 m, 70 °C	± SD
***TNM* sweet**										
Oxalic	0.117	0.002	0.108	0.002	0.100	0.002	0.104	0.000	0.073	0.002
Tartaric	0.638	0.008	0.481	0.006	0.512	0.013	0.621	0.016	0.532	0.015
Formic	0.126	0.007	0.156	0.004	0.141	0.006	0.142	0.006	0.249	0.008
Malic	4.541	0.018	4.010	0.011	3.666	0.016	3.760	0.005	3.885	0.008
Lactic	0.250	0.007	0.302	0.013	0.325	0.014	0.350	0.008	1.588	0.011
Acetic	1.108	0.003	0.890	0.019	0.824	0.007	0.878	0.004	1.773	0.006
Citric	0.214	0.005	0.166	0.005	0.146	0.005	0.183	0.010	0.206	0.005
Succinic	0.091	0.002	0.071	0.002	0.082	0.003	0.069	0.003	0.132	0.006
Total	7.09		6.18		5.80		6.11		8.44	
***TNM* dry**										
Oxalic	0.046	0.000	0.040	0.001	0.041	0.002	0.044	0.003	0.037	0.001
Tartaric	1.123	0.014	0.480	0.010	0.527	0.012	0.522	0.001	0.331	0.006
Formic	0.228	0.008	0.256	0.008	0.224	0.009	0.231	0.012	0.223	0.010
Malic	3.901	0.017	3.731	0.039	3.519	0.015	3.522	0.007	3.532	0.057
Lactic	0.912	0.018	0.938	0.022	0.924	0.007	0.911	0.009	0.937	0.046
Acetic	1.328	0.019	1.569	0.009	1.635	0.034	1.840	0.013	1.979	0.008
Citric	0.360	0.003	0.267	0.015	0.228	0.009	0.223	0.001	0.210	0.006
Succinic	0.359	0.001	0.330	0.001	0.154	0.001	0.089	0.003	0.193	0.003
Total	8.26		7.61		7.25		7.38		7.44	
Malvasia										
Oxalic	0.047	0.002	0.047	0.001	0.047	0.001	0.040	0.002	0.036	0.001
Tartaric	0.806	0.003	0.597	0.013	0.666	0.006	0.481	0.011	0.434	0.003
Formic	0.189	0.004	0.222	0.006	0.215	0.011	0.186	0.008	0.268	0.009
Malic	4.028	0.010	3.871	0.015	3.619	0.014	3.189	0.002	4.069	0.028
Lactic	0.362	0.010	0.425	0.005	0.460	0.020	0.435	0.004	1.089	0.034
Acetic	1.062	0.012	1.309	0.015	1.269	0.016	1.096	0.006	1.842	0.016
Citric	0.170	0.004	0.123	0.005	0.133	0.007	0.123	0.007	0.193	0.003
Succinic	0.079	0.002	0.022	0.000	0.025	0.000	0.028	0.001	0.096	0.004
Total	6.74		6.62		6.44		5.58		8.03	

Acetic acid levels were also high, 0.824 - 1.979 g/L. Mato et al. (2007) suggested that acetic acid levels superior than 1 g/L could change the wine quality. In this sense, acetic acid attributes (vinegar-like odour) may be perceptible in these samples, but due to Madeira wine complexity is very difficult predict it. Indeed, some authors support the idea that acetic acid odour perception depends on wine type and style (Ugliano & Henschke, 2009). During the heating process, acetic acid did not seem to have a clear tendency: in sweet *TNM* decreased about 21% but in dry *TNM* increased 39%, while in *Malvasia* first increased and then decreased to values very close to the initial. However, the overheating conditions seem to potentiate the increase of the acetic acid levels, always more than 49%, eventually due to the thermal degradation of sugars as proposed by Ginz et al. (2000).

Regarding to tartaric acid contents, 0.331 - 1.123 g/L, were low when comparing with other wines: fortified (0.824 – 2.752 g/L (Cunha, et al., 2002; Esteves, et al., 2004)), red (1.088

– 3.8 g/L (Casella & Gatta, 2001; Peres, et al., 2009; Villiers, et al., 2003)) and white (0.964 – 2.4 g/L (Casella & Gatta, 2001; Peres, et al., 2009; Villiers, et al., 2003). These results might be associated with the tartaric levels already present in grapes used for the preparation of these wines or be related with previous precipitation of tartrates salts. In general, tartaric acid concentration decreased during the heating of the studied Madeira wines, especially in dry *TNM* (53%), although some oscillations were verified. The temperature increase seems to amplify this effect since the decrease raised up to 71% in one month (dry *TNM*).

Usually, the malolactic fermentation is not encouraged in Madeira winemaking, but anyway, was found considerable levels of lactic acid, 0.250 - 1.588 g/L, maybe derived from alcoholic fermentation through the action of yeasts. Indeed, lactic acid levels in the initial wines were higher in the *TNM* dry wines which were more fermented. Interestingly, lactic acid concentration increased during Madeira wine baking especially in sweet wines up to 40% when the heating was conducted at standard conditions and up to 6-fold when the heating was performed at overheating conditions.

Citric acid derives from grapes and in the initial wines its amount ranged from 0.170 to 0.360 g/L comparable with those found for Port wines (0.235 – 0.312 g/L) by Cunha et al. (2002). This acid generally decreased during the thermal processing.

Succinic acid is derived from the alcoholic fermentation and for that reason the initial *TNM* dry wine presented the highest levels (0.359 g/L). This acid decreased during the standard baking process up to 75%, representing in fact the greatest decrease at these conditions. The decrease was not always observed at overheating conditions.

Formic acid ranged from 0.126 to 0.256 g/L and increased up to 97% when the baking process was performed at 70 °C during 1 month, but only in sweet wines. As aforementioned, this growth may be related with sugar degradation promoted by temperature.

Finally, oxalic acid was the acid present at lowest concentrations, never exceeding the 0.117 g/L, and slightly decreased during the thermal processing.

8.4 Conclusions

The current study showed that *estufagem* introduced significant changes in the volatile composition of Madeira wines. The heating process promoted the increase of the volatile fraction of both Madeira wine types (dry and sweet), especially in sweet wines up to 88%, increasing the complexity of these wines. At least 190 volatile compounds were identified, 53 of which exclusively encountered in wines after baking. In quantitative terms, the volatile profile of baked Madeira wines was mostly represented by esters (more than 38.4%) and alcohols (more than 15.1%). Furan compounds can also play a significant role in the volatile fraction of sweet wines, especially if baking was performed at overheating conditions (up to 40.6%). It was also observed that most chemical families increased after baking, especially furan compounds and esters, specifically esters of organic acids. Interestingly, 6 norisoprenoids were developed during baking, particularly *β*-damascenone which exceeded

the limit of olfactory perception, at least 70-fold more. Contrarily, alcohols and fatty acids presented a slight decrease after heating. Similarly, most acetates declined after baking contributing for the loss of fruitiness. Additionally, several varietal aromas, such as monoterpenic alcohols usually related to the floral character of some wines and especially encountered in *Malvasia* wine, disappear after baking. In terms of odorant impact, the obtained results showed that *estufagem* favoured the development of some volatiles previously reported as typical aromas of Madeira wines, particularly phenylacetaldeyde, *β*-damascenone and 5-ethoxymethylfurfural. Additionally, *estufagem* also promoted the development of positive potential contributors to the global aroma of baked wines such as ethyl butyrate, ethyl 2-methylbutyrate, ethyl hexanoate, ethyl isovalerate, 5-hydroxymethylfurfural and γ-decalatone. In contrast, promoted the increase of volatile phenols usually considered off-flavours such as guaiacol, especially when higher temperatures were used.

Furthermore, the analysis of organic acids indicated that Madeira wines are especially rich in malic acid, representing in average 55% of the acids, probably because grapes contain high values of this acid at the time of harvest. Malic, tartaric, citric, succinic and oxalic acids decreased during the thermal processing confirming their involvement in the production of the corresponding volatile esters. Succinic acid showed the greatest decreased, up to 75%. However, lactic acid clearly increased up to 6-fold during the baking at 70 °C. Acetic and formic acids clearly increased only when overheating conditions were performed, probably because of thermal sugar degradation.

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CHAPTER 9

Volatile degradation products of sugars in model systems simulating the Madeira wine heating

This chapter is based on the following publication:

Volatile degradation products of sugars obtained in model systems under the same conditions of thermally processed wines

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(Submitted)

Abstract

Following the previous chapters, the current one presents a study where the purpose was to evaluate the profiles of volatiles developed in glucose and fructose model systems prepared under similar conditions of Madeira wines submitted to baking (*estufagem*), in order to elucidate the occurrence of Maillard reaction vs. acidic sugar degradation. To do so, 16 different model systems (MS) were prepared in synthetic wine (ethanolic solution with acid pH) containing fructose and glucose, with or without the amino acids: arginine, cysteine, γ-aminobutyric acid and aspartic acid. To simulate Madeira wine baking, model systems were heated at 50 °C during 4 months. The results clearly showed the development of several volatile compounds (up to 29 compounds were identified), particularly in the model systems containing fructose which presented the highest amounts. The identified compounds belong to four different chemical families: carbonyls, ethyl esters, pyrans and mainly furans. The main component was 5-hydroxymethylfurfural, representing about 84% of the compounds formed during the heating of model systems containing fructose. Additionally, was evaluated the antioxidant capacity of the prepared model systems, which ranged from 3.03 mg/L to 65.11 mg/L GAE. The antioxidant potential was more expressive in fructose model systems. Moreover, the current experiment could confirm that the development of colour, aroma and chemical composition is strongly associated with sugar degradation due to baking, particularly when the sugar is fructose. The fructose model systems originated darker (brown colour) and richer volatile profiles.

9.1 Introduction

It is commonly known that sugar in acidic media can be degraded into several low-molecular weight compounds, such as furans and pyrans (Belitz, Grosch, & Schieberle, 2009; Sanz & Martínez-Castro, 2009) and brown-coloured compounds can be formed. Furthermore, sugar in presence of amino compounds can undergo Maillard reaction. This complex reaction is known to develop non-enzymatic browning due to the reaction of carbonyl compounds and free amino groups (usually amino acids). This reaction is of prime importance in food quality, mainly in heat-processed foods, affecting colour (inducing browning), flavour, taste and nutritional value. This reaction can eventually have toxicological implications, such as acrylamide formation, but in contrast, also produces high antioxidant capacity products namely melanoidins (Bressa, Tesson, Dalla Rosa, Sensidoni, & Tubaro, 1996; Osada & Shibamoto, 2006; Wagner, Derkits, Herr, Schuh, & Elmadfa, 2002; Yilmaz & Toledo, 2005). It is widely stated on the literature that Maillard reaction encompasses a series of subsequent and parallel reactions, which can be divided into three stages, starting with the condensation between an amino group and a reducing sugar, leading to an Amadori product from an aldose (or Heyns product, if the reducing sugar is a ketose) (Belitz, et al., 2009). The Amadori/Heyns product leads to the formation of deoxyosones which are transformed into sugar fragmentation products. It is commonly accepted that in the initial stage of Maillard reaction, the amino group acts as catalyst increasing the reaction rate, resulting higher levels of very reactive intermediate products. In the final stage of Maillard reaction, amino groups

participate again, but this time, are integrated in molecules to originate dehydration, fragmentation, cyclization and polymerisation products. Parallel to the Maillard reaction, sugar degradation reactions (in the absence of amino groups) can occur, leading to similar products. The formation of flavoured, coloured or colourless Maillard products is influenced by several factors such as temperature, pH, time, water activity and the amount and type of reactants (Ames, Bailey, & Mann, 1999; van Boekel, 2006). In general, the sugar molecule determines the flavour compounds formed and amino acids affects the kinetics. Coloured Maillard products can be divided into two main groups: the low-molecular weight compounds (MW < 1000) and the macromolecules, also known as melanoidins (Rizzi, 1997).

Since Maillard reaction develops complex intermediate and final reaction products, researchers commonly use model systems to limit the scope of this reaction. These studies usually use water as solvent (Adams, Polizzi, van Boekel, & De Kimpe, 2008; Ames, et al., 1999; Osada & Shibamoto, 2006; Venskutonis, Vasiliauskaite, Galdikas, & Setkus, 2002; Yilmaz & Toledo, 2005) and only a limited number of reports have dealt with non-aqueous systems (Hofmann, 1998; Pripis-Nicolau, de Revel, Bertrand, & Maujean, 2000; S.-C. Shen, Tseng, & Wu, 2007; S. C. Shen & Wu, 2004). Shen and Wu (2004) used ethanolic systems and proved that the browning extent and the HMF content rise with the ethanol increase. They also found different product profiles in aqueous and ethanolic model systems indicating some differences in the Maillard reaction mechanisms (S.-C. Shen, et al., 2007). Furthermore, there are some studies that highlight the formation of flavour components in wine model systems, at low pH but at low temperatures. According to Sanz & Martínez-Castro (2009), Kroh (1994) studied wine model systems containing glucose with alanine, arginine and proline. Pripis Nicolau and co-workers (2000) researched the reaction of carbonyl (acetoin and acetol) and dicarbonyl (glyoxal, methylglyoxal, diacetyl and pentan-2,3-dione) compounds with 14 amino acids. They realized that this reaction gave rise to many products, including pyrazines, methylthiazoles, acetylthiazoles, acetylthiazolines, acetylthiazolidines, trimethyloxazole, and dimethylethyloxazoles, especially due to the cysteine presence. Moreover, they also found out that these compounds have a remarkable odour, with notes resembling to sulphur, corn, pungent, nut, popcorn, roasted hazelnut, toasted, roasted, and ripe fruits. Later, Marchand et al. (2002) have indeed proved the occurrence of a Maillard intermediate in wine model systems: N-(2-sulfanylethyl)-2-oxopropanamide, the intermediate in the formation of 2-acetylthiazole from methylglyoxal and cysteine. On the other hand, Cutzach et al. (1999) studied the formation mechanisms of some volatile compounds during ageing of sweet fortified wines and conclude that the majority is formed by Maillard mechanisms.

Taking into consideration that Maillard reaction takes place at 50 °C, favoured at pH 4 – 7 and that caramelisation requires higher temperatures but is favoured at pH 3 – 9 (Kroh, 1994; Morales & Jiménez-Pérez, 2001), it is reasonable to admit that these reactions can eventually occur during the heating process traditionally applied to Madeira wines, contributing to browning, flavouring and antioxidant activity. Thus, the aim of the current study was to elucidate the sugar availability (fructose and glucose) to develop Maillard reaction and/or acidic sugar degradation though the preparation of 16 different model systems (MS), under the same conditions of baked Madeira wines, evaluating the colour, the antioxidant activity and especially the development of volatile compounds.

9.2 Experimental

9.2.1 *Chemicals*

D(+)-Glucose, *D(-)*-fructose and *L(+)*-tartaric acid were obtained from Merck Co. (Darmstadt, Germany), the amino acids *L*-arginine, *L*-cysteine, *L*-aspartic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and *γ*-aminobutyric acid was supplied by Fluka BioChemika AG (Buchs, Switzerland). Ethanol was obtained from Panreac (Barcelona, Spain). Ethyl acetate was supplied by Lab-Scan (Dublin, Ireland). All chemicals had a purity grade higher than 98%. 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the crystallized diammonium salt form (≈ 98.0%), gallic acid monohydrate (≥ 98.0%) and potassium persulphate were obtained from Fluka BioChemika AG (Buchs, Switzerland).

9.2.2 *Preparation of model systems (MS)*

TABLE 9.1 – Composition of the 16 model systems.

Model System	Sugar (g/L)	Amino acid (mg/L)
GluArg	Glucose, 125	Arginine, 100
GluCys		Cysteine, 100
GluGABA		GABA, 100
GluAspac		Aspartic acid, 100
FruArg	Fructose, 125	Arginine, 100
FruCys		Cysteine, 100
FruGABA		GABA, 100
FruAspac		Aspartic acid, 100
FruGlu(4aa)	Fructose, 62.5 Glucose, 62.5	Arginine, 25
		Cysteine, 25
		GABA, 25
		Aspartic acid, 25
Glu	Glucose, 125	
Fru	Fructose, 125	
FruGlu	Fructose, 62.5 Glucose, 62.5	
FruArg(×2)	Fructose, 125	Arginine, 200
FruCys(×2)		Cysteine, 200
FruGABA(×2)		GABA, 200
FruAspac(×2)		Aspartic acid, 200

For the preparation of the 16 MS, four amino acids were chosen: arginine (Arg), cysteine (Cys), *γ*-aminobutyric acid (GABA) and aspartic acid (Asp), since they are important

amino acids present in Madeira wines, as shown in Chapter 5. The selected sugars were fructose (Fru) and glucose (Glu) as they usually are the main sugars present in wines. The 16 model systems were prepared according to the composition described in Table 9.1. All model systems were prepared in synthetic wine containing 6 g/L of tartaric acid, 18% of ethanol and pH adjusted to 3.5 (Madeira wine typical conditions). The first nine models intended to simulate Maillard reaction, the following three to simulate acidic sugar degradation and the last four intend to evaluate the influence of the amino acid concentration. The models systems were heated at 50 °C during 4 months to simulate *estufagem*.

9.2.3 *Liquid-Liquid Extraction*

The extraction procedure was adapted from Ortega et al. (Ortega, López, Cacho, & Ferreira, 2001). 5 mL of sample (model system) were added to 3 mL of distilled water. Then, 2 g of ammonium sulphate and 5 µL of the internal standard (422 mg/L of 3-octanol, prepared in synthetic wine) were added. The extraction was carried out with 1 mL of ethyl acetate. This mixture was mechanically agitated during 30 min and finally the extract was separated from the aqueous phase and analysed. The extraction was carried out in triplicate (3 extractions / MS).

9.2.4 *Gas Chromatography–Mass Spectrometry Analysis*

GC–MS analysis was carried out on an Agilent 6890N (Palo Alto, CA, USA) gas chromatograph coupled to an Agilent 5975 quadrupole inert mass selective detector. The column was a BP–20 (WAX) from SGE (Austin, TX, U.S.A.), 30 m × 0.25 mm i.d., with 0.25 µm film thickness. The carrier gas was helium (helium N60, Air Liquid, Portugal) at 1 mL/min (column-head pressure of 13 Psi). 1 µL of extract was vaporized in the injector port maintained at 250 °C in splitless mode (1 min). The oven temperature was then raised from 40 °C to 220 °C at 3 °C/min and finally held at 220 °C for 5 min. The quadrupole ion source and transfer line temperatures were maintained at 230 and 250 °C, respectively. The ionization energy was set to 70 eV. The mass range 30–300 m/z were recorded in full-scan mode.

The identification of the compounds was made by comparison of the mass spectra of the compounds formed with those present in the NIST05 MS library database.

9.2.5 *Spectrophotometric Analysis*

The colour development of the several MS after the heating step was evaluated by spectrophotometric analysis. UV spectra of the different MS were recorded on a Perkin Elmer Lambda 2 spectrophotometer, covering the wavelength range 240 – 600 nm and using a 1 cm path length quartz cell.

9.2.6 *Determination of the total antioxidant capacity*

Additionally, the MS antioxidant potential was also determined. The determination of the antioxidant capacity, according to the reaction with a stable ABTS radical cation ($ABTS^{\bullet+}$), was based on the method reported by Re et al. (Re et al., 1999). Briefly, $ABTS^{\bullet+}$ was obtained by the reaction of 2 mM ABTS diammonium salt with 70 mM potassium persulphate in 50 mL of phosphate buffered saline (PBS). The mixture was left to stand in the dark at room temperature for about 16 hours before use. For the antioxidant capacity evaluation, the $ABTS^{\bullet+}$ solution was diluted with PBS to obtain the absorbance of 0.800 ± 0.030 at 734 nm. Then 12 µL of MS were mixed with 3 mL of $ABTS^{\bullet+}$ solution. The absorbance was recorded at room temperature during 20 min. PBS solution was used as the blank sample. The decrease percentage of the absorbance at 734 nm was calculated by the formula I = [(AB -AA)/AB] × 100, where I = $ABTS^{\bullet+}$ inhibition (%), AB = absorbance of the blank sample (t = 0 min), AA = absorbance of a tested wine at the end of the reaction (t = 20 min). The results were expressed as mg/L of gallic acid equivalents (GAE).

9.3 Results and discussion

9.3.1 *Volatile composition of MS*

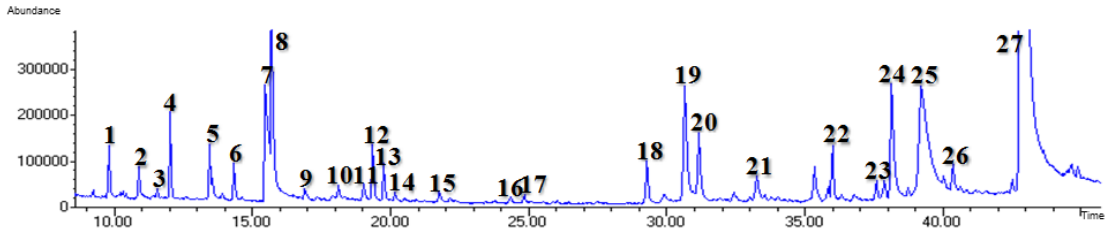

FIGURE 9.1 – Typical gas chromatogram of the FruArg ethyl acetate extract. Peak identification: 1 - ethyl pyruvate; 2 – hydroxyacetone; 3 – methyl 3-hydroxybutanoate; 4 – ethyl L(-)-lactate; 5 – octan-3-ol (IS); 6 – ethyl glycolate; 7 – Acetic acid; 8 – furfural; 9 – acetylfuran; 10 – propanoic acid; 11 – 5-methylfurfural; 12 – 2-cyclopentene-1,4-dione; 13 – ethyl 3-hydroxybutanoate; 14 – ethyl levulinate; 15 – furfuryl alcohol; 16 – 2(5H)-furanone; 17 – 2-hydroxy-2-cyclopenten-1-one; 18 – 5-ethoxymethylfurfural; 19 – 2,5-furandicarboxaldehyde; 20 – methyl 2-furoate; 21 – dihydroxyacetone; 22 – 5-acetoxymethyl-2-furaldehyde; 23 – 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; 24 – 3,5-dihydroxy-2-methyl-4H-pyran-4-one; 25 – diethyl tartrate; 26 – ethyl hydrogen succinate; 27 – 5-hydroxymethylfurfural.

All MS, sampled after 2, 3 and 4 months of heating, were extracted with ethyl acetate and analysed by GC-MS to evaluate the volatile compounds developed during this period. Figure 9.1 depicts a typical chromatogram of the ethyl acetate extracts, in the case is shown the chromatogram of a FruArg extract. The chromatographic peaks were integrated and the relative areas (relative to internal standard) were measured with an average RSD of 7.2%. The applied chromatographic procedure, allowed the identification of a total of 29 chemicals compounds in all heated MS (see Table 9.2). The chemical families found involve carbonyls, ethyl esters, furans and pyrans. Most of them can be found in Madeira wines, namely some of

the ethyl esters and furans. In general, these compounds contribute to the aroma with sweet, caramel, almond or even burnt notes.

TABLE 9.2 – Relative areas of the compounds identified in the MS at the end of the heating step, according to the applied methodology.

t_R (min)	Compounds	GluArg	GluCys	GluGABA	GluAspac	FruArg	FruCys	FruGABA	FruAspac
9.8	ethyl pyruvate	1.66 ± 0.30	2.18 ± 0.09	1.96 ± 0.08	2.06 ± 0.28	1.43 ± 0.10	1.27 ± 0.13	1.08 ± 0.17	1.25 ± 0.06
10.3	ethyl ethoxyacetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.24 ± 0.02
10.9	hydroxyacetone	0.38 ± 0.02	0.37 ± 0.07	0.41 ± 0.10	0.34 ± 0.09	0.87 ± 0.08	0.88 ± 0.07	0.80 ± 0.11	0.83 ± 0.13
11.6	methyl 3-hydroxybutanoate	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.03	0.22 ± 0.03	0.20 ± 0.02	0.49 ± 0.05
12.0	ethyl lactate	n.d.	n.d.	n.d.	n.d.	1.82 ± 0.00	1.23 ± 0.06	1.55 ± 0.03	1.79 ± 0.06
14.3	ethyl glycolate	0.10 ± 0.01	0.34 ± 0.03	0.12 ± 0.01	0.15 ± 0.01	0.90 ± 0.09	1.12 ± 0.03	0.89 ± 0.01	1.47 ± 0.10
15.5	acetic acid	0.82 ± 0.02	7.15 ± 0.71	1.00 ± 0.25	0.73 ± 0.14	3.56 ± 0.12	3.89 ± 0.42	4.05 ± 0.50	7.02 ± 1.61
15.7	furfural	n.d.	2.14 ± 0.13	1.07 ± 0.18	n.d.	5.65 ± 0.15	4.20 ± 0.53	5.80 ± 0.54	5.17 ± 0.72
16.9	acetylfuran	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.02	0.24 ± 0.01	0.25 ± 0.03	0.27 ± 0.04
18.1	propanoic acid	n.d.	0.35 ± 0.07	n.d.	n.d.	0.34 ± 0.04	0.65 ± 0.04	0.53 ± 0.04	0.88 ± 0.13
19.0	5-methylfurfural	n.d.	n.d.	n.d.	n.d.	0.72 ± 0.02	0.69 ± 0.03	0.59 ± 0.03	0.54 ± 0.03
19.3	2-cyclopentene-1,4-dione	n.d.	0.36 ± 0.03	n.d.	n.d.	1.85 ± 0.07	3.53 ± 0.32	1.41 ± 0.07	2.65 ± 0.06
19.8	ethyl 3-hydroxybutanoate	n.d.	n.d.	n.d.	n.d.	1.10 ± 0.04	0.92 ± 0.05	0.97 ± 0.03	1.30 ± 0.08
20.2	ethyl levulinate	n.d.	n.d.	n.d.	n.d.	0.32 ± 0.03	0.43 ± 0.03	0.23 ± 0.02	0.29 ± 0.03
21.7	furfuryl alcohol	n.d.	n.d.	n.d.	n.d.	0.43 ± 0.02	0.41 ± 0.04	0.36 ± 0.01	0.42 ± 0.08
22.2	diethyl butanoate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.36 ± 0.01
24.4	2(5H)-furanone	n.d.	n.d.	n.d.	n.d.	0.28 ± 0.03	0.36 ± 0.03	0.26 ± 0.01	0.43 ± 0.03
24.9	2-hydroxy-2-cyclopenten-1-one	n.d.	0.22 ± 0.04	n.d.	n.d.	0.30 ± 0.01	0.24 ± 0.04	0.24 ± 0.01	0.27 ± 0.05
29.3	5-ethoxymethylfurfural	n.d.	n.d.	n.d.	n.d.	1.96 ± 0.06	1.44 ± 0.08	1.39 ± 0.09	1.27 ± 0.08
29.9	2-furoic acid	n.d.	n.d.	n.d.	n.d.	0.63 ± 0.13	n.d.	n.d.	n.d.
30.7	2,5-furandicarboxaldehyde	n.d.	n.d.	n.d.	n.d.	6.34 ± 0.07	5.86 ± 0.49	5.65 ± 0.21	5.76 ± 0.22
31.2	methyl 2-furoate	n.d.	n.d.	n.d.	n.d.	3.54 ± 0.12	3.31 ± 0.18	2.92 ± 0.11	3.21 ± 0.27
33.3	dihydroxyacetone	0.61 ± 0.05	0.63 ± 0.03	0.46 ± 0.09	0.34 ± 0.11	1.66 ± 0.01	1.18 ± 0.18	1.28 ± 0.10	1.83 ± 0.50
36.0	5-acetoxymethylfurfural	n.d.	n.d.	0.17 ± 0.03	0.19 ± 0.00	1.53 ± 0.05	1.36 ± 0.19	1.58 ± 0.10	1.11 ± 0.06
36.3	dihydro-6-methyl-2H-Pyran-3(4H)-one	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
37.6	DDMP [a]	n.d.	n.d.	n.d.	n.d.	0.83 ± 0.02	0.57 ± 0.14	0.65 ± 0.04	0.72 ± 0.18
38.2	hydroxymaltol	n.d.	n.d.	n.d.	n.d.	6.61 ± 0.10	2.74 ± 0.23	5.90 ± 0.28	4.08 ± 0.45
39.2	diethyl tartrate	15.61 ± 0.89	17.01 ± 0.73	11.07 ± 0.60	11.42 ± 2.02	20.16 ± 0.48	17.76 ± 1.03	15.19 ± 0.36	19.24 ± 2.03
40.4	ethyl hydrogen succinate	n.d.	n.d.	n.d.	n.d.	1.24 ± 0.05	0.70 ± 0.07	1.02 ± 0.06	2.02 ± 0.16
43.0	HMF [b]	16.71 ± 0.94	9.10 ± 0.16	10.28 ± 0.59	8.92 ± 0.99	383.38 ± 38	288.27 ± 12.71	309.70 ± 8.15	278.83 ± 11.91
	Number of Compounds	**7**	**11**	**9**	**8**	**27**	**26**	**26**	**28**

TABLE 9.2 – (continued)

t_R (min)	Compounds	FruGlu(4aa)	Glu	Fru	FruGlu	FruArg(×2)	FruCys(×2)	FruGABA(×2)	FruAspac(×2)
9.8	ethyl pyruvate	1.18 ± 0.16	1.81 ± 0.17	1.56 ± 0.07	1.62 ± 0.08	1.49 ± 0.29	1.13 ± 0.17	0.96 ± 0.07	1.27 ± 0.24
10.3	ethyl ethoxyacetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10.9	hydroxyacetone	0.50 ± 0.09	0.26 ± 0.03	0.73 ± 0.03	0.62 ± 0.04	0.89 ± 0.17	0.60 ± 0.13	0.76 ± 0.16	0.70 ± 0.07
11.6	methyl 3-hydroxybutanoate	0.19 ± 0.02	n.d.	0.16 ± 0.03	n.d.	n.d.	0.13 ± 0.01	0.14 ± 0.01	0.19 ± 0.03
12.0	ethyl lactate	0.76 ± 0.01	n.d.	1.53 ± 0.05	0.87 ± 0.04	1.45 ± 0.04	0.92 ± 0.02	1.30 ± 0.07	1.31 ± 0.09
14.3	ethyl glycolate	0.74 ± 0.02	n.d.	0.93 ± 0.03	0.55 ± 0.04	0.73 ± 0.04	0.84 ± 7.07	0.66 ± 0.07	0.78 ± 0.04
15.5	acetic acid	3.07 ± 0.33	0.55 ± 0.13	2.71 ± 0.43	1.95 ± 0.75	3.60 ± 0.22	4.35 ± 0.15	3.70 ± 0.80	3.54 ± 0.17
15.7	furfural	3.74 ± 0.23	n.d.	4.71 ± 0.53	4.13 ± 0.30	4.64 ± 0.42	3.54 ± 0.66	5.43 ± 0.51	4.93 ± 0.24
16.9	acetylfuran	0.17 ± 0.03	n.d.	0.26 ± 0.05	n.d.	0.23 ± 0.02	0.20 ± 0.02	0.29 ± 0.03	0.25 ± 0.02
18.1	propanoic acid	0.63 ± 0.04	n.d.	0.48 ± 0.05	0.16 ± 0.02	0.38 ± 0.07	0.47 ± 0.03	0.41 ± 0.05	0.52 ± 0.01
19.0	5-methylfurfural	0.41 ± 0.02	n.d.	0.61 ± 0.03	0.38 ± 0.04	0.62 ± 0.04	0.49 ± 0.02	0.53 ± 0.05	0.54 ± 0.07
19.3	2-cyclopentene-1,4-dione	1.95 ± 0.04	n.d.	1.53 ± 0.09	0.61 ± 0.04	1.40 ± 0.07	2.75 ± 0.06	1.32 ± 0.11	2.28 ± 0.21
19.8	ethyl 3-hydroxybutanoate	0.58 ± 0.02	n.d.	0.89 ± 0.05	0.45 ± 0.02	0.86 ± 0.03	0.61 ± 0.05	0.83 ± 0.05	0.77 ± 0.06
20.2	ethyl levulinate	0.19 ± 0.04	n.d.	0.25 ± 0.02	n.d.	0.24 ± 0.01	0.38 ± 0.03	0.20 ± 0.03	0.22 ± 0.02
21.7	furfuryl alcohol	0.22 ± 0.05	n.d.	0.34 ± 0.02	0.24 ± 0.05	0.33 ± 0.02	0.33 ± 0.03	0.34 ± 0.03	0.37 ± 0.03
22.2	diethyl butanoate	n.d.	n.d.	0.18 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
24.4	2(5H)-furanone	0.33 ± 0.01	n.d.	0.26 ± 0.00	0.15 ± 0.02	0.25 ± 0.01	0.30 ± 0.02	0.24 ± 0.02	0.33 ± 0.02
24.9	2-hydroxy-2-cyclopenten-1-one	0.23 ± 0.02	n.d.	0.24 ± 0.02	0.25 ± 0.04	0.26 ± 0.04	0.23 ± 0.03	0.25 ± 0.03	0.25 ± 0.02
29.3	5-ethoxymethylfurfural	0.62 ± 0.01	n.d.	1.32 ± 0.12	0.58 ± 0.06	1.47 ± 0.07	1.03 ± 0.02	1.17 ± 0.03	1.17 ± 0.02
29.9	2-furoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.43 ± 0.02
30.7	2,5-furandicarboxaldehyde	3.47 ± 0.19	n.d.	6.05 ± 0.20	3.45 ± 0.18	5.76 ± 0.27	4.39 ± 0.20	5.19 ± 0.32	5.19 ± 0.31
31.2	methyl 2-furoate	1.47 ± 0.09	n.d.	2.77 ± 0.18	1.44 ± 0.12	2.80 ± 0.13	2.94 ± 0.11	2.71 ± 0.18	2.94 ± 0.13
33.3	dihydroxyacetone	0.83 ± 0.10	n.d.	1.19 ± 0.06	0.85 ± 0.10	1.40 ± 0.26	0.93 ± 0.07	1.21 ± 0.16	1.35 ± 0.05
36.0	5-acetoxymethylfurfural	1.01 ± 0.08	n.d.	1.59 ± 0.10	1.18 ± 0.13	1.75 ± 0.07	1.17 ± 0.09	1.49 ± 0.10	1.24 ± 0.10
36.3	dihydro-6-methyl-2H-Pyran-3(4H)-one	n.d.	n.d.	n.d.	n.d.	n.d.	0.44 ± 0.07	n.d.	0.36 ± 0.04
37.6	DDMP [a]	0.27 ± 0.05	n.d.	0.56 ± 0.09	0.38 ± 0.03	0.58 ± 0.07	0.45 ± 0.03	0.67 ± 0.05	0.59 ± 0.03
38.2	hydroxymaltol	1.65 ± 0.15	n.d.	5.28 ± 0.51	2.08 ± 0.21	4.96 ± 0.60	2.50 ± 0.31	5.76 ± 0.45	5.02 ± 0.19
39.2	diethyl tartrate	15.14 ± 0.61	11.62 ± 0.43	15.31 ± 0.76	14.40 ± 1.11	15.13 ± 0.71	16.01 ± 0.69	13.02 ± 0.84	16.30 ± 0.86
40.4	ethyl hydrogen succinate	0.71 ± 0.05	n.d.	1.00 ± 0.16	0.54 ± 0.09	0.71 ± 0.09	0.47 ± 0.09	0.79 ± 0.07	1.05 ± 7.43
43.0	HMF [b]	153.54 ± 2.36	3.93 ± 0.12	298.43 ± 12.61	178.38 ± 10.09	303.20 ± 3.75	227.30 ± 7.93	289.52 ± 15.88	272.05 ± 15.87
	Number of Compounds	**26**	**5**	**27**	**23**	**25**	**27**	**26**	**28**

Values are means of triplicate determination (n=3) ± SD
n.d. - not detected

[a] 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one

[b] 5-hydroxymethylfurfural

In acidic medium, fructose more easily opens its ring structure than glucose, therefore is more reactive. Thus, this can be a good explanation for fructose MS developed a large number of volatiles, up to 27, comparatively to those formed in glucose MS (about 8 compounds) (see Table 9.2). These results may also indicate that fructose in wines might be chemically more reactive than glucose. Göğüş et al. (1998) also found that fructose was more reactive than glucose when studied the kinetics of Maillard reactions through the preparation of MS containing the major sugars (fructose and glucose) and amino acids (glutamine and arginine) of boiled grape juice.

Additionally, we could confirm that it is sugar that determines the volatile promotion, rather than the amino acid type, since MS prepared with different amino acids originated similar volatile profiles (see Table 9.2).

Nursten (1981) verified that sugars by themselves show similar reactions to those produced between sugars and amino acids. Indeed, the current study showed that MS with or without amino acids developed similar compounds, especially those prepared with fructose (see Table 9.2). In this sense becomes difficult to elucidate if the Maillard reaction takes place. At this pH, the Maillard pathway more likely to happen it is the 1,2-enolisation that gives essentially the same products of acidic sugar degradation. Moreover, the change in the amino acid content did not revealed significant effect in the volatile profile (see Table 9.2).

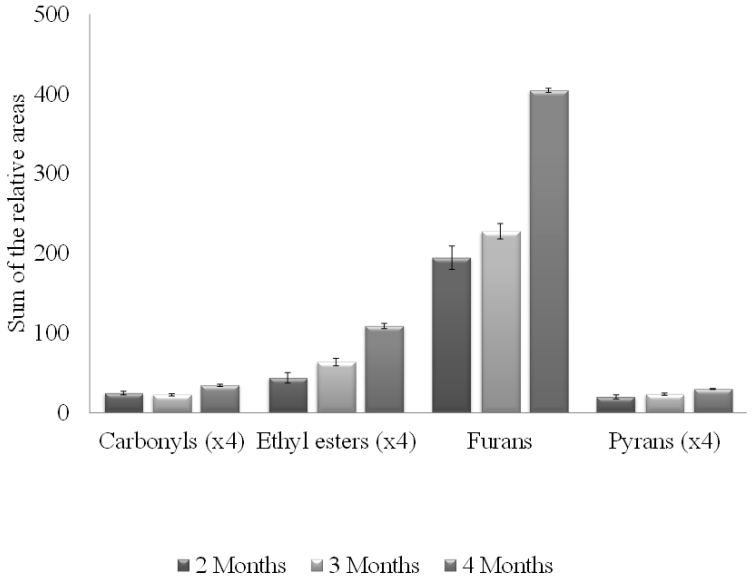

FIGURE 9.2 – Evolution of the chemical families during the heating of the FruArg model system.

The obtained data show that, in general, the concentration of all chemical families increase during the heating step, especially furans, as can be seen for FruArg in Figure 9.2. Furans represent about 90% of the total compounds formed for the MS containing fructose, being HMF the most representative, about 95%, followed by 2,5-furandicarboxaldehyde (≈ 2.1%) and furfural (≈ 1.5%) - for FruArg MS see Table 9.3. As it can be seen for FruArg, MS containing fructose did not show significant variation in the composition along the period of heating, with HMF representing about 84.2% of the total compounds formed.

TABLE 9.3 – Normalized amounts (%) of chemical families found in FruArg during its heating process. Distribution of the detected furans.

%	2 Months	3 Months	4 Months
Carbonyls	2.8 ± 0.3	2.2 ± 0.2	1.9 ± 0.1
Ethyl esters	5.0 ± 0.8	6.2 ± 0.5	6.1 ± 0.2
Furans	89.8 ± 6.7	89.3 ± 3.8	90.4 ± 0.6
Pyrans	2.3 ± 0.3	2.3 ± 0.1	1.7 ± 0.0
Furfural	1.9 ± 0.1	1.4 ± 0.1	1.4 ± 0.0
Acetylfuran	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
5-Methylfurfural	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Furfuryl alcohol	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
2(5H)-Furanone	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
5-Ethoxymethylfurfural	0.0 ± 0.0	0.3 ± 0.0	0.5 ± 0.0
2-Furoic acid	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
2,5-Furandicarboxaldehyde	2.5 ± 0.2	2.2 ± 0.2	1.6 ± 0.0
Methyl 2-furoate	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.0
5-Acetoxymethyl-2-furaldehyde	0.2 ± 0.0	0.1 ± 0.0	0.4 ± 0.0
HMF	94.1 ± 7.1	94.7 ± 3.9	94.7 ± 0.5

It seems that the fructose is an important factor in the formation of HMF, given that, for example, FruGlu(4aa) and FruGlu, with the same amount of fructose (62.5 g/L) and glucose (62.5 g/L) showed higher percentages (about 81%) relative to glucose MS (125 g/L, about 33%).

The predominance of furans can be explained by the fact that the heating of hexoses in an acid medium leads, after enolization, to the elimination of water molecules, originating furanic derivatives, essentially HMF (from a 1,2-endiol). Additionally, the dehydration of Amadori/Heyns compounds via 1,2-enolisation can contribute to the formation of furans, in the MS in which amino acids are present. Indeed, the 1,2-enolization is favoured by acid conditions (low pH) instead the 2,3-enolization. The 2,3-enolization originates carbonyl compounds, namely furanones and pyranones (Mottram, 2007). Although 2,3-enolization is not so favoured in acidic medium, it could be verified that it occurs, in less extension, since that DDMP and hydroxyacetone, typical markers of this pathway, were identified (Davidek, Clety, Devaud, Robert, & Blank, 2003; Martins, Jongen, & van Boekel, 2000). DDMP was found only in MS containing fructose and hydroxyacetone was found in all MS.

Cutzach et al. (1999) found out that 5-ethoxymethylfurfural is formed during the ageing process of sweet fortified wines and suggested that its presence can be justified by the reaction between ethanol and HMF. In fact, we can confirm the presence of this compound in the MS. This furan was previously found in baked Madeira wines. In addition, 5-ethoxymethylfurfural has been identified in wood-aged Madeira wines by Câmara et al. (2006).

Organic acids were found in the current MS, and its formation can follow the scheme suggested by Ginz et al. (2000) (Figure 9.3). Actually, formic acid and acetic acid have been frequently found in glucose and fructose MS (Ginz, et al., 2000; Martins, et al., 2000). Acetic

acid, propanoic acid and formic acid (detected but not measured) could be found in the present MS. The group of ethyl esters found in the analysed MS may result from organic acid esterification in the presence of ethanol, namely the ethyl esters from pyruvic acid, lactic acid, glycolic acid and levulinic acid.

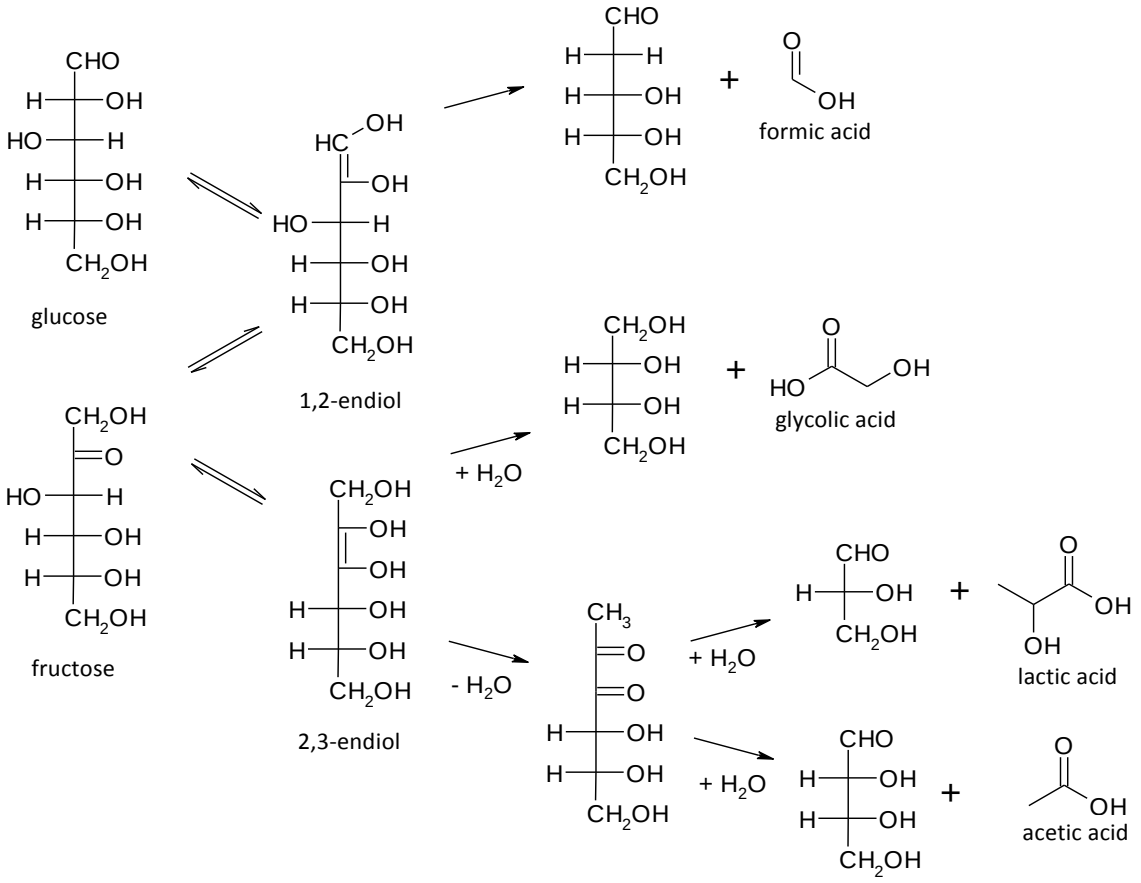

FIGURE 9.3 – Formation of organic acids by sugar degradation.

The total amount of the compounds formed during the heating step is exhibited in Figure 9.4. Other than the large number of compounds formed by fructose MS, these model systems also present a higher amount when compared with glucose MS. Moreover, fructose MS also showed a greater increase with heating. The effect of doubling the amino acid amount does not affect significantly the kinetics of production of volatile compounds.

The experimental results were complemented with a preliminary olfactometric test performed by persons involved in the production of Madeira wines. MS prepared with fructose and cysteine (FruCys) revealed the presence of aromas usually found in Madeira wine, namely caramel and dried fruits.

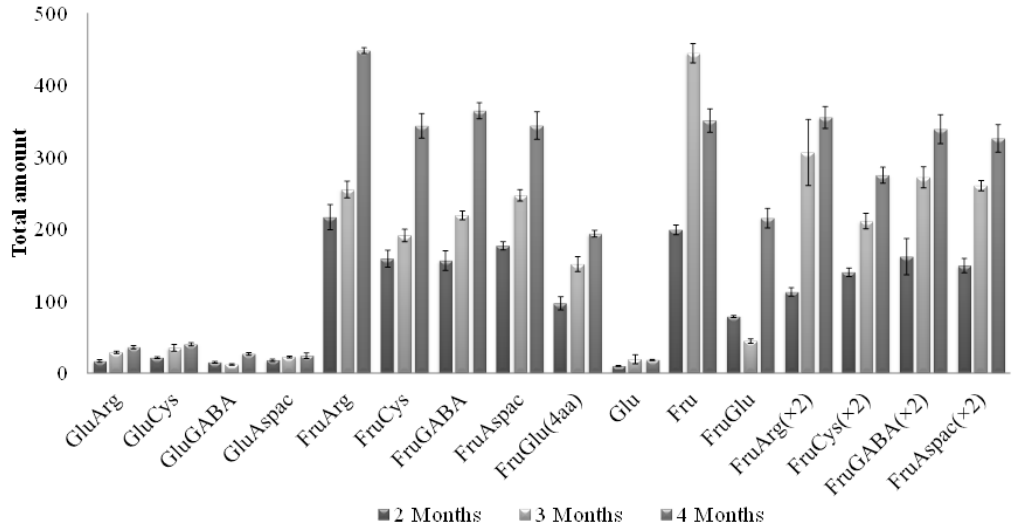

FIGURE 9.4 – Evolution of the total amount, relative to internal standard, of the volatile compounds formed in all MS, during the heating step.

9.3.2 *Colour development*

The colour developed by the several MS after the heating step was evaluated from the UV spectra recorded between 240–600 nm. At the initial stage, all MS were colourless but along baking became yellowish. As it can be seen in Figure 9.5, all MS absorb mainly bellow 300 nm, after the heating step. The absorbance is higher whenever fructose is present in the MS. This absorption may be principally due to furans formation, which absorb below 300 nm. Actually, the yellowish colour developed by the MS containing only fructose was less intense than the developed by MS containing fructose and an amino acid.

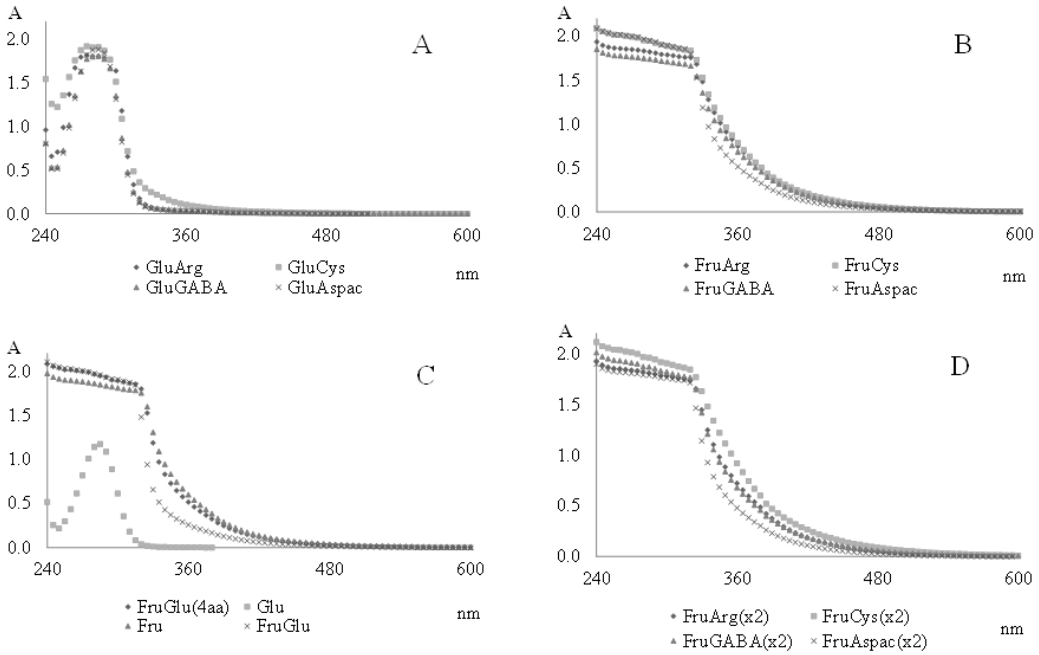

FIGURE 9.5 – UV spectra recorded between 240 – 600 nm of **A**: GluArg, GluCys, GluGABA, GluAsp; **B**: FruArg, FruCys, FruGABA, FruAspac; **C**: GluFru(4aa), Glu, Fru, GluFru; **D**: FruArg(×2), FruCys(×2), FruGABA(×2), FruAspac(×2).

The extent of browning of the several MS along heating was measured by the absorbance determination at 420 nm (Figure 9.6). It can be observed that MS in which fructose exists presented higher absorbance, generally showing a significant increase during the heating period. Furthermore, Figure 9.6 also shows that the presence of amino acids tends to favour the browning, perhaps by occurrence of the Maillard mechanism together with acidic sugar degradation. The absorbance increase seems to be slightly more pronounced in MS where the amino acid content was doubled, probably due to the increasing of the reaction kinetic, through the amino acids action. Indeed, the MS containing cysteine, mainly FruCys and FruCys(×2), attained higher browning values at the current heating conditions.

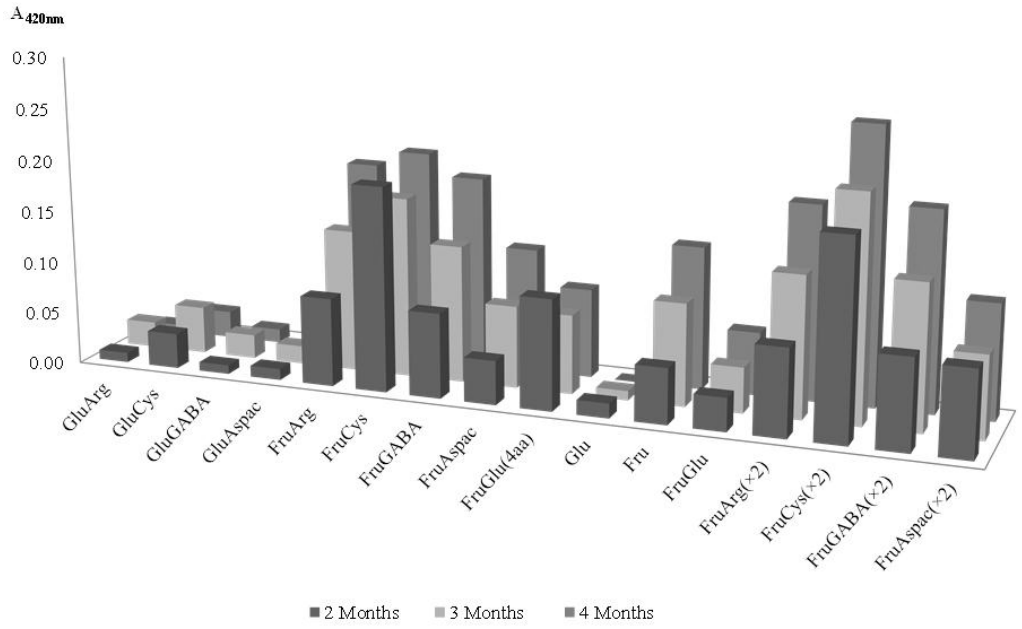

FIGURE 9.6 – Absorbance at 420 nm of the MS during the heating step at 50 °C.

9.3.3 *Total antioxidant capacity*

The antioxidant activity of Maillard products has been studied by numerous investigators (Chawla, Chander, & Sharma, 2007; Moreno, Peinado, & Peinado, 2007; Osada & Shibamoto, 2006; Yilmaz & Toledo, 2005). The total antioxidant capacity (TAC) of the MS was determined by bleaching the pre-formed ABTS radical cations. The addition of free radical-scavengers to a solution containing ABTS-derived radical cations leads to a decrease in the absorbance of the MS at 734 nm. Table 9.4 shows that MS presented antioxidant capacity. The obtained results varied from 3.03 mg/L (GluGABA) to 65.11 mg/L (FruArg). The fructose MS presented higher TAC, averaging 56.39 ± 8.40 mg/L, than glucose MS, 6.40 ± 4.45 mg/. These results indicate that antioxidant potential is dependent on the sugar type. Moreover, the TCA values obtained in the present study, showed minor differences between the MS with or without amino acids, suggesting that the presented antioxidant potential is exclusively from products formed from sugar degradation.

TABLE 9.4 – Total antioxidant activity (TAC) of the MS after 4 months of heating at 50 °C, expressed as mg/L of gallic acid equivalents (GAE).

Model systems	TAC (mg/L GAE)
GluArg	8.80 ± 0.34
GluCys	13.18 ± 0.43
GluGABA	3.04 ± 0.24
GluAspac	3.75 ± 0.28
FruArg	65.33 ± 9.54
FruCys	40.37 ± 0.72
FruGABA	61.00 ± 0.02
FruAspac	58.77 ± 3.57
FruGlu(4aa)	28.77 ± 0.26
Glu	3.31 ± 0.32
Fru	61.66 ± 0.47
FruGlu	33.00 ± 0.99
FruArg(×2)	62.14 ± 2.51
FruCys(×2)	44.89 ± 0.13
FruGABA(×2)	59.44 ± 0.07
FruAspac(×2)	54.87 ± 6.30

9.4 Conclusions

This work revealed that an important number of volatiles are formed through sugar degradation in model systems prepared under the same conditions of baked Madeira wines. The sugar derived-products found belongs to different chemical families, like carbonyls, ethyl esters, pyrans and furans. Furans are the main family of compounds formed in the conditions of the experiment, namely HMF, which represent about 84% of the major volatiles of the fructose MS. The prepared MS showed that the volatile formation is independent from amino acids, but strongly dependent from sugar type. In this sense fructose MS develop more volatile compounds than glucose MS, indicating that fructose is more reactive than glucose. Nevertheless, the amino acids (namely cysteine) seem to have a greater impact in the colour formation. In terms of antioxidant activity fructose MS presented higher levels (about 56.4 mg/L GAE) than glucose MS (about 6.4 mg/L GAE).

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PART 4

Conclusions and perspectives

CHAPTER 10
Conclusions

The main aim of this thesis was to explore the effects of *estufagem* on some chemical constituents of Madeira wines. From the overall work, some conclusions can be drawn:

(a) Two validated analytical methods were developed to overcome the need of monitoring target compounds, namely amino acids, biogenic amines, polyphenols and organic acids, combining precision and reproducibility to execution effectiveness. The methodology for the simultaneous quantification of amino acid an biogenic amine in wines was achieved through a RP-HPLC-FLD method, using a pre-column *o*-phthaldialdehyde (OPA) derivatization procedure, performed in the sample injection loop, simplifying the derivatization procedure and achieving reproducible results (average RSD of 2%). The developed method allowed the quantification of 19 amino acids and 6 amines within 80 min. Moreover, the methodology for the quantification of monomeric polyphenols and organic acids in wines was achieved through a RP-HPLC-DAD procedure, which allowed the quantification of 8 organic acids, 22 polyphenols and also 2 furanic compounds simply by direct injection of wine samples, ensuring sensitivity and reproducibility (RSD below 9.0%). The elution was performed in 12 min for the organic acids and in 60 min for the phenolic and furan compounds.

(b) Now, regarding the effect of *estufagem* on Madeira wines, it was clearly demonstrated that there is a strong relation between HMF, sugar content and baking temperature/time, ie, the amount of HMF tends to increase with heating, and important amounts (greater than 1 g/L) can be formed in sweet wines if heated above standard conditions (more than 50 °C). The results also suggest that HMF levels can be easily controlled when Madeira wines are submitted to adequate conditions of heating during *estufagem* (about 45 °C during 4 months; HMF levels up to about 150 mg/L). Furthermore, different temperatures/periods for the baking of sweet and dry wines may be considered.

(c) Most amino acids decreased with heating, suggesting their participation in the formation of aromas (Strecker degradation). The decrease was more pronounced in sweet wines (about 109 mg/L of amino acids were transformed), which can be associated with the development of the wine bouquet with ageing. This can explain the lower evolution of dry wines during the heating process, showing once again that the conditions for heating sweet and dry wines are not necessarily the same, as it is currently done.

(d) The total concentration of biogenic amines never exceeded 12 mg/L, indicating that the Madeira wines appear to be produced under adequate hygienic conditions and safe from a healthy point of view, including when wines are submitted to heating. Moreover, the results indicate that amines practically remained constant during the heating period, so that, it can be concluded that temperature and time do not promote their development.

(e) It seems that *estufagem* do not greatly affect the total polyphenolic composition of the Madeira wines, moderately decreasing up to 25%, with at least 434.42 mg (GAE)/L of total polyphenols present after heating, which is comparable with most white wines. Similarly, the antioxidant potential of baked wines (0.94 – 1.64 mM) is also comparable to white wines.

(f) The most abundant class of phenolics in Madeira wines were hydroxycinnamates and hydroxybenzoates, even in baked wines. Most individual polyphenols decline after *estufagem* be applied, with the exception of caffeic, ferulic, *p*-coumaric, gallic and syringic acids.

(g) The results showed that all baked wines tend to the same chromatic characteristics: white wine turns to brownish colour and red wines become clearer, loosing the red tones since the monomeric anthocyanins decline, and also acquire yellow tones.

(h) The analyses of organic acids showed that Madeira wines are especially rich in malic acid (about 55%) and that *estufagem* promote the decline of most acids, excepting for lactic, acetic and formic acids.

(i) *Estufagem* introduced important changes in the volatile composition of dry and sweet Madeira wines, especially promoting its increase. The increase is especially important in furans and esters. On the contrary, some chemical families tend to the opposite trend, namely varietal aromas, such as monoterpenic alcohols often encountered in *Malvasia* wine, which disappear after baking. It was also verified that *estufagem* favoured the development of some volatiles usually reported as typical aromas of Madeira wines, particularly phenylacetaldeyde, *β*-damascenone and 5-ethoxymethylfurfural. Additionally, *estufagem* also promoted the development of positive potential contributors to the global aroma of baked wines such as ethyl butyrate, ethyl 2-methylbutyrate, ethyl hexanoate, ethyl isovalerate, 5-hydroxymethylfurfural and *γ*-decalactone. In contrast, promoted the increase of volatile phenols usually considered off-flavours such as guaiacol, especially when higher temperatures were used.

(j) Finally, according to glucose and fructose model systems prepared under similar conditions of Madeira wines submitted to baking it was observed that several volatile compounds are developed (up to 29 volatiles were identified). The identified compounds enclose carbonyls, ethyl esters, pyrans and mainly furans, most of them

usually identified in baked Madeira wines. HMF represented about 84% of the compounds formed principally during the heating of model systems containing fructose. It was also demonstrated that the development of colour, aroma and chemical composition is highly associated with thermal degradation of sugars, particularly when the sugar is fructose.

CHAPTER 11
Future perspectives

The conclusions presented in the current thesis satisfy the objectives which were initially outlined, but raise new questions and request additional studies in order to increase the knowledge about the impact of *estufagem* on Madeira wines. New tests are fundamental to elucidate and reinforce some encountered trends, as well as other issues and prospects for future work have to be done. As future work we consider important to:

(a) Improve the developed methodologies - whenever possible, increase their sensitivity and reduce the analysis time/chemical consumption, namely using shorter columns.

(b) Get a better insight about the involvement of amino acids in the development of specific ageing aromas formed during the heating step, particularly by continuing to study model systems involving the reaction of sugars with amino acids, according to the Strecker degradation. Preliminary studies were already performed, but different extraction procedures should be applied in order to obtain a better response to minor volatiles.

(c) Confirm the identification of the volatiles formed in the performed model systems preparing new model systems. Elucidate their odorant importance through GC-O studies.

(d) Pursue with the identification of the several volatile compounds that remain unknown with the intention of improving the understanding of their origin and specific role in the aroma of the baked Madeira wines.

(e) Apply a convenient methodology to evaluate the presence of sotolon in the studied Madeira wines.

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