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Olfactory Perception Threshold Assessment of Volatile Acidity in Madeira Wines

MASTER DISSERTATION

Andreia Fátima Santos Miranda

MASTER IN APPLIED BIOCHEMISTRY



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SUPERVISOR

José Carlos Antunes Marques

CO-SUPERVISOR

Vanda Nulita Gomes Pereira

Dedicado aos meus pais, à minha avó, aos meus irmãos e ao Jorge

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RESUMO

O vinho Madeira é um vinho fortificado com impacto na economia da região. Similarmente a outros vinhos, a acidez deve ser controlada de modo a assegurar a sua qualidade, sobretudo a acidez volátil. Devido à sua complexidade é crucial obter um melhor conhecimento acerca do impacto desta acidez nas suas características, ou seja, determinar o limite de percepção do ácido acético e acetato de etilo, visto serem os principais responsáveis pela acidez volátil.

Avaliou-se o limiar de percepção olfativa da acidez volátil em amostras de 5 e 10 anos (Sercial e Malvasia), utilizando um painel treinado e outro não-treinado. Neste trabalho também determinou-se a evolução dos ácidos orgânicos, ácido acético e acetato de etilo durante 540 dias de envelhecimento do vinho Madeira (Malvasia, Bual, Verdelho e Sercial), nos dois processos tradicionais de envelhecimento: canteiro e estufagem. Amostras de vinhos envelhecidos em cascos no mínimo 5 anos, foram também avaliadas. HS-SPME seguida de análise por GC-MS foi a técnica usada na determinação da concentração do acetato de etilo, enquanto o IEC-HPLC-DAD na determinação dos ácidos orgânicos (incluindo ácido acético).

Os resultados demonstraram que o limiar de percepção olfativa do ácido acético e acetato de etilo dependem essencialmente da idade do vinho. O limiar de ácido acético no painel não-treinado foi em média 5,45 g/L (5 anos) e 6,22 g/L (10 anos). Formando o painel treinado para reconhecer o odor do acético, os valores diminuíram para 1,44 g/L (5 anos) e 1,87 g/L (10 anos), continuando contudo acima dos limites estabelecidos para a acidez volátil. O limiar do acetato de etilo foi similar em ambos os painéis (em média 327,97 mg/L). Ambos os compostos tendem a aumentar exponencialmente com a idade, especialmente em vinhos doces. Os ácidos orgânicos nos vinhos jovens dependem sobretudo das castas, sendo esta diferença minimizada com o envelhecimento.

Palavra-chave: Envelhecimento do vinho, Acidez volátil, Limiar de percepção olfativa, Ácidos orgânicos.

SUMMARY

Madeira wine is a fortified wine with impact in the Madeira Island's economy. Similarly to other wines, its acidity should be well controlled in order to ensure Madeira wine quality, mostly the volatile acidity. Due to Madeira wine complex flavour, it is crucial to get a better knowledge about the volatile acidity impact in its features, namely determine the perception limit of acetic acid and ethyl acetate, as both are the main contributors for volatile acidity.

Firstly, the olfactory perception threshold of volatile acidity was assessed by a trained and an untrained panel, using 5 and 10 years-old Sercial and Malvasia wines. Moreover, the current work also presents the evolution of organic acids, acetic acid and ethyl acetate during 540 days of ageing of Madeira wines (Malvasia, Bual, Verdelho and Sercial), comparing the same wines aged by both traditional ageing processes: *canteiro* and *estufagem*. Other wine samples, aged in wood casks (*canteiro*) for at least 5 years, were also evaluated. HS-SPME followed by GC-MS analysis was used to determine ethyl acetate concentration and IEC-HPLC-DAD was used for the organic acids determination, including acetic acid.

The results indicated that acetic acid and ethyl acetate olfactory perception threshold depends essentially on wine's age. Concerning acetic acid, the untrained panel was in average 5.45 g/L (5 years-old) and 6.22 g/L (10 years-old). Training the expert panel to recognize acetic acid odour, the values decreased for 1.44 g/L (5 years-old) and 1.87 g/L (10 years-old), but still remained higher than the established volatile acidity legal limits. Ethyl acetate threshold was similar for both panels (in average 327.97 mg/L). Both compounds tend to increase exponentially with age, being more evident in sweet wines. Organic acids in young Madeira wines depend mostly on the nature of grape varieties, but this difference is minimized with wine ageing.

Keywords: Wine ageing, Volatile acidity, Olfactory perception threshold, Organic acids.

Contents

PART I - GENERAL INTRODUCTION	1
1. MADEIRA WINE	3
1.1 Vinification process and ageing	5
1.2 Scientific overview	7
1.2.1 Volatile profile	7
1.2.2 Analytical characterization	8
1.2.3 Chemometric studies: wine ageing prediction	9
1.2.4 Other studies	9
2. WINE ACIDITY	10
2.1 Total acidity	11
2.1.1 Inorganic acids	12
2.1.2 Organic acids	12
2.2 Volatile acidity	16
2.2.1 Acetic acid	17
2.2.2 Ethyl acetate	19
2.3 Volatile acidity in wines	20
2.3.1 Volatile acidity in Madeira wines	23
3. SENSORY ANALYSIS	23
3.1 Paired comparison test	26
3.2 Triangle test	27
4. GOAL	27
PART II - EXPERIMENTAL	29
5. EXPERIMENTAL	31
5.1 Samples	31
5.2 Basic oenological parameters	32
5.3 Sensorial analysis – olfactory perception threshold of volatile acidity	33
5.3.1 Chosen panels	33

5.3.2	Basic training - untrained panel	33
5.3.3	Preliminary tests	34
5.3.4	Olfactory perception threshold assay	36
5.4	Determination of organic acids by IEC-HPLC-DAD	36
5.4.1	Chemicals and standards	36
5.4.2	Apparatus and conditions	37
5.5	Determination of ethyl acetate by GC-MS.....	37
5.5.1	Chemicals	37
5.5.2	Preparation of samples	38
5.5.3	HS-SPME/GC-MS analysis	38
<i>PART III - RESULTS / DISCUSSION.....</i>		<i>41</i>
6.	RESULTS / DISCUSSION.....	43
6.1	Analytical methodologies.....	43
6.2	Sensorial analysis	43
6.3	Acetic acid evolution during vinification and ageing processes	50
6.4	Ethyl acetate evolution during ageing	53
6.5	Organic acids determination during ageing process.....	56
<i>PART IV – CONCLUSIONS/ FUTURE PERPECTIVES</i>		<i>63</i>
7.	CONCLUSIONS	65
8.	FUTURE PERsPECTIVES.....	66
9.	REFERENCES.....	67
10.	APPENDIX	77

List of Figures

Fig. 1 – Main viticultural regions of Madeira Island.	3
Fig. 2 - Conduction system: <i>latada</i> (a) and <i>espaldeira</i> (b).....	4
Fig. 3 – Grape varieties: Sercial (a), Verdelho (b), Bual (c), Malvasia (d) and Tinta Negra (e).	4
Fig. 4 – Madeira wine vinification process.	5
Fig. 5 - Chemical structure of the main organic acids in wines.	12
Fig. 6 - Formation of acetic acid in wines in the presence of <i>Acebobacter</i>	19
Fig. 7 - Formation of ethyl acetate in the presence of acetic acid and ethanol.	20
Fig. 8 – Volatile acidity removal using a reverse osmosis coupled with ion-exchange technology.	22
Fig. 9 - The sense of smell and taste	24
Fig. 10 – Tongue taste map.	25
Fig. 11 – Scheme of assayed paired comparison and triangle tests (wine with the standard solution (A) and without (B))......	35
Fig. 12 – Paired comparison test vs. triangle test.	44
Fig. 13 – Paired comparison test vs. triangle test in a stress free environment (trained panel).	45
Fig. 14 – Olfactory perception threshold for acetic acid (untrained panel).....	46
Fig. 15 - Olfactory perception threshold for acetic acid (after training the trained panel).....	47
Fig. 16 - Olfactory perception threshold for ethyl acetate (untrained panel).....	48
Fig. 17 - Olfactory perception threshold for ethyl acetate (trained panel).	49
Fig. 18 – Acetic acid evolution during the vinification process.	50
Fig. 19 – Acetic acid evolution: <i>estufagem</i> vs. <i>canteiro</i>	52
Fig. 20 – Acetic acid evolution with long oak ageing.	53
Fig. 21 – Ethyl acetate evolution: <i>estufagem</i> vs. <i>canteiro</i>	54
Fig. 22 – Ethyl acetate evolution with long oak ageing.....	55
Fig. 23 – Linearity between acetic acid and ethyl acetate formation.....	55
Fig. 24 – Organic acids levels found in the monitored Madeira wine samples.....	58
Fig. 25 - Organic acids levels found in Madeira wine cask samples.	60

List of Tables

Table 1 -Total sugar content in Madeira wines.....	4
Table 2 – Selected old Madeira wines aged only by <i>canteiro</i>	32
Table 3 – Validation parameters of the used routine organic acids methodology.	43
Table 4 – Intrinsic amount of acetic acid and ethyl acetate in Madeira wine samples.	43
Table 5 – Basic oenological parameters of Madeira wines sample casks.....	78

List of abbreviations

AAB – Acetic acid bacteria.

AOAC – Association of Official Analytical Chemists.

CV – Coefficient of variation.

Da – Daltons.

EU – European Union.

GC-MS – Gas chromatography - mass spectrometry.

GC-O – Gas chromatography – olfactometry.

ha – Hectare.

He – Helium.

HMF – 5-Hydroxymethylfurfural.

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

HPLC – High performance liquid chromatography.

IEC-HPLC-DAD – Ion exchange - high performance liquid chromatography - diode array detection.

IR – Infrared spectroscopy.

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

ISO – International Organization of Standardization.

I3N – Instituto de Nanoestruturas, Nanomodelação e Nanofabricação de Aveiro.

K₂S₂O₅ – Potassium metabisulphite.

LAB – Lactic acid bacteria.

LOD – Limit of detection.

LOQ – Limit of quantification.

M0 – Grape juice.

MAF – Before must fortification.

MEPS – Microextraction by packed sorbent.

MIF – Begin of must fermentation.

NaCl – Sodium chloride.

NaOH – Sodium hydroxide.

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

RP-HPLC-FLD – Reversed phase - high performance liquid chromatography - fluorescence detection.

R^2 – Correlation coefficient.

SO₂ – Sulfur dioxide.

TCA – 2,4,6-Trichloroisole.

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

TIC – Total ion count.

UK – United Kingdom.

USA – United States of America.

UV – Ultraviolet

VAT – After wine fortification.

Vis – Visible.

VT – After wine post-fermentation treatments.

Short curriculum vitae

Andreia Fátima Santos Miranda was born in 1989, in Calheta, Portugal. She is graduated in Biochemistry (2010) by University of Madeira. In 2010 she performed a professional internship for 12 months at Madeira Wine Company, S.A. From 2012 until the present, she integrates a research project (IMPACT II) between Madeira Wine Company, S.A. and the University of Madeira. During this period she had the opportunity to follow 5 wine harvests and to be involved in the quality control of Madeira wine production, from grape to the product to be marketed. Furthermore, she participated in the training about sensory analysis and was also responsible for the preparation and assembly of sensory analysis. In addition, her work also resulted in 6 poster presentations, mostly in international scientific meetings and 1 submitted publication as a co-author in an international peer-reviewed journal.

COLLABORATION IN PROJECTS

IMPACT II – Impact of production technologies in Madeira wine quality. Funding through FEDER - Intervir+, QREN.

ESTUFA – Madeira wine estufagem monitorization. Funding through FCT.

LIST OF PUBLICATIONS

REFEREED JOURNAL ARTICLES:

Carvalho, M.J., Leça, J.M., **Miranda, A.**, Pereira, V., Pereira, A.C., Marques, J.C. An exploratory study to adjust the vinification practices of fortified wines based on a comprehensive physicochemical characterization. (Submitted)

POSTER COMMUNICATIONS:

Carvalho, M.J., Leça, J.M., **Miranda, A.**, Pereira, V., Pereira, A.C., Albuquerque, F., Marques, J.C. Evaluating the polyphenolic content and colour of Tinta Negra must at different fermentation extensions, XX ENCONTRO LUSO-GALEGO DE QUÍMICA, Porto, Portugal, 26–28 novembro, 2014.

Miranda, A., Leça, J.M., Pereira, V., Albuquerque, F., Marques, J.C. Volatile profile comparison between Madeira traditional varieties during winemaking process, EUROANALYSIS XVII, Varsóvia, Polónia, 25-29 agosto, 2013.

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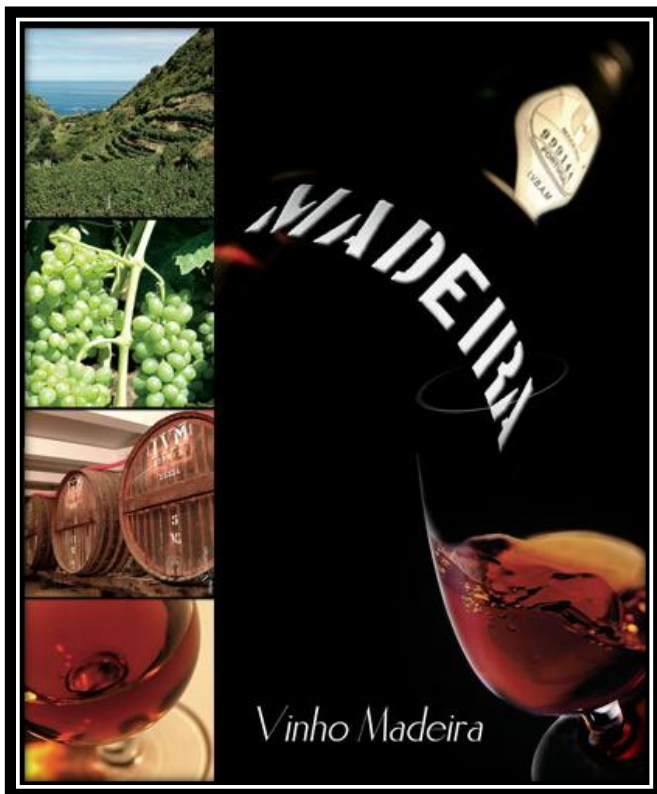
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PART I

GENERAL INTRODUCTION



1. MADEIRA WINE

Madeira wine is a fortified wine (17 - 22%, v/v) essentially served as an aperitif or digestive due to its intense flavour and complexity. From the 15th century until nowadays, Madeira wine has an important role in the region's economy (1). This wine is an internationally well-recognized product not only by its History (well documented in several papers (1-5)) but also by its unique characteristics. The commercialization of Madeira wine has risen from 637.904 €, in 1976, to 16,815.757 €, in 2013. In 2014, Madeira wine sold 3.37 million litres, mainly for European Union (EU) (79.7%), especially Portugal (17.6%), France, Germany, United Kingdom (UK) and Belgium. Outside the EU, countries like Japan, United States of America (USA) and Switzerland have a significant importation rate, especially of sweet and medium-sweet wines (1).

The vineyard is practiced throughout the islands of Madeira and Porto Santo with a total of 400 hectares (ha), mainly in the councils of Câmara de Lobos (125 ha), followed by São Vicente (122 ha) and Santana (82 ha) (Fig. 1) (1).

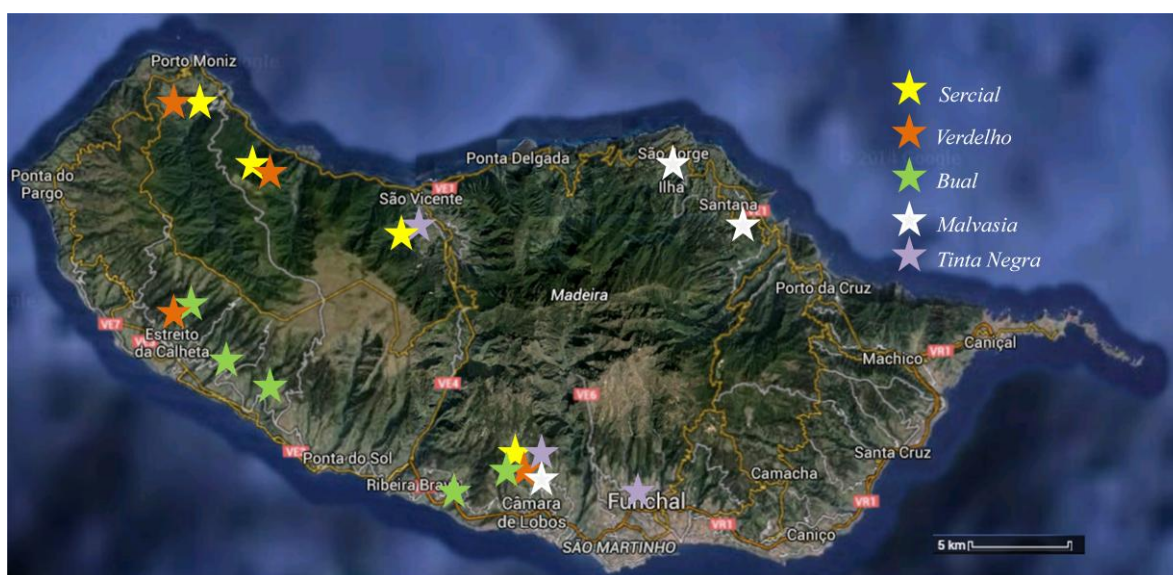


Fig. 1 – Main viticultural regions of Madeira Island [adapted from (6)]

The vine is usually conducted using the *latada* or *pergola* system (Fig. 2a). In this system the vines are horizontally disposed on wires and suspended off the ground by stakes with a height that varies between 1 to 2 m. The densities of the plantations can also vary between 2500 to 4000 plants/ha (1, 7). In the second half of the 20th century, a new conduction system was introduced: the

espaldeira system (Fig. 2b). This vertical conduction system can only be used on lands with soft slopes and plantation densities ranging between 4000 to 5000 plants/ha (1, 7).



Fig. 2 - Conduction system: *latada*(a) and *espaldeira* (b).

According to the style, Madeira wine can vary from extra dry to sweet, depending on the total sugar content (Table 1).

Table 1 -Total sugar content in Madeira wines (1).

Type of wine	Total sugar content (minimum limit) g/L	Total sugar content (maximum limit) g/L
Extra-dry	Do not exist	49.1
Dry	49.1	64.8
Medium-Dry	64.8	80.4
Medium Sweet	80.4	96.1
Sweet	96.1	Do not exist

All varieties used in the production of Madeira wines are *Vitis vinifera* L. species and can be divided in recommended and authorized varieties. The varieties commonly used are called the traditional varieties, namely: Sercial, Verdelho, Bual and Malvasia (white varieties) producing dry, medium-dry, medium-sweet and sweet wines, respectively. However, the most used is the red variety Tinta Negra (about 80%), from which can be obtained all sweetness degrees (Fig. 3) (1, 8) . Regarding colour, the wine can vary between very pale to dark brown, passing through golden tones. Note that usually sweet wines tend to a darker tone (1).



Fig. 3 – Grape varieties: Sercial (a), Verdelho (b), Bual (c), Malvasia (d) and Tinta Negra (e).

1.1 Vinification process and ageing

Madeira wine traditional winemaking process is unique, mainly its ageing processes that permit to acquire the typical bouquet. The vinification process of Madeira wine is schematized in Fig. 4.

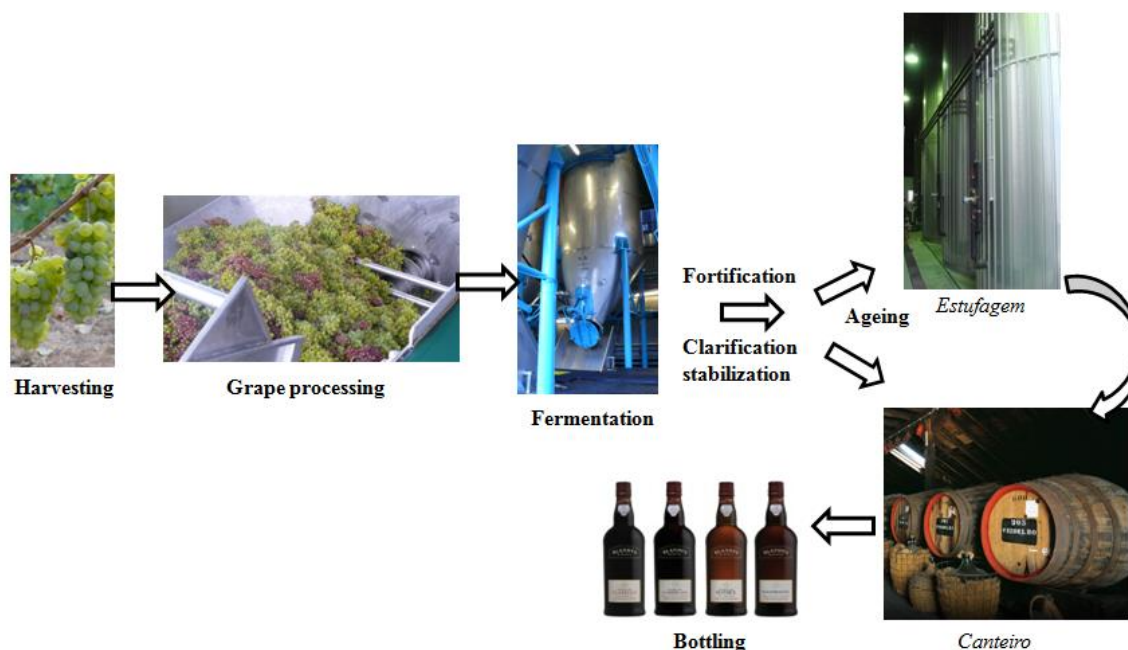


Fig. 4 – Madeira wine vinification process.

Harvesting – usually begins in the end of August up to the middle of October (when grapes are at their maturity peak). In this period the grapes with a minimum alcohol potential of 9% are picked and placed in boxes (25 and 50 kg) and then transported to the cellars, wherein a triage is firstly performed in order to verify the sanitary conditions. Then, after weighed and verified the probable degree of alcohol with a refractometer, the grapes are selected according to the type of wine desired (1, 7, 9).

Grape processing – Before crushing, the grapes are generally destemmed. At this stage, it is usually added an aqueous solution of 5% of potassium metabisulphite ($K_2S_2O_5$) to inhibit the growth of natural microbial flora (bacteria). Some wine producers also add pectolytic enzymes to promote a better extraction of the aroma and intensify the colour of the musts. For the production of Madeira wine two types of fermentation process can be performed: *bica aberta* or *curtimento*. In the first one, the resulting masses of crushing are pressed and the juice (must) is separated to ferment without grape solids (mainly used for the production of dry and medium-dry wines). In the

curtimento process, the fermentation occurs with grape solids and the pressing only takes place after reaching the desired degree of sweetness (essentially used for the production of sweet and medium-sweet wines) (1, 9).

Fermentation – In this stage, fermentable sugars (mostly fructose and glucose) present in grape juices are essentially converted into ethanol and carbon dioxide by yeast, promoting heat generation (10, 11).

Madeira wine fermentations typically occur in large stainless steel tanks on the wine producing-cellars. Fermentation is spontaneously developed due to the presence of indigenous yeasts. During this process the temperature is controlled, usually below 25 °C. The duration of the fermentation process depends on the type of desired wine (dry, medium-dry, medium-sweet or sweet). In this sense, sweet wines undergo a soft fermentation in order to retain high sugar content, while dry wines undergo a long fermentation so that low sugar levels can be obtained (9, 12).

Fortification – Once the desired levels of sweetness are obtained (usually below 130 g/L), fermentation is stopped by the addition of natural grape spirit, containing 95% (v/v) of ethanol, raising the wine ethanolic content between 17 - 22%. This step is important to stop fermentation and can also prevent the metabolism of lactic acid bacteria (transformation of malic acid into lactic acid) that can lead to the formation of acetic acid (1, 9, 11).

Clarification/ stabilization – After the fortification, fining agents (bentonite and gelatins) are added to the wine to gradually promote precipitation of suspended particles. This procedure takes about 30 days and aims to improve the final appearance of wines. In this stage some corrections can be done in order to correct some oenological parameters, namely alcohol degree. Finally, the wine is decanted into a new tank to be submitted to the traditional ageing process (1, 9).

Ageing – Madeira wine can be subjected to two types of ageing process: *canteiro* and *estufagem*. In the first one, the wines are aged in casks in the top floor of cellars, where the temperature is higher, for at least 2 years. This ageing process promotes the development of complex and intense flavours, resulting from the oxidative ageing in wood casks. These wines can only be commercialized after three years of ageing. Other Madeira wine ageing process is *estufagem*, where the wine is heated at 45 °C for three months. Then, these wines usually undergo maturation in wood casks at wine cellar lofts at least 90 days, as regulated by the official authority for the regulation and production of Madeira Wine, IVBAM. After this time, the oenologist evaluates the wine characteristics and decides if the maturation process in wood casks continues or

if it is ready to be commercialized with the required quality. Madeira ageing process is unique, providing the peculiar and unique characteristics of Madeira wines (1, 7, 9).

Bottling – Before bottling, the wines are submitted to several procedures to ensure that all analytical and sensorial parameters are within legal limits required for each type of wine. At this stage, some corrections can be done, namely the addition of natural grape spirit up to 17-22% to compensate some loss of alcohol content during the ageing process. Also, it is added small amounts of sulphite. Additionally, the wines are filtered and collected for the IVBAM verification. After wine approval by this official regulatory entity, it can be finally bottled, generally in 37.5, 50 or 75 cL brown, dark green or black bottles (1, 9).

1.2 Scientific overview

In the last decades, scientific investigation about these wines gradually increased in order to promote a better understanding about the wine composition and/or some particular phenomena that occurs during winemaking, especially during ageing. Also, these scientific studies allow maintain the high quality required to merchandise these fortified wines. In this sense, up to date there are dozens of publications about Madeira wines, essentially related with its volatile profile. Madeira wine's investigation also resulted in four doctoral thesis, namely from Câmara (13), Pereira V. (14), Pereira AC. (15) and Perestrelo (16). Câmara mainly focused on the volatile profile of Madeira wines made from white grape varieties (13). Pereira V. studied the effect of the *estufagem* process on the chemical constituents of Madeira fortified wines, evaluating the following compounds: volatile compounds, organic acids, biogenic amines, furans, amino acids and polyphenols (14). The studies performed by Pereira AC. (15) results in the development of reliable tools to predict Madeira wine age based on its intrinsic features. Finally, Perestrelo (16) work was based on the volatile profile and phenolic compounds of *Vitis vinifera* L. grapes used to produce Madeira wines.

1.2.1 Volatile profile

In 2004, Câmara (17) published the first scientific paper about Madeira wine volatile profile, where they determined the levels of one of the most powerful odorant in wines, sotolon, and concluded that its concentration linearly increased with age. In the same year, after characterizing the varietal aromas of Madeira's musts made from white grape varieties, they also verified that the

terpenoids profile depends on variety and that Malvasia showed a higher concentration of these compounds when compared to other varieties (18). Alves (19) after analysing the volatile profile of 33 monovarietal Madeira wines with different sweetness degree and ages, observed that the volatile fraction of these wines were mainly composed by esters (> 80%), but also by other minor compounds such as alcohols (< 8%), C₁₃-norisoprenoids (< 6.5%), carboxylic acids (< 4%), aldehydes (< 4%), lactones (< 3%), pyrans (< 2%), monoterpenes (< 1%) and sesquiterpenes (< 1%). They also found out that some aromas were intensified with age such as vanilla, coconut, woody and chocolate notes (19). Additionally, Perestrelo (20) evaluated the volatile profile of Tinta Negra variety of young Madeira wines and identified more than 90 compounds, especially on dry wines due to the fermentation time that is more extensive. Also in the same year, Campo (21) analysed 10 years-old Madeira wines made from white varieties by GC-O (gas chromatography-olfactometry) and determined that 41 odorants have a significant contribution for its aroma complexity, with nutty, toasty, woody and dried fruit notes.

Recently, Pereira V. (22) identified at least 190 volatile compounds in young Madeira wines, 53 of which were only found in wines after being submitted to the *estufagem* process. They concluded that the heating process promotes significant changes in the volatile composition of Tinta Negra and Malvasia wines, increasing its abundance and profile variety. In addition, some varietal aromas (essentially derived from grapes) found in Malvasia disappear after heating.

1.2.2 Analytical characterization

Some studies were performed, in order to understand the chemical composition that involves the complexity of Madeira wines. In this sense, Nogueira (23) firstly determined the physiochemical and sensorial parameters of 52 Madeira wines samples with different ages and types. They concluded that these fortified wines represent a high quality beverage without toxicological risks, within the parameters required by EU regulations. Later, some studies related with the development of methodologies for the determination of Madeira wine chemical constituents were performed. Pereira V. (24) developed a RP-HPLC-FLD method for the simultaneous quantification of free amino acids and biogenic amines in wines. This procedure allowed the quantification of 19 amino acids being arginine present at higher concentration. They also observed that biogenic amines were only present in residual levels, indicating the absence of toxicological effects. In the same year, Paixão (25) have conducted studies regarding the study of

polyphenols with antioxidant potential properties in Madeira wines, concluding that gallic acid was the most predominant acid, representing more than 65% of all phenolics. On the other hand, the major stilbene found in these wines was *trans*-resveratrol. Similarly, Pereira V. (26) developed a simple and sensitive HPLC method with photo-diode array detection in order to analyse monomeric polyphenols as well as organic acids and furanic compounds commonly found in wines. These previous studies were important to promote rapid and reliable results of Madeira wine chemical constituents. Thus, similar scientific studies were performed to determinate the impact of *estufagem* process in the chemical composition of these fortified wines. In this sense, heating seems to promote a significant decrease in the total amount of amino acids, indicating its involvement in the development of ageing aromas (14). Still, *estufagem* process does not appear to affect significantly the polyphenols content and antioxidant potential of Madeira wines. The colour of these wines tend to similar chromatic characteristics after this ageing process (27). In addition, studies about organic acids revealed that Madeira wines are mainly rich in malic acid and that the heating process promotes a decline in some acids, affecting essentially succinic acid. On the other hand, acetic, lactic and formic acids tend to increase when Madeira wines are submitted to overheating conditions (14).

1.2.3 Chemometric studies: wine ageing prediction

In order to explore the large volume of information provided by analytical instrumentation, some studies involving the development of advanced statistical methods (chemometric analysis) were realized. Scientific work show that it can be possible to predict Madeira wine age taking advantage of chemometric studies based on volatile compounds (chemical reactions that occurs during the ageing process modifying its profile), phenolic composition and measuring the absorbance in UV and Visible regions (28-31). Similarly, Rudnitskaya (32) also predicted the age of these wines with accuracy by using an electronic tongue multisensor system calibrated using data obtained by HPLC analysis (organic acids, phenolic and furan compounds).

1.2.4 Other studies

Other studies involving the content in metallic ions and copper in Madeira wines were also performed (33, 34). Furthermore, the current concerns in food safety and quality lead to the need to identify possible contaminants that may be present in commercial products. Thus, in the case of

Madeira wine, due to its unique ageing involving a heating process (typical of these wines), some compounds need a special attention namely 5-hydroxymethylfurfural (HMF) and ethyl carbamate. Although HMF is formed mainly due to the presence of sugars when submitted to heating, scientific studies regarding these fortified wines revealed that it can be easily controlled when these wines are submitted to adequate temperature conditions during *estufagem* and storage process (35). Likewise, Ferreira (36) evaluated the ethyl carbamate behaviour during the production of Madeira wines and found that this compound slightly increase during fermentation and *estufagem* process. Recently, Leça (37) developed a new methodology to quantify the ethyl carbamate in fortified wines using microextraction by packed sorbent (MEPS) and GC-MS detection.

2. WINE ACIDITY

Acidity is one of the most important characteristics of wines, contributing directly and indirectly for its quality. Therefore, acids confer a refreshing taste and can also modify the taste perception, namely reducing the sweetness perception. Wines with insufficient acidity usually originate flat or insipid tasting wines while the opposite, gives sour tasting wines (10, 38). Even though must or wine acidity depends essentially on grape varieties, other factors contribute for the acid composition, namely, soil fertility, pruning, irrigation, virus injection and the maturation degree of grapes. Thus, the wines from grapes that grow in warm climates are usually low in acidity and high in alcohol. Contrarily, grapes from cooler climates generally have acidity too high balanced with low sugar contents (10, 39).

Several important aroma compounds (such as phenolics, monoterpenes, benzyl alcohol, 2-phenylethanol and C₁₃-noriprenoids) may be present in grapes as acid non-volatile glycosides. These acids are important for the aroma preservation. During fermentation and ageing processes, acids are involved in reactions that promote the release of these aromas, contributing for the wine bouquet (38). Besides the acid composition being essential for the flavour and aroma, they are also involved in the wine stabilization and treatments (promoting the precipitation of proteins and pectins), in the colour stability and have beneficial antimicrobial effects, since most bacteria do not grow at low pH values (10, 38, 39).

The acidity of wines can be measured by the pH, representing the quantity and the strength of the acids. The strength is obtained by the constant dissociation, in other words, by the proportion of hydrogen ions liberated. The wine pH can range between 2.9 and 3.9 depending on the type.

Commonly, for most white wines the pH range from 3.1 to 3.4 and for red wines from 3.3 and 3.6 (38, 40).

Additionally to pH, acidity in wine is generally divided into two categories, namely the volatile acidity and total acidity. Volatile acidity is the free and combined forms of volatile acids that can be easily removed by steam distillation. Total acidity consists in all types of acids present in musts or wines, mostly organic acids and inorganic acids, but also amino acids. In addition, the fixed acidity (that includes those that are poorly volatile, namely all non volatile organic acids) can be calculated by the difference between total and volatile acidity (38, 40, 41). Generally in grapes and wines, the fixed acidity is essentially constituted by two main organic acids that are tartaric and malic acids. This acidity can vary from less than 2 g/L and more than 5 g/L depending on the grape ripeness and climate conditions (38).

2.1 Total acidity

Total acidity, also known as “titratable acidity”, can be determined by several procedures. However, the standard method of *Office International de la Vigne et du Vin* (OIV), used in Europe, is based on the potentiometric titration, by the addition of a base to wine for neutralization (with NaOH 0.1M) to bring the pH to 7.0 (42, 43). Total acidity can be expressed in terms of tartaric, malic, lactic, citric, sulfuric or acetic acid equivalents. Although, since tartaric acid is the main acid found in wines and has an important role on the pH, as well in the sensorial characteristics of wine, the total acidity is often expressed in terms of tartaric acid (g/L). Other countries as France, usually express these acidity in grams of sulfuric acid (40, 41, 44). Generally, the desired range of total acidity in wines lies between 5.5 to 8.5 g/L. Higher levels of acidity are preferred in white wines than in the red wines (45).

Total acidity together with sugar and alcohol contents contributes for the flavour wine balance (41):

Sweet taste	↔	Acid taste
(sugar, alcohols)		(organic and inorganic acids)

2.1.1 Inorganic acids

The main inorganic acids found in wines are dissolved gases, namely carbonic and sulphurous acids. Once they have an important role in wines as gases, they do not influence either the wine pH or the acidity perception (38). For that reason and according to OIV, these inorganic acids are not included in the expression of total acidity (42). Furthermore, other inorganic acids that can be present in wine are chloride, sulfuric, nitric and phosphoric acids. However, these acids are in trace amounts and consequently do not affect in a significant way the total acidity of the wine (41).

2.1.2 Organic acids

Total acidity is defined as the concentration of organic acids present in grapes or wines. These acids are relatively weak with dissociation constants usually in the order of 10^{-4} or even smaller (42). In wines, the most common organic acids are tartaric, malic and citric provided from grapes and also succinic, lactic and acetic acids formed during alcoholic and malolactic fermentation (Fig. 5). Moreover, some other acids derived from ethanol oxidation can be present in traces amounts (7, 26, 46).

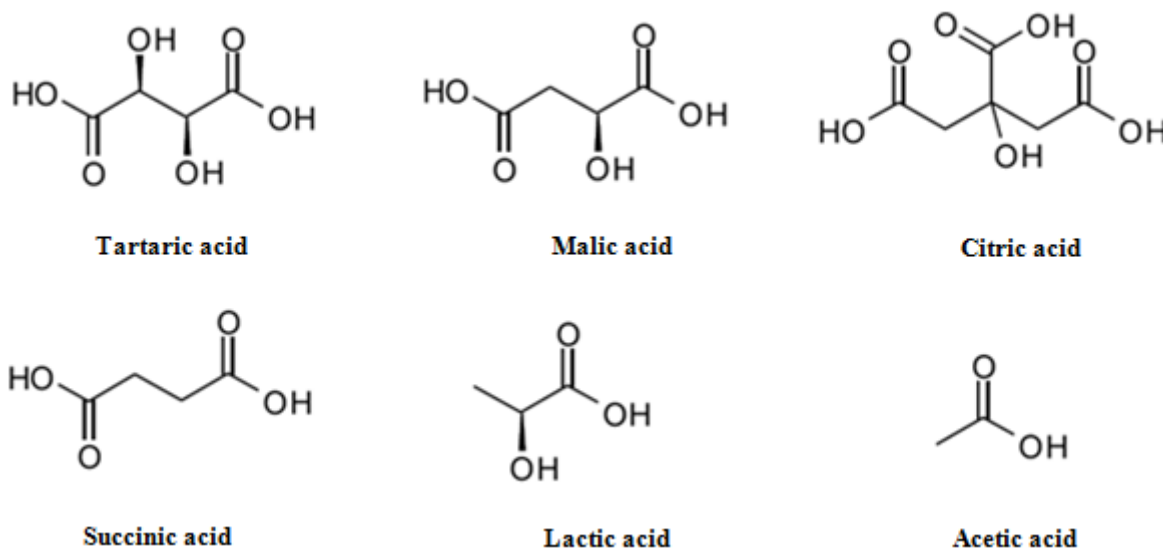


Fig. 5 - Chemical structure of the main organic acids in wines.

The determination of organic acids is useful for monitoring the maturation process, control the evolution of the acidity during winemaking and ageing processes and also to detect eventual wine alterations or illness. Tartaric, malic and citric acids are the predominant acids in wines, representing about 90% of the total acidity. Therefore, these acids have an important role on the flavour balance, microbiological control and chemical stability. There are also organic acids that have enough volatility, contributing to its odour such as acetic acid. The organic acids present in grapes and wines are essentially affected by several factors such as variety, region, ripening, atmospheric conditions and also by the wine production techniques (7, 10, 26, 46).

The predominant organic acids in Madeira wines are the same as aforementioned for other wines, particularly tartaric, malic and lactic acids. The overall range varied between 0.06 to 6.27 g/L and their amounts are usually bellow the odour threshold (7).

The main non-volatile organic acids present in grapes and wines are discussed below.

Tartaric acid

Tartaric acid is the strongest organic acid and one of the most relevant acid present in grapes, must and also in wines. Despite of this organic acid do not be very widespread in nature, it is synthesized and accumulated in significant quantities in plants of few genera, especially on *Vitaceae*. For this reason, tartaric acid is commonly present in grapes and is usually called the “wine acid” (7, 41).

Tartaric acid concentration may be 15 g/L in unripe grapes, decreasing to 6 g/L in musts, from the northerly vineyards, or to 2-3 g/L in the south, due to the higher temperature. In wines, tartaric acid concentrations generally range between 2 to 5 g/L, giving wine a pH that lies between 3.0 and 3.5. Tartaric acid content of table red wine (as well as total acidity) shows to be substantially higher than white wines. Thus, due to its relevance in wine acidity, several wine characteristics are affected by this organic acid, such as colour, flavour, chemical and microbiological stability of the final product (10, 41, 47, 48).

According to the European Community legislation it is possible to add tartaric acid (known as “tartrating”) up to a maximum of 1.5 g/L in must and 2.5 g/L in wines, in order to correct some natural acidity deficiencies. Tartaric acid addition promotes the pH reduction, however it is necessary to ensure that pH cannot be reduced bellow 3.0 (41, 49).

The tartaric acid found in wines can be present as a free acid or as tartrates salts, when combined with potassium or calcium ions. The tartrates salts tend to precipitate due to the alcohol content rising during fermentation and ageing processes. However, before bottling, wine is usually submitted to cold stabilization in order to accelerate the precipitation process, preventing the deposition of crystals in the bottle. When the precipitation of calcium and potassium salts of tartaric acids is verified generally occurs a loss in the total acidity and consequently a pH increase (14, 41, 50) .

Tartrates derived from wine industry are the main source of tartaric acid largely used in several industries such as food (chocolates, cakes), beverage (soft drinks), medicine (as a laxative) and dyeing. Some of them can be used for several purposes, for example, tartrazine (diazoic derivative of tartaric acid) is generally applied as food colouring (E102) as well as yellow colouring matter in silk and wool (41).

Malic acid

L(-)-Malic acid is found in living organisms and is called the “apple acid” since it is abundant in green apples. This organic acid is also one of the most important acid in grapes, constituting about half of the total acidity in wine and grapes (38, 41).

Malic acid content in grapes is one of the prime indicators in determining the harvest date, once its concentration tends to decrease as grapes mature. Therefore, grapes may contain about 25 g/L of malic acid before changing colour. During the two weeks following the first signs of colour change, grapes size increase and promote a dilution in the concentration of this organic acid that drops to half. The concentrations of malic acid in grapes matured in southerly regions are usually about 1-2 g/L and sometimes can lead to the production of wine with flat taste and more susceptible to microbial spoilage. On the other hand, if the maturation happens in cool conditions, malic acid content may remain high (generally 4-6.5 g/L), giving a sour taste to wine (38, 41).

During malolactic fermentation, the malic acid (di-acid) is transformed in lactic acid (monoacid). In this step, occurs a loss of acidity due to the loss of acidic equivalent, since there is a replace of a strong green taste of malic acid to a less aggressive taste of lactic acid. Furthermore, some aromas revealed during the alcoholic fermentation may disappear or change during this type of fermentation. So, malolactic fermentation can favour the wines where fruity aromas attributed to

grape variety are not so important. These wines are often submitted to ageing for a long time in barrels, representing a more complex taste (51, 52).

The concentration of *L*(-)-malic acid is essential for the wine sensory properties and also has an important role as an indicator of its microbiological stability, once this acid is responsible for the malolactic fermentation. Thus, fermentation must be controlled in order to avoid undesirable effects such as the reduction of acidity that leads to the risk of spoilage, colour changes and formation of undesirable flavours and amines (53).

Citric acid

Citric acid (tri-acid) is very widespread in some citrus fruits found in nature, such the case of lemons. It is used in the industry for the acidification of several foods and beverages and is also utilized in photography and pharmaceuticals. The presence of this organic acid in ripe grapes is scarce, appearing for this reason, in small quantities in wines (0.5 to 1 g/L). Despite its low concentration, citric acid is very important for wine taste (41, 54).

Citric acid decay during must fermentation, since yeasts promote its conversion into acetic acid, being the most important oenological effect associated to the formation of acetonc compounds (such as diacetyl, acetoin and 2,3-butanediol) that influence wine aroma (51, 54).

The formation of acetic acid, acetoin and diacetyl depends on the rate of malolactic fermentation. In this sense, when the fermentation is fast, citric acid produces high amounts of acetic acid and acetoin and diacetyl is low. On the other hand, when the malolactic fermentation is slower, less acetic acid is produced and more acetoin and diacetyl is formed (54).

Succinic acid

Succinic acid (1,4-butanedioic acid) also called succinate, is a di-acid produced by all living organisms. In grapes it is only present in trace amounts, but is one of the predominant non-volatile organic acids formed during alcoholic fermentation since it is the main carboxylic acid produced during fermentation by wine yeast (41, 55). The amount of succinate in wines can change depending on the yeast strain, for example *Saccharomyces cerevisiae* strain produces variable amounts up to 2 g/L, whereas *Saccharomyces bayanusluvarum* produces higher concentrations (54). Succinate in wines can also be obtained by Krebs cycle during anaerobic fermentation (41, 55).

Succinate is very resistant to bacterial attack under anaerobic conditions and is particularly stable in wines. However, can affect wines quality because it has an intense bitter and salty taste (38, 41, 56). Moreover, there are several factors that have an effect on the accumulation of succinic acid during fermentation, namely the fermentation temperature, yeast strain, pH, acidity, SO₂ concentration, nutrient content, must composition and clarification (also including the sugar concentration) (41, 55).

Lactic acid

Lactic acid found in wines can be originated during the alcoholic or malolactic fermentation. However, the main source of lactic acid is provided from the malolactic fermentation, which is desirable mainly in red wines and in some white wine styles. This stage involves the presence of lactic acid bacteria (LAB), producing an enzyme that promotes the malic acid decarboxylation directly into lactic acid (12, 57).

LAB affect wine aroma and has a great impact in wine quality. LAB from grapes, musts or wines belong to two families, namely Lactobacillaceae (genus *Lactobacillus*) and Streptococcaceae (genus *Oenococcus* and *Pediococcus*) (41). These bacteria can also metabolize citric acid, producing lactic acid, acetoin, diacetyl and acetic acid. The amount obtained from the products of this metabolism depends on the concentration of glucose involved (58).

The content of lactic acid depends on the type of the wine. Thus, the ones that undergo malolactic fermentation process show significantly higher concentrations (900-2600 mg/L). Italian (34–205 mg/L) and Bordeaux (7–55 mg/L) wines have demonstrated lower amounts (10).

2.2 Volatile acidity

Volatile acidity in wines is surely one of the most important physicochemical parameter for its quality. Although this parameter is an integral part of the wines total acidity, it is considered separately, representing a small fraction in quantitative terms (41).

One way of volatile acidity determination consists on the wine acidification with tartaric acid (approximately 0.5 g/20 mL) and once this organic acid is stronger than the volatile acids, it can displaced them from their salts (41). According to AOAC, volatile acidity determination can also be performed via steam-distillation where the distillate is collected in a container and then is

titrated with a base (such as NaOH). The amount of the base used for the neutralization of the acidic sample is directly proportional to the amount of volatile acids present in the sample (59).

The principal volatile acid present in wine is acetic acid. For this reason, volatile acidity is usually expressed in terms of this acid. Other volatile acids can also be found namely formic, butyric and propionic. Despite these acids are found in wines, they rarely occur above their threshold levels. All of these have marked odours: acetic acid is characterized by the vinegar odour; formic acid has a strong pungent odour; butyric acid has a rancid butter scent and propionic acid has fatty note (38, 60). Although formic acid concentration is invariably very low, it occurs as a metabolite in many biochemical reactions. This acid is a small part of the volatile acids presents in wines and can also be formed in baking processes, as it occurs in beers. Even at low concentration, formic acid contributes positively, having both fungicidal and bactericidal effects (61).

Other important compound involved in volatile acidity is ethyl acetate (nail varnish odour), which is formed as a result of the acetic acid esterification (62). Therefore, the determination of volatile acidity in wines takes into account mostly both compounds: acetic acid and ethyl acetate. The presence of these compounds above perceptible levels can be considered undesirable and may evidence microbiological problems. However, when present at low amounts it can enhance the fruitiness flavour and add complexity to wine (63).

2.2.1 Acetic acid

Acetic acid is the main volatile acid in wines and affects both its acidity and odour, being for that reason one of the most important analytical parameter in oenology. In this sense, acetic acid can be beneficial or detrimental depending on its concentration. The ideally concentration of this organic acid, in table wines, should be around 0.3 g/L in order to contribute to wine aroma and taste through the production of acetate esters (fruity notes). On the other hand, high concentration of acetic acid gives a vinegar odour and a disagreeable mouthfeel. So, the concentrations of this compound must be controlled to avoid compromise the wine quality. However, and according to some authors, the odour perception of this acid in wines depends on the type and style (10, 54).

The formation of acetic acid in wines is generally associated to the presence of yeasts, LAB and acetic acid bacteria (AAB). Acetic acid is formed by most yeast species during fermentation, but the amount of acid produced depends on the involved strains (64). In this sense, during, alcoholic fermentation occurs always the formation of small quantities of acetic acid (usually

between 0.1-0.3 g/L) by the yeast *Saccharomyces cerevisiae*. However, in certain winemaking conditions (even without bacterial contamination) *S. cerevisiae* produces high levels of acetic acid. The production of this volatile acid is closely related to the initial sugar level in must, independently of the quantity of sugars fermented. This event occurs due to the mechanism of the yeast for adapting to a medium with a high sugar amount. Thus, as higher is the concentration of sugars present on must greater is the production of acetic acid (and also glycerol) by yeast during fermentation (41, 54). Other factors involved in the production of acetic acid by *S. cerevisiae* are mostly related with pH (below 3.1 or up to 4), anaerobiosis, temperature (25-30°C) and also amino acid or vitamin deficiencies in musts. Yeasts seem to metabolize the acetic acid at the beginning of alcoholic fermentation (during the fermentation of the first 50-100 g of sugar). This acid is not used in the second half of fermentation, accumulating up to the end of the fermentation (41).

LAB perform the malolactic fermentation in some white wines and in almost all red wines, producing a small quantity of acetic acid. However, when alcoholic fermentation stop and the lactic bacteria act on unfermented sugar in the must, occurs an increase in the production of acetic acid (62). This volatile acid can be formed during the metabolism of tartaric, citric, malic and gluconic acids as well as pentoses, hexoses and also glycerol. Furthermore, the amount of acetic acid synthesized depends on the strain and also in the conditions involved. For instance, anaerobic conditions do not favour the acetic acid formation (38).

The increase of acetic acid concentration after fermentation is attributed essentially to the presence of AAB. These bacteria are the main responsible for higher volatile acidity in wines and are characterized by the ability to oxidize ethanol to acetic acid in an aerobic environment (38, 59, 62). These bacteria can influence wine quality in three phases: by grapes contaminations; by possible growth during alcoholic fermentation and finally by its growth during wine storage in cellars (50), favoured in environments rich in sugar and alcohol (62).

There are two genera of AAB present in grapes and wines: *Acetobacter* and *Gluconobacter*. The most frequently species found are *A. aceti*, *A. pasteurianus* and *G. oxydans*. Studies show that these bacteria can remain viable in wine under anaerobic conditions for years and only when the environment is favourable occurs its growing. The key for its biology is the presence of oxygen that promotes bacteria growing. So, at any stage, if the level of dissolved oxygen rises, the bacteria will also grow producing acetic acid. Furthermore, *Gluconobacter* commonly occurs on grapes or musts and are characterized for the ability to oxidize the sugars to ketonic compounds. On the other hand,

Acetobacter is mainly present in wines and in the presence of oxygen, oxidizes ethanol to acetaldehyde and finally to acetic acid (Fig. 6). Acetic acid can also be transformed into ethyl acetate in the presence of this bacteria (38, 62).

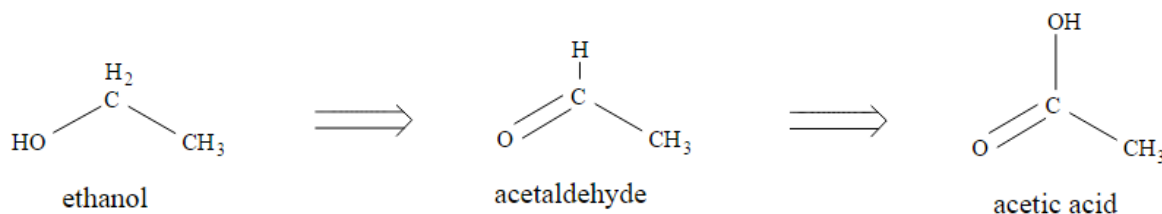


Fig. 6 - Formation of acetic acid in wines in the presence of *Acetobacter*.

AAB are present in all ripe grapes and for that reason the wines contain at least a small population of these bacteria. If the grapes are damaged before picked (such as skin broken), there is oxygen around, promoting the oxidization of alcohol into acetic acid and consequently the levels of volatile acidity will be high, even before the beginning of fermentation. As aforementioned, yeast normally formed 0.1-0.3 g/L of acetic acid while AAB usually contributes with 0.2-0.4 g/L. However, the levels of acetic acid in most finished wines typically round 0.3-0.5 g/L. These levels are below the aroma threshold, in other words, the lowest concentration at which a substance can be detected (62). The levels of acetic acid tend to increase to about 0.5-0.7 g/L in wines that were submitted to wood cask ageing (59).

Although, volatile acidity is usually measured by steam-distillation, the determination of acetic acid levels in wines can be performed by chromatographic techniques such as GC (gas chromatography) and HPLC (high-performance liquid chromatography) and also by enzymatic methods (59). Despite the fact that acetic acid level is never equal to volatile acidity, they are well correlated in juice, must and young wines. During wine ageing, other volatile acids can probably migrate from wood to wine, also contributing for its volatile acidity (59).

2.2.2 Ethyl acetate

Ethyl acetate is an ester well abundant in wines and even though is not an acid, has an important role on wine volatile acidity, affecting the organoleptic properties. All wines have small amounts of ethyl acetate formed during the alcoholic fermentation and ageing by non-enzymatic reaction between acetic acid and ethanol (Fig. 7) (10, 41, 60, 65). Large amounts of this compound

can be observed during wine ageing in wood casks due to the presence of AAB, which are capable of esterifying the acetic acid into ethyl acetate. Apparently, LAB do not seem to have capability to synthesize this ester (10, 41). The determination of ethyl acetate levels in wines is usual performed by GC (63, 66).

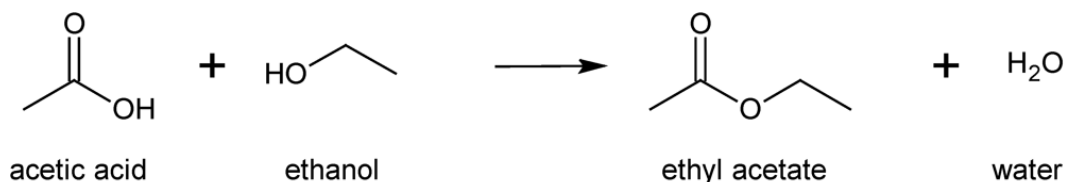


Fig. 7 - Formation of ethyl acetate in the presence of acetic acid and ethanol.

The perception threshold of ethyl acetate in table wines (usually around 150 mg/L) is much lower than the acetic acid (approximately 750 mg/L), having a positive impact on wine quality at very low doses (50-80 mg/L) especially in sweet wines, contributing with fruity notes. Contrarily, above the odour threshold may be undesired, since produces an odour similar to fingernail polish remover (41, 59, 67). Notice that, the sensory threshold of ethyl acetate can vary depending on the style and type of wine, appearing higher mostly in white (4.50 – 180 mg/L) than in red wines (22- 90 mg/L) (67, 68). In case of ice wine, studies show that the mean concentration of ethyl acetate for this type of wine is 240 mg/L (69).

Currently, there is no legal limit for the ethyl acetate concentration in wines. Contrarily, the concentration of acetic acid is regulated. Thus, the measurement of acetic acid content is usually done while ethyl acetate is not always controlled (57, 70). However, since the production of ethyl acetate occurs simultaneously with acetic acid, essentially due to the bacterial action, the concentration of both compounds in wine are interdependent. Therefore, at equilibrium it may be possible to calculate the concentration of one of them if the concentration of the other is known, together with the wine density, ethanol content and extract values (equilibrium constant, K) (63).

2.3 Volatile acidity in wines

In spite of winemakers attempt to minimize the volatile acidity present in wines (to avoid perceptible levels) it is not possible to produce wine without at least some amount, due to microbes involved in fermentation, as the example of AAB, that promote the production of acetic acid and

ethyl acetate. Therefore, to keep volatile acidity levels down, already in harvesting, only grapes in good conditions must be picked. During the wine vinification the levels can increase by the presence of oxygen conjugated with high temperature and low pH, promoting the growth of AAB. In winery, it is important to maintain a good hygiene and also protect the wine from oxygen. Furthermore, for wines that are bottled young (kept in stainless tanks), volatile acidity can be easily controlled by the addition of the antimicrobial/antioxidant compound SO₂ (generally 25-30 mg/L), maintaining the pH low and also using an inert gas cover. However, for the wines that are aged in casks is more difficult to avoid an increase of volatile acidity levels since the goal of this ageing process is to allow controlled exposure to oxygen. In this case, it is essential to fill up the casks and also control the SO₂ levels (62).

Wines should not exceed certain limits of volatile acidity in order to ensure their quality. However, if the wines show high volatile acidity problems there are two ways to reduce it: blend it with a wine with low volatile acidity ensuring that the final product has the level below the aroma threshold or use reverse osmosis coupled with ion-exchange technology (62, 69, 71, 72). The reverse osmosis technology was invented and patented in 1996 by Clark Smith of Californian Company, Vinovation, Inc. and is known as the Smith's process (69). This technology involves two stages: the first is a reverse osmosis and the second is an ion exchange (Fig. 8). Firstly, the wine circulates at high pressure through a membrane. The type of membrane is important to ensure a good separation, ideally with a molecular size of about 100 Daltons (Da), since allow the passage of ethyl acetate (MV=88) and also acetic acid (MV=60). Other acids present in wine are rejected by the membrane, being shortly retained because have greater molecular size, such as tartaric, malic and lactic, tannins and other important molecules for colour and aroma. On the other hand, the permeated molecules (acetic acid and ethyl acetate) pass into a charged anion exchange column, being adsorbed on the selected resin. Finally, once these compounds are removed, permeate and retentate are recombined and treated wine return to tank. This process continues until the levels of volatile acidity are within required limits (62, 69, 70, 72). Smith's process can also be used in wines that stuck during fermentation. In this case, after remove acetic acid, the fermentation process usually starts again (69).

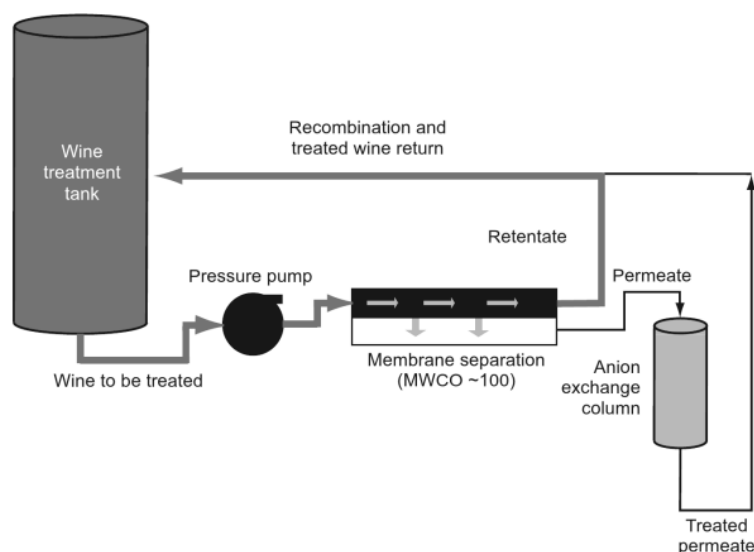


Fig. 8 – Volatile acidity removal using a reverse osmosis coupled with ion-exchange technology (69).

The maximum limit of this organoleptic parameter is not the same for all countries and types of wines (it can also depend on the sweetness degree). In this sense, in EU and California the limit allowed is 1.1 g/L (express in terms of acetic acid) for rosés and white wines and 1.2 g/L for reds. In Australia the limit is 1.5 g/L for all wines, while in United States the volatile acidity legal limits depends of the sweetness degree. For wines made from grapes with less than 28°Brix the limits are 1.2 g/L for white wines and 1.4 g/L for reds (expressed in terms of acetic acid). On the other hand, when wines are produced from grapes with °Brix higher than 28 (dessert wines), the maximum volatile acidity allowed increase for 1.5 g/L and 1.7 g/L for white and red wines, respectively (59, 62). In Canada, the legal limits are 1.3 g/L for table wines and 2.1 g/L in case of the ice wines (63).

However, in some cases the legal volatile acidity limits are contested once the type and style of wine can in a certain way mask its perception. One example is the ice wine (high intrinsic concentration). Cliff (63) studies, based on organoleptic test (paired comparison method), demonstrated that in this type of wine, the odour threshold for ethyl acetate was 0.198 g/L and 3.185 g/L for acetic acid (approximately three times higher than in table wines). So, they concluded that the legal limit for volatile acidity should be higher than the currently in force and that these two compounds must be separately considered.

2.3.1 Volatile acidity in Madeira wines

One of Madeira wine ageing process (*canteiro*) consists on ageing the wine in oak casks. Usually, AAB (namely *Acetobacter*) may be present in old wooden barrels and tend to grow in this type of oxygenated environment, producing acetic acid (67). However, there are evidences that another pathway for acetic acid formation may be purely chemical, since there are some old mature wines with high volatile acidity without signs of spoilage (10). Furthermore, in fortified wines (such as Madeira wines), the *Acetobacter* activity is inhibited due the higher alcohol content (73, 74). So, Wildenrad (75) showed that during oxidative wine maturation occurs the production of aldehydes from ethanol and that the ethanol oxidation leads to the acetic acid formation. Additionally, acetic acid can also be formed during wood-ageing by chemical hydrolysis of hemicelluloses (45).

According to the latest decree law (2011), the maximum legal limit of Madeira wine volatile acidity (expressed in terms of acetic acid) are: 1.2 g/L for wines with equal or less than 10 years old; 1.5 g/L for wines between 10 to 20 years and 1.8 g/L for wines equal or greater than 20 years (76). Nonetheless, since this fortified wine is characterized by its intense and complex flavour acquired during its ageing process, it can easily mask the presence of acetic acid and ethyl acetate especially in old wines. However, until today there is not any scientific study about volatile acidity limits for Madeira wine. For that reason, this work pretends to elucidate about its odour perception limits.

3. SENSORY ANALYSIS

Sensory tests have been conducted for many years in order to evaluate some product such as water, foods, drinks or everything else that can be consumed or used (77). In fact, sensory analysis is one of the most important tools used in wine industry. Both, analytical and sensorial analysis ensures the wine quality during the winemaking process as well as in the final product. While analytical analysis allows the control of the chemical parameters and wine stability, sensorial analysis is mainly used to guide winemakers in their choice about the wine flavour (78, 79).

The senses used on wine sensorial analysis are: smell, taste, touch and sight. Even though we can hear the fizz of a glass of champagne or a pop of a cork, this sense is not significant to evaluate wine flavour. Sight gives information about the visual appearance such as the colour and

turbidity (sensory evaluation) and touch is involved in the wine texture. The most relevant senses used for evaluate wine flavour are the smell (aroma) and the sense of taste (79-81).

Wine is characterized by a complex chemical composition resulting from several compounds present in grapes or formed during fermentation and ageing processes. In this sense, more than 800 volatile compounds have been found in wines at different concentrations, being responsible for wine's aroma. For that reason, smell is the most important and developed sense. Notice that the human nose can identify thousands of different aromas (79). The volatile compounds inhaled through the nose reach the olfactory epithelium (located to either side of nasal septum), dissolving in the nasal mucus and then interacting with the odorants receptor on the cilia of olfactory neurons that are present in these membranes. These neurons are bipolar nerve cells formed by a single axon through the bony cribriform plate to the olfactory bulb (located above the nasal cavities). Then, the axon forms synapses with the olfactory bulb neurons that are capable to relay signals to the olfactory cortex, wherein all the information is processed (Fig. 9). The description of the aroma is usually formed in our brain based on the comparison to other aromas that we remember to have smelled before. So that, it is evident that in the general population, there is a great variation in the aroma's perception that depends on each person. On the other hand, it can elucidates that with training is possible to increase the ability to distinguish different aromas (79, 82).

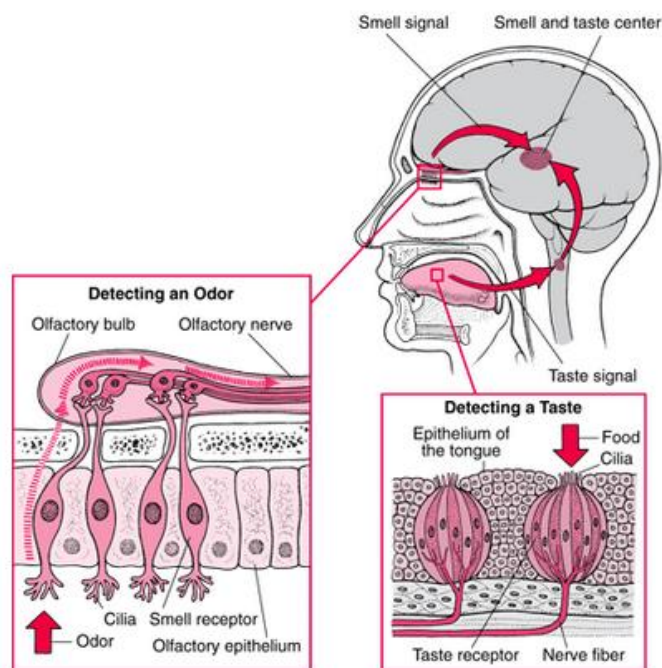


Fig. 9 - The sense of smell and taste (83).

The taste is one of the most important senses to evaluate the wine flavour. For many years it was thought that different areas of the tongue were sensitive to different tastes (bitter, salty, sweet and sour), known as “tongue taste map”. However, this concept was overthrown by Dr. Virginia Collings work (in 1974) that showed that these four tastes can be detected in any place of the tongue

due to taste receptors presence in all parts of the tongue (Fig. 10). After the compounds react with the receptors, these send signals to our brain in order to process all the information (79, 84, 85).



Fig. 10 – Tongue taste map (79).

The interaction between the smell and the taste provide the possibility to experience more than the four basic flavours. This fact occurs since most flavours that we taste come from the compounds that enter at the back of the mouth through the retronasal pathway (Fig. 9). The retronasal perception is usually responsible for the ability of a person to identify a flavour or an aroma (77, 79). Additionally, several studies showed that with age there are a significantly reduction in the number of taste and olfactory receptors resulting in the loss of sensory acuity. The loss of acuity is usually associated with an increase in the threshold levels (12).

In wine industry, sensory analysis has been increasingly used to ensure the quality of the wine and also its acceptance by the consumers. Depending on the purpose of the analysis, the type of sensorial tests, panel and number of members involved may change (60). There are different types of panels: trained, untrained and consumer panel:

- A **trained panel** is formed by people who had an intensive training during several periods and had the capability of describing differences between two samples. The people that form a trained panel must have a good acuity and also the ability to recall, recognize and describe consistently the characteristics of the product over time. Training (repeat exposure) show to improve detection and for that reason the panel is periodically submitted to training in order to refresh the memories (60, 80).
- An **untrained panel** is formed by people that tend to have less sensitivity to small differences and are usually less consistent in the assessment, even in replicate products. For that reason, they are typically requested for difference tests, even though they can be use in descriptive tests. Furthermore, this type of panel can also receive some basic training in order to be more effective (80).

- A **consumer panel** is recruited, not based on their sensory acuity, but essentially to obtain some detailed information about the product by the consumers. Thus, this type of panel is not recommended for descriptive testing (80).

Furthermore, tasting conditions are very important to obtain good results. Taste requires concentration and for that reason the sensory analysis should be in an environment with a minimum of distractions. The place must be fresh and quiet and should have no distracting smells that could interfere with wine aroma (79).

Threshold detection is defined as the minimum value of a sensory stimulus needed to give rise to a sensation (86). It was firstly use in the identification of the compounds that contributes to wine varietal aromas, since these can occur at or above their threshold values, affecting wine aroma. The determination of the threshold is complicated once it depends on the factors such as solubility and volatility of the compound and can also depend on the acuity of each person (60).

There are several tests that can be performed in wine sensorial analysis. However, since that in the current study we wanted to determine the odour detection threshold of two compounds (acetic acid and ethyl acetate) it will be focused only two tests (paired comparison test and triangle test).

3.1 Paired comparison test

Paired comparison test is very simple and is used to compare two samples. This test may be simple or directional. The purpose of the simple paired comparison test is to determine if there is some difference between two samples (e.g, if sample A is different from sample B). On the other hand, in directional paired comparison test (also called 2-alternative force-choice) two samples are presented to the tasters and they must determine which of them differ in a specific sensory attribute (80, 87-89). In these tests is necessary a panel size with a minimum of 20 members (80). Additionally, the probability of correct guessing is 50% and because of that the detection of a legitimate differentiation between samples is usually considered when the correct response is higher than 75% (above the probability to guess) (60, 88). The advantages of paired comparison test are its simplicity, to be quite sensitive, low in confusion error and also the fact that fatigue is minimized due to the small number of samples (88).

Paired comparison tests have been often used in the sensorial evaluation of several beverages, including wines. Several studies use this sensorial test in order to determine some

differences in wine appearance, flavour, taste and aroma (90-92). Additionally, studies performed by Cliff (63) applied this sensorial test to determine the thresholds levels of acetic acid and ethyl acetate in ice wine. Other studies also used paired comparison for the perception threshold of 2,4,6-trichloroanisole (TCA) in white wines (93), ethyl phenylacetate and phenylacetic acid in Spain red wine (94) and also eucalyptol in Australian red wines (95).

3.2 Triangle test

In the triangle test, three samples are presented to the tasters. Two of them are the same and the other one is different. The taster is asked to choose the different sample. In this test, the probability of correct guessing is 33.3%, lower than the paired comparison test. So that, the number of a panel member can be reduced, reducing also the time and sample costs. Moreover, since this type of test requires a larger number of samples when compared to a paired comparison test, there is a higher risk of loss of sensitivity and also fatigue, which can compromise the tasting results (80, 88, 96).

Triangle tests are also often used in the sensorial evaluation of wines. Several studies (97-99) used this sensorial test to discriminate wine samples namely in flavour, taste or aroma in order to improve wine quality. Mazzoneli (100) studies applied triangle tests for the detection threshold levels of TCA while Lorrain (101) applied these tests for the detection of isoamyl acetate, ethyl isobutyrate, ethyl butyrate and ethyl octanoate. Additionally, other studies based in triangle tests were also performed to determine the perception threshold levels of lyoni-resinol (102), 1,1,6-trimethyl-1,2-dihydronaphthalene (TND) (103) and geosmin (104).

4. GOAL

The main goal of this work was to assess the odour detection threshold for volatile acidity (acetic acid and ethyl acetate) in Madeira wines (5 and 10 years-old Sercial and Malvasia) through a trained and untrained panel. Additionally, in this study it was performed an evaluation of Madeira wines made from white varieties in order to estimate the evolution of acetic acid, ethyl acetate and other organic acids during both processes commonly used in Madeira wine ageing. And also evaluate their evolution with wine ageing in wood casks for several years.

PART II

EXPERIMENTAL



5. EXPERIMENTAL

5.1 Samples

This study was performed based on Madeira wines made from four *Vitis vinifera* L. white varieties: Sercial, Verdelho, Bual and Malvasia. Three sample groups were evaluated:

➤ Madeira wines assessed through sensory analysis

For the determination of the volatile acidity olfactory perception threshold it was chosen 4 Madeira wines from different styles and sweetness degrees, taking into account wines with different ages: Sercial with 5 and 10 years-old as well as Malvasia with 5 and 10 years-old. These commercial wines were kindly provided by a local Madeira wine-producer.

➤ Young Madeira wines aged by both traditional processes: *canteiro* vs. *estufagem*

Four Madeira wines were prepared from about 1000 kg of four *Vitis vinifera* L. white grape varieties (Sercial, Verdelho, Bual and Malvasia), collected in 2011 from different locals of Madeira Island. The wines were produced using the winemaking practices of a local Madeira wine-producer. The elaboration of each wine was conducted in separated stainless steel tanks. Sulphite solution (solution of 100 g/L) was added to the must up to about 60 mg/L. Diammonium phosphate (solution 0.1 g/L) and pectins were also added to the initial grape juice. The grape juice together with skins followed 24 hours of maceration. The alcoholic fermentation (without grape solids) was conducted under controlled temperature ($20\text{ }^{\circ}\text{C} \pm 3$), without adding any commercial yeast. The fermentation process for Sercial, Verdelho, Bual and Malvasia was stopped by the addition of natural grape spirit (containing 95% (v/v) of ethanol) when the must specific gravity reached 1.000, 1.021, 1.026 and 1.046 g/cm^3 , respectively. After fortification the wines were submitted to a clarification process through albuminicol gelatins and bentonite clays. Then, each wine was divided in two 200 L fractions in order to represent the two ageing processes: *canteiro* and *estufagem*. Consequently, one fraction was placed directly into oak casks (*canteiro* process) while the other was firstly submitted to *estufagem* (45 °C during 120 days) and then transferred to the oak casks. These wines were monitored during 540 days (18 months) and three sample replicates of each variety were collected at different stages of both ageing processes.

The following stages were assessed during the vinification process: grape juice (M0), begin of must fermentation (MIF), before must fortification (MAF), after wine fortification (VAT) and after wine post-fermentation treatments (VT). The ageing processes (*canteiro* and *estufagem*) were also monitored in the following stages: V0, V30, V60, V90, V120, V180, V360 and V540. The stage V0 correspond to the initial wine before ageing, while the others stages indicate the days of ageing, i.e. 30, 60, 90, 120, 180, 360 and 540 days of ageing.

➤ Old Madeira wines aged by *canteiro*

It was also selected a set of 32 Madeira wine samples made from white grape varieties that were aged in casks for at least 5 years (*canteiro*), in order to study the acidity evolution with wine age. These samples are described in Table 2.

Table 2 – Selected old Madeira wines aged only by *canteiro*.

Wine	Sercial	Verdelho	Bual	Malvasia
Age	6	5	6	6
	12	9	9	9
	18	14	15	12
	22	16	18	19
	24	24	23	22
	37	26	25	23
	42	32	27	33
	45	35	36	
		38		
No. of samples	8	9	8	7

5.2 Basic oenological parameters

Some basic oenological parameters were evaluated for the Madeira wine samples. These parameters included: alcohol (v/v), specific gravity (g/cm³), total acidity (g/L express in terms of tartaric acid), pH, and reducing sugars (g/L). The determination of these parameters was carried out by a spectrophotometric analysis through the Bacchus 3 analyser (UV/Vis/IR spectroscopy combined with chemometrics) from Thermo Scientific (Hudson, NH, USA). This equipment gives

the result of an analytical parameter in a fast and reliable way, after being conveniently calibrated for the wine. The used calibration had the following correlation coefficients (R^2): 0.987 for alcohol; 0.999 for specific gravity; 0.982 for total acidity; 0.842 for pH and 0.998 for reducing sugars. Notice that, the low R^2 value obtained for pH is related to the fact that the working range is reduced (Madeira wine typical pH ranges around 3.5). The calibration is performed taking into account the reference values (values obtained from the laboratory analysis according to ISO standards) and the UV –visible and IR spectrum areas.

Madeira wine samples were simply filtered through 0.45 μm syringe filters Acrodisc GHP (Pall Gelman Sciences, Ann Arbor, MI, USA) before being placed at the equipment autosampler.

5.3 Sensorial analysis – olfactory perception threshold of volatile acidity

5.3.1 Chosen panels

The evaluation of the olfactory perception threshold of Madeira wine volatile acidity took into account two different panels: one trained and other untrained. The untrained panel was formed by 23 participants from the University of Madeira while the trained panel consisted of 12 wine tasters from Madeira Wine Company, S.A. Both panels evaluated two dry wines made from Sercial grapes and two sweet wines made from Malvasia grapes (5 and 10 years-old).

5.3.2 Basic training - untrained panel

In order to obtain more reliable results, we proceeded with a basic training for the untrained panel. Two types of tests were performed: paired comparison and ranking tests (intensity).

➤ Paired comparison tests

Test 1:

The first training test consisted in two sample groups (I and II). Both groups were formed by a set of 5 samples: water, water + ethanol, water + acetic acid, 5 years-old Malvasia and 5 years-old Sercial. The aim was to match in pairs the samples from group I with those from group II. Additionally, it was asked to describe the presented samples.

Test 2:

The second paired comparison test consisted once again in two sample groups (I and II), but this time the group I was formed by a set of 4 samples: water + ethanol, water + acetic acid, 5 years-old Malvasia and 5 years-old Sercial. In group II it was presented 6 samples with the same sensation described in group I but 2 of them showed a repeated sensation. The aim was to match the samples from group I with those from group II, and also described the samples.

Test 3:

This paired comparison test was similar to Test 2, however the solution water + acetic acid was replaced by the solution water + ethyl acetate.

➤ Ranking tests (intensity)

Test 4:

This test was formed by 4 groups. Each group had 4 samples at different concentrations of acetic acid (0, 1, 3 and 5 g/L of acetic acid acetic) dissolved in the following: water, water + ethanol, 5 years-old Malvasia or 5 years-old Sercial. In a first stage it was asked to identify each group. The acetic acid solutions were randomly placed and it was asked to rank in ascending order of intensity (where 1 was the control and 4 was the most concentrated solution).

Test 5:

This ranking test was similar to Test 4, but in this case, ethyl acetate was tested in the following concentrations: 0, 100, 200 and 400 mg/L.

5.3.3 Preliminary tests

To determine the appropriate concentration range for the olfactory perception threshold, acetic acid (99.7%) from Panreac Química S.A. (Barcelona, Spain) and ethyl acetate from Fisher Scientific (Loughborough, UK) standards were added to a 5 years-old Malvasia and 5 years-old Sercial. The concentration was gradually increased until the odour of the evaluated standard was detected by at least 4 staff members of the lab. The concentration obtained was the upper-end of the concentration range. Therefore, five wine solutions at different concentrations of acetic acid (1, 2, 3, 5 and 8 g/L) were prepared dissolving the acid into the chosen Madeira wines, in order to determine

the olfactory threshold. The same was done for the ethyl acetate evaluation, but decreasing the concentration to 50, 100, 200, 300 and 400 mg/L.

After choosing the concentration range it was necessary to select the most appropriated test. For that, to determine the olfactory perception threshold of acetic acid and ethyl acetate in Madeira wines, two types of olfactory tests were evaluated: paired comparison and triangle test. Both panels participated in this preliminary test and only acetic acid was evaluated. The solutions were prepared in 250 mL volumetric flasks using 5 years-old Malvasia, approximately 2 hours before the sensory analysis being performed. The tests were performed according to Fig. 11.

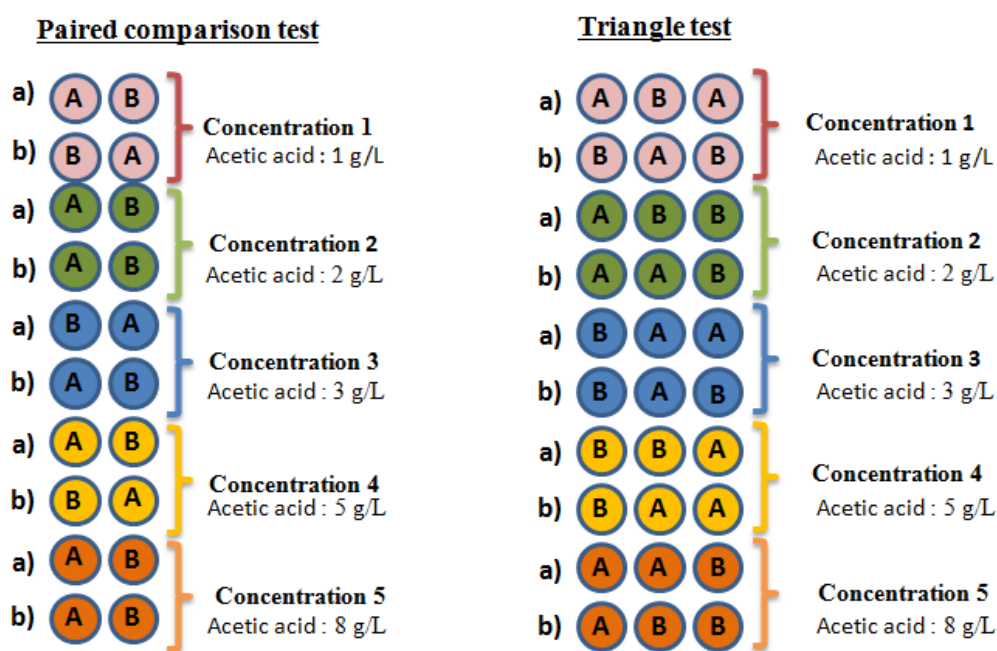


Fig. 11 – Scheme of assayed paired comparison and triangle tests (wine with the standard solution (A) and without (B)).

Both tests consisted of five groups with wine solutions at different concentrations of acetic acid. Groups were arranged in increasing order of concentration. 30 mL of wine solution were placed in glasses and then covered with small petri-dishes. The assays were conducted at room temperature between 10:00 to 12:00 am.

It was firstly provided a standard sample of acetic acid in order to the panel subjects familiarize with its odour. In the paired comparison test, subjects were asked to select between two samples which were pairing, wherein one had the odorant involved. On the other hand, in the

triangle test, subjects must choose between the three samples and pointed out which was the different one, that could be the odorant or the base wine. Samples were assessed only by sniffing.

Furthermore, paired comparison and triangle tests were evaluated in a stress free environment by the trained panel. Subjects carried out the tests individually in a predefined hour, calmly and without any distractions (sounds and smells).

5.3.4 Olfactory perception threshold assay

After choosing the adequate test, the olfactory perception threshold of acetic acid and ethyl acetate was evaluated in four Madeira wines (5 and 10 years-old Malvasia and Sercial). The concentrations of the acetic acid wine solutions used in the tests performed by the untrained panel were 1, 2, 3, 5 and 8 g/L. However, in the case of the trained panel it was necessary to decrease its concentration for: 0.3, 0.6, 1, 2 and 2.5 g/L. The concentration of ethyl acetate wine solutions was 50, 100, 200, 300 and 400 mg/L. The sensorial test is presented in the appendix.

The intrinsic levels of acetic acid and ethyl acetate in each wine were quantified, and were accounted for the total concentration. The accounted data took into consideration the percentage of correct responses for each concentration. The linear regressions were calculated and R^2 were determined using the Excel from Microsoft Office. The olfactory perception threshold limits were established when the percentage of correct response was 50% for triangle test and 75% for paired comparison test.

5.4 Determination of organic acids by IEC-HPLC-DAD

5.4.1 Chemicals and standards

Organic acids standards were obtained from different suppliers: *L*-malic (99.5%), *L*-tartaric (99.5%) and succinic (99.5%) from Merck (Darmstadt, Germany); acetic (99.7%) and lactic (85%) from Panreac Química S.A. (Barcelona, Spain); formic (99.7%) from Fisher Scientific (Loughborough, UK) and citric (99.5%) from Fluka BioChemika AG. Type 1 ultra-pure water was obtained from Simplicity UV apparatus from Millipore (Milford, MA, USA) and was used for the preparation of standards and mobile phases. Sulfuric acid (95-97%) was supplied by Riedel-de-Haën. The eluents were previously filtered with a membrane filters supplied from Pall (0.20 mm, Ann Arbor, MI, USA).

5.4.2 Apparatus and conditions

The analysis of organic acids (including acetic acid) was performed using the Waters Alliance liquid chromatographer (Milford, MA, USA) equipped with the Waters 2695 separations module and the Waters 2996 photodiode array detector. The configuration and processing data was driven by Empower Pro software from Waters Corporation. This assay was performed based on a routine methodology implemented in the laboratory where the work was performed. This procedure quantifies the organic acids by direct injection of wine samples, after being previously filtered through 0.45 µm syringe filters from Acrodisc GHP (Pall Gelman Sciences, Ann Arbor, MI, USA). The calibration curves range between 50-5000 mg/L for lactic acid; 100-5000 mg/L for acetic, malic and tartaric acids; 20-1000 mg/L for citric and succinic acids; 100-1000 mg/L for formic and.

Briefly, the column used for the separation of organic acids was a Hi-Plex H (300 × 7.7 mm; 8µm; Agilent Technologies, U.S.A). The chromatographic separation of the compounds was carried out using an isocratic elution with the following mobile phase: 0.0025M of sulphuric acid. The flow rate was set to 0.6 mL/min, the column thermostated at 65 °C and the injection volume was set to 10 µL. All wine replicates were injected in duplicate.

Organic acids were detected at 210 nm and the individual compounds were identified based on the peaks UV-Vis spectra, elution order and retention time, as well as by spiking the samples with the standards compounds. The quantification was performed using the standard calibration method. The coefficient of variation (% CV) between wine replicates was in average 2% (citric, tartaric and malic), 5% (succinic), 6% (lactic), 7% (formic) and 4% (acetic acid).

5.5 Determination of ethyl acetate by GC-MS

5.5.1 Chemicals

Ethyl acetate (99.98%) standard was from Fisher Scientific (Loughborough, UK). Ethyl acetate stock solution of 10 g/L was prepared in synthetic wine and then stored at 4 °C. The working solutions (used for internal calibration) were prepared by diluting the stock solution in synthetic wine. The ethyl acetate calibration range was set between 50 and 500 mg/L.

Synthetic wine was composed by 6 g/L of tartaric acid (99.5%, Merck, Darmstadt, Germany), 18% ethanol (99.5%, Sigma-Aldrich, St. Louis, MO, USA) and pH adjusted to 3.50 with

NaOH 1M (98%, Panreac Química S.A., Barcelona, Spain). The internal standard (IS) was prepared by the addition of 0.010 g of 3-octanol standard (97%, Acros Organics, Loughborough, UK) to 20 mL of synthetic wine. The solution was vortexed and then preserved at 4 °C until being used. For SPME analysis, NaCl (99.5%) from Panreac Química S.A. (Barcelona, Spain) was used.

5.5.2 Preparation of samples

10 µL of internal standard (IS, 3-octanol 500 mg/L) were added to 20 mL of each sample, before diluting 5 mL of this solution into 5 mL of ultra-pure water, in a 20 mL headspace vial containing 3 g of NaCl, to promote salting-out. Then the vial was immediately capped and vortexed prior to automated HS-SPME/GC-MS analysis. Three replicates of each sample were analysed.

5.5.3 HS-SPME/GC-MS analysis

SPME was the selected technique for the ethyl acetate extraction. It was developed by Pawliszyn and co-workers and has many advantages, such as the high sensitivity and reproducibility, ease to use, low cost, does not require solvents or previous sample preparation and can be easily automated (19). Furthermore, it has been successfully applied to wine and beverage characterization (19, 105, 106). The headspace SPME (HS-SPME) mode consists in firstly expose the fiber indirectly to the sample through the vial headspace (19). This indirect mode provides longer lifetimes of the fiber, shorter equilibration times and allows the extraction of minor compounds. In the last few years, this extraction mode is the most common and has been considered the easiest method for food quality control, being widely applied for the study of compounds present in wine flavour (105). Then, the second step consists in the desorption of the trapped compounds into the injector port of the GC (19, 105).

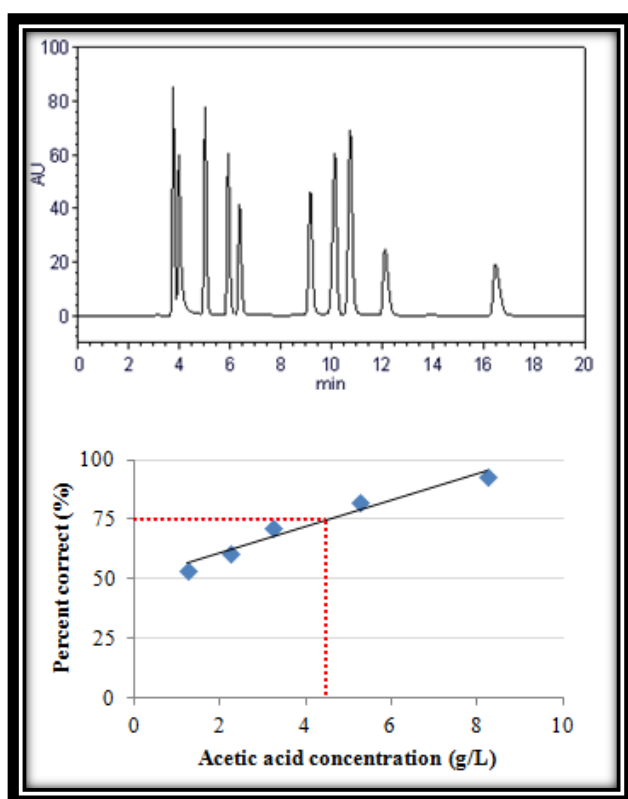
After preparing the samples, the extraction was performed by exposing the SPME fiber 50 µm/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxan (DVB/CAR/PDMS, bipolar adsorbent) into the vial for 30 min at 60 °C, keeping the sample under continuous stirring.

The compounds were desorbed for 5 min at 240 °C inserting the fiber into the GC injection port of the TRACE GC Ultra, equipped with the TriPlus autosampler (SPME mode) and the mass spectrometer detector ISQ single quadrupole (electronic impact ionization mode) from Thermo Scientific (Hudson, NH, USA). The column was a DB-WAXetr, 60 m × 0.250 mm and 0.50 µm of film thickness from Agilent J&W (Folsom, CA, USA). The carrier gas was He at 1 mL/min. The

transfer line and ion source temperatures were both kept at 230 °C. The oven temperature program started at 40 °C for 2 min then increased up to 230 °C at 4 °C/min and finally kept at 230 °C for 15 min. The standard and samples were performed in total ion count (TIC), recording the mass range 30-400 m/z. However, for quantifying it was used ion m/z 61 for ethyl acetate and the ion m/z 101 for the IS. The calibrations curves were obtained by ethyl acetate relative area (ethyl acetate area ÷ IS area), against the corresponding ethyl acetate concentration. The linearity (R^2) was determined based on the linear regression results. And the quantification of the ethyl acetate in wine samples was performed according to the obtained calibration curve. The % CV between wine replicates was in average 4%.

PART III

RESULTS / DISCUSSION



6. RESULTS / DISCUSSION

6.1 Analytical methodologies

The validation parameters of organic acids methodology by IEC-HPLC-DAD are described in Table 3.

Table 3 – Validation parameters of the used routine organic acids methodology.

Organic acid	R ²	LOQ (mg/L)
Citric acid	0.999	10.27 ±2.05
Tartaric acid	0.999	96.96 ±2.86
Malic acid	0.999	60.77 ±2.30
Succinic acid	0.999	13.90 ±2.88
Lactic acid	0.999	44.18 ±3.60
Formic acid	0.998	33.42 ±3.61
Acetic acid	0.999	79.84 ±1.57

The resulting calibration for ethyl acetate by GC-MS showed good linearity with a R² of 0.999 (LOD = 8.40 mg/L; LOQ = 25.44 mg/L).

6.2 Sensorial analysis

For the determination of the olfactory perception threshold of acetic acid and ethyl acetate in Madeira wines intrinsic levels, in each wine, were quantified in order to be accounted for the total concentration (Table 4).

Table 4 – Intrinsic amount of acetic acid and ethyl acetate in Madeira wine samples.

Madeira Wine	Acetic acid (g/L)	Ethyl acetate (mg/L)
5 years-old Malvasia	0.23 ±0.06	67.55 ±3.58
5 years-old Sercial	0.17 ±0.04	112.46 ±5.76
10 years-old Malvasia	0.29 ±0.07	64.34 ±1.39
10 years-old Sercial	0.28 ±0.03	114.74 ±5.93

For the olfactory perception threshold assessment of volatile acidity in Madeira wines, first it was necessary to choose the type of test: paired comparison or triangle test. 5 years-old Malvasia was the sample used in these tests and only the acetic acid threshold was evaluated. The results are described below (Fig. 12).

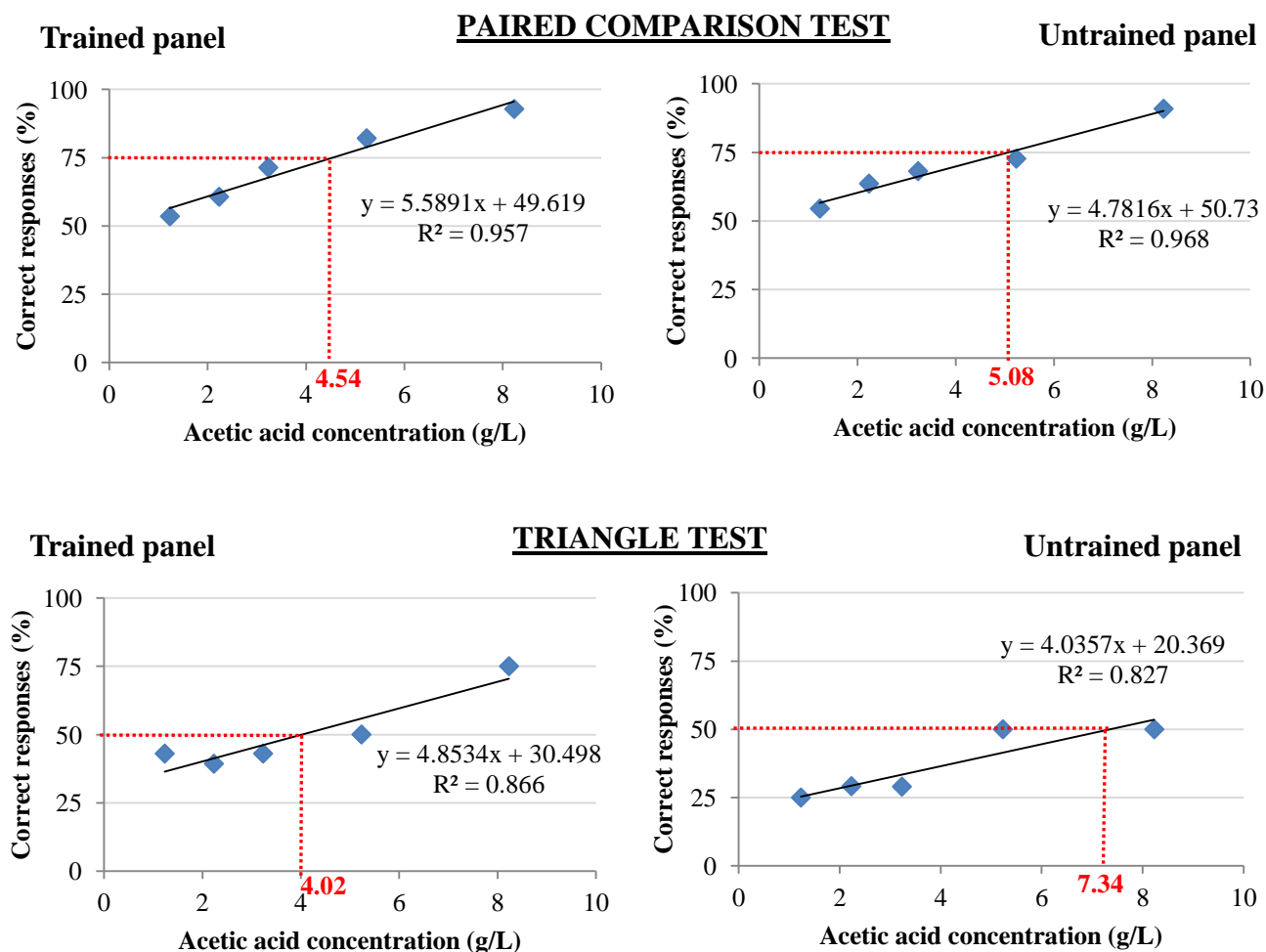


Fig. 12 – Paired comparison test vs. triangle test.

According to Fig. 12, the olfactory perception threshold of acetic acid for 5 years-old Malvasia obtained from paired comparison test (correct response >75%) was 4.54 g/L for the trained panel and 5.08 g/L for the untrained panel. In the triangle test, the threshold levels of acetic acid (>50% correct response) was 4.02 g/L and 7.34 g/L for trained and untrained panel, respectively. In both tests and panels, the threshold levels were higher than the legal limit established for the volatile acidity of 5 years-old Madeira wines (1.2 g/L) (76).

Furthermore, the paired comparison test showed better R^2 (0.957 for the trained panel and 0.968 for the untrained panel) than the triangle test (0.866 for the trained panel and 0.827 for the untrained panel). This can be associated with the higher number of samples used in the triangle test

which promotes greater fatigue and loss of sensitivity, especially in complex samples (88), as is the case of Madeira wines.

The same tests were also performed, in a stress free environment, by the trained panel in order to verify if there were significant differences between both sensorial tests (Fig. 13).

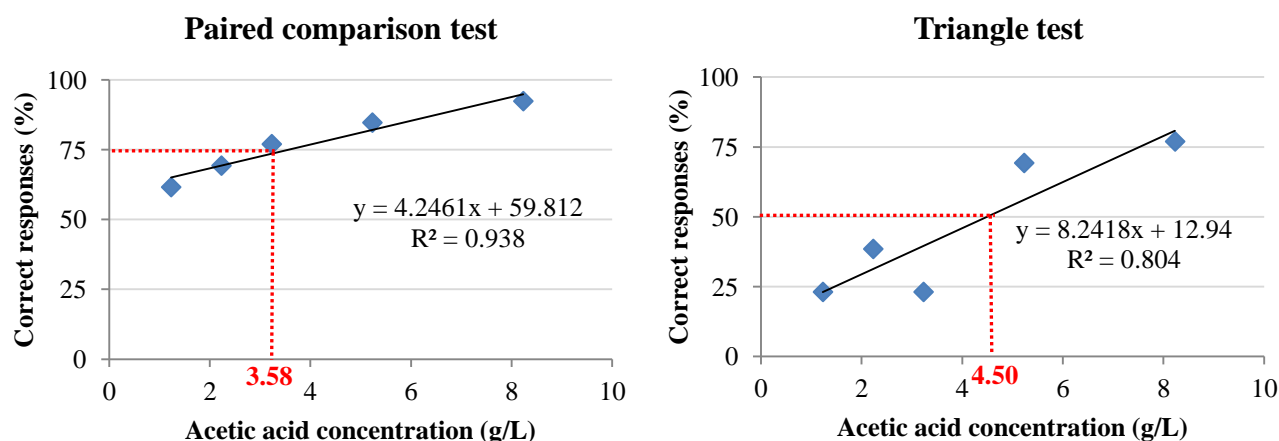


Fig. 13 – Paired comparison test vs. triangle test in a stress free environment (trained panel).

The results were similar to those described before in Fig. 12, suggesting that a stress free environment did not have a significant impact in the results. In other words, the paired comparison test revealed a better R^2 (0.938) than the triangle test ($R^2 = 0.804$) since the triangle test is more difficult to perform due to the panel fatigue originated by the complexity of Madeira wines samples (88), as mentioned above. For this reason, we chose to use the paired comparison test for the determination of acetic acid and ethyl acetate olfactory perception threshold in Madeira wines samples.

As also described in Fig. 13 the acetic acid threshold levels for this expert panel in the paired comparison test was 3.58 g/L, decreasing when compared to the initial sensorial test (4.54 g/L) described in Fig. 12.

After these preliminary tests, for the determination of acetic acid and ethyl acetate threshold levels four Madeira wines were used, namely Sercial and Malvasia, with 5 and 10 years-old. Paired comparison tests were performed and the criterion selected for the linearity range comprised a range of correct responses between 50 and 90%.

The acetic acid olfactory perception threshold for the untrained panel is described bellow (Fig. 14).

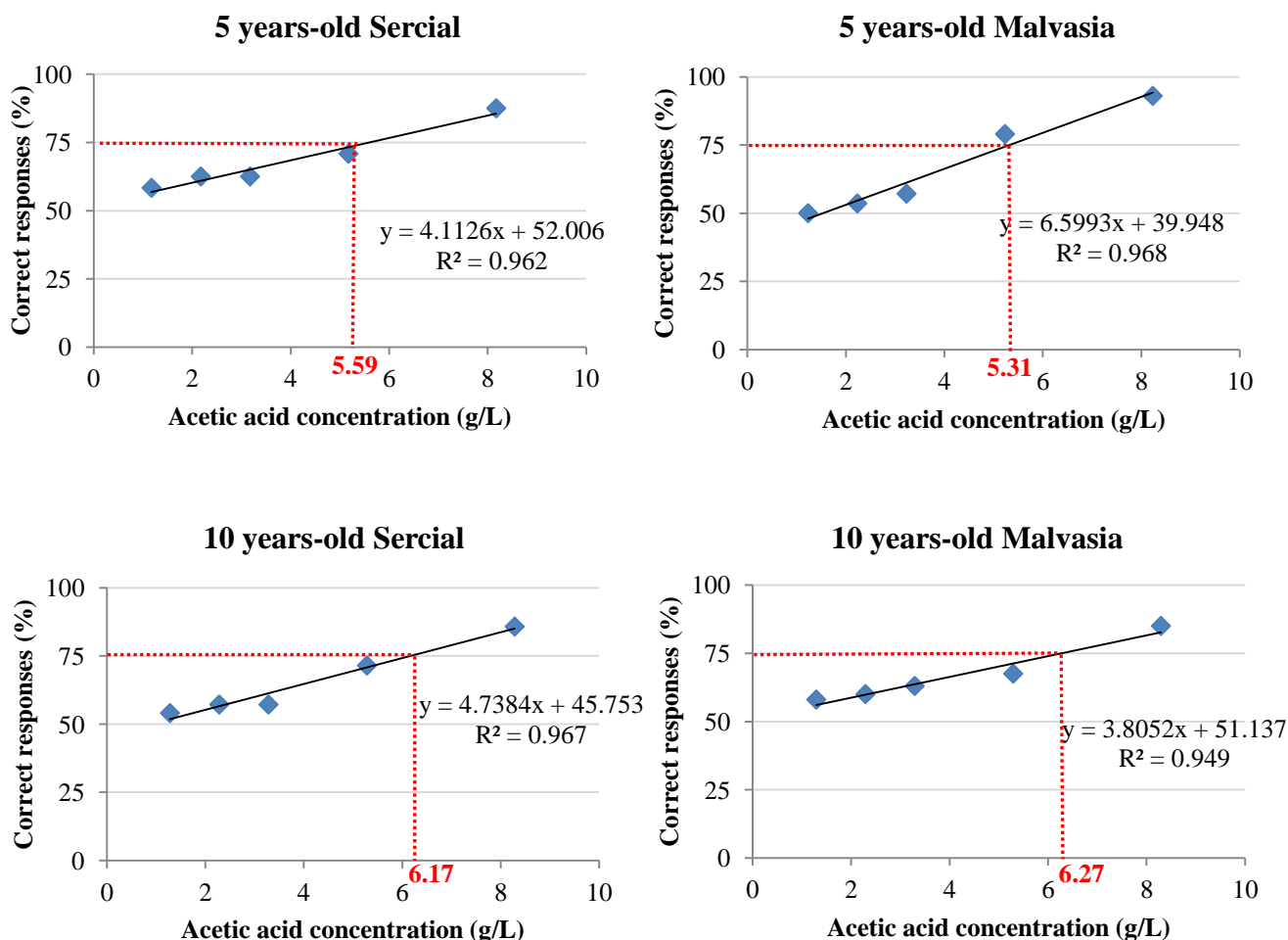


Fig. 14 – Olfactory perception threshold for acetic acid (untrained panel).

The untrained panel results revealed that acetic acid threshold levels for Madeira wine samples were much higher than the legal limits established for the volatile acidity of 5 and 10 years-old Madeira wines (1.2 g/L) (76): 5.59 g/L (5 years-old Sercial), 5.31 g/L (5 years-old Malvasia), 6.17 g/L (10 years-old Sercial) and 6.27 g/L (10 years-old Malvasia). These threshold levels were also higher than those found by Cliff (63) for ice wine (3.19 g/L), probably due to the complexity of Madeira wines acquired during the maturation process. Interestingly, both Malvasia and Sercial had similar acetic acid perception thresholds for the same age. Once residual sugar is known to mask the perception of the volatile acidity (63), it was expected that Malvasia showed higher threshold levels. However, the acidity of Sercial probably also mask the presence of acetic acid. Thus, the perception

threshold of acetic acid seems to mainly dependent from the Madeira wine age and not from the wine style.

For the trained panel there was a notorious decrease in the acetic acid perception threshold along the sensorial tests due to the taster's experience. For that reason, we trained the panel (with training in sensorial analysis) in order to improve their ability to detect the acetic acid in Madeira wines, so that we could determine its maximum olfactory threshold, as described in Fig. 15.

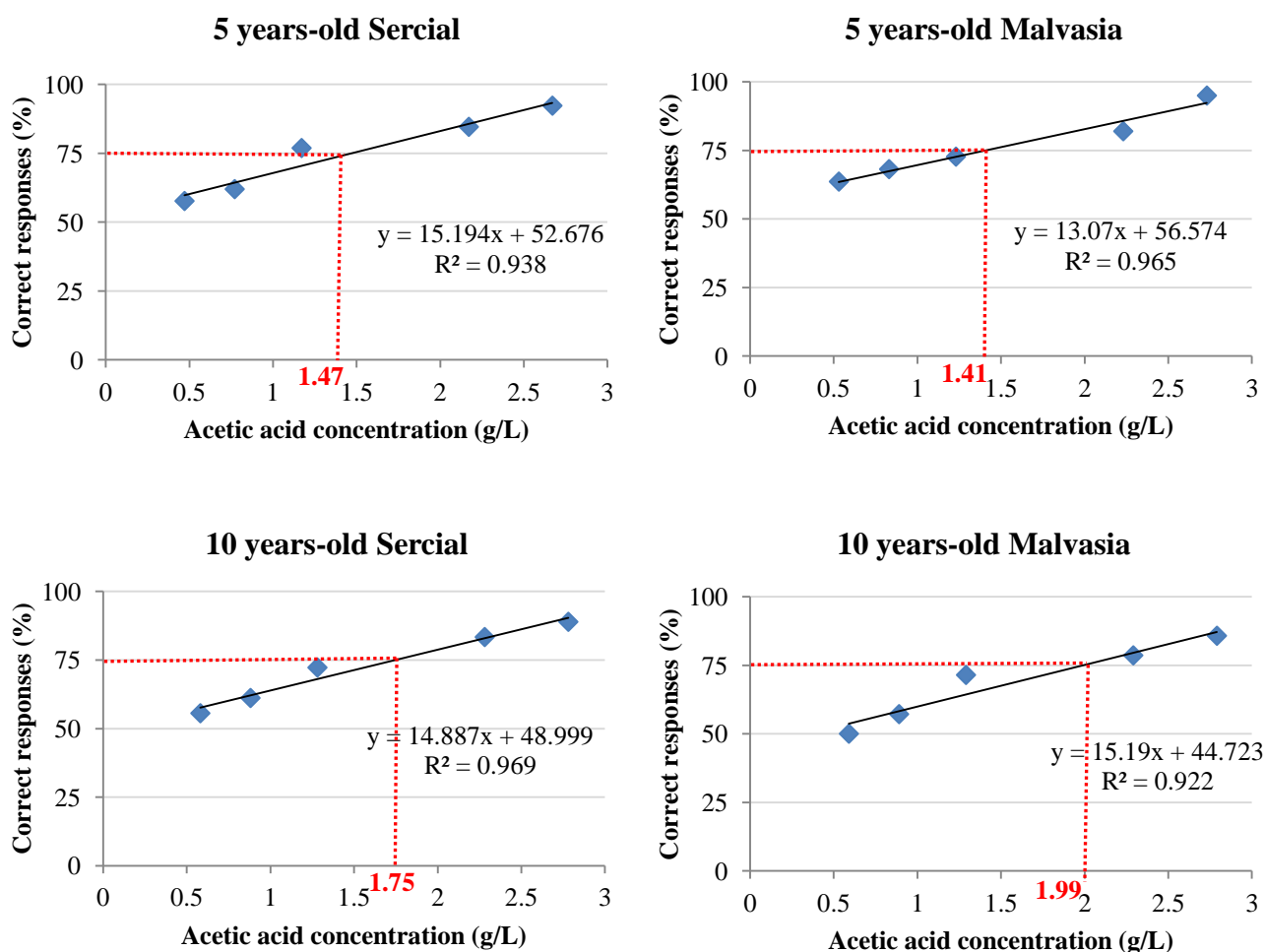


Fig. 15 - Olfactory perception threshold for acetic acid (after training the trained panel).

As expected, Fig. 15 showed that the acetic acid perception threshold obtained from the trained panel was much lower than those found for the untrained panel, namely 1.47 g/L (5 years-old Sercial), 1.41 g/L (5 years-old Malvasia), 1.75 g/L (10 years-old Sercial) and 1.99 g/L (10 years-old Malvasia).

years-old Malvasia). And also, lower than in the initial paired comparison test for 5 years-old Malvasia (4.54 g/L). So, training (repeat exposure) improved the ability to recognize the acetic acid (60). Nevertheless, the perception threshold for the trained panel was still higher than the legal limits established for the volatile acidity of Madeira wines (5 and 10 years-old) (76). Furthermore, and comparatively with the described in the Fig. 14 for the untrained panel, the acetic acid olfactory perception threshold seems to depend essentially on the wine's age.

The results of ethyl acetate olfactory perception threshold for the untrained panel are presented below (Fig. 16).

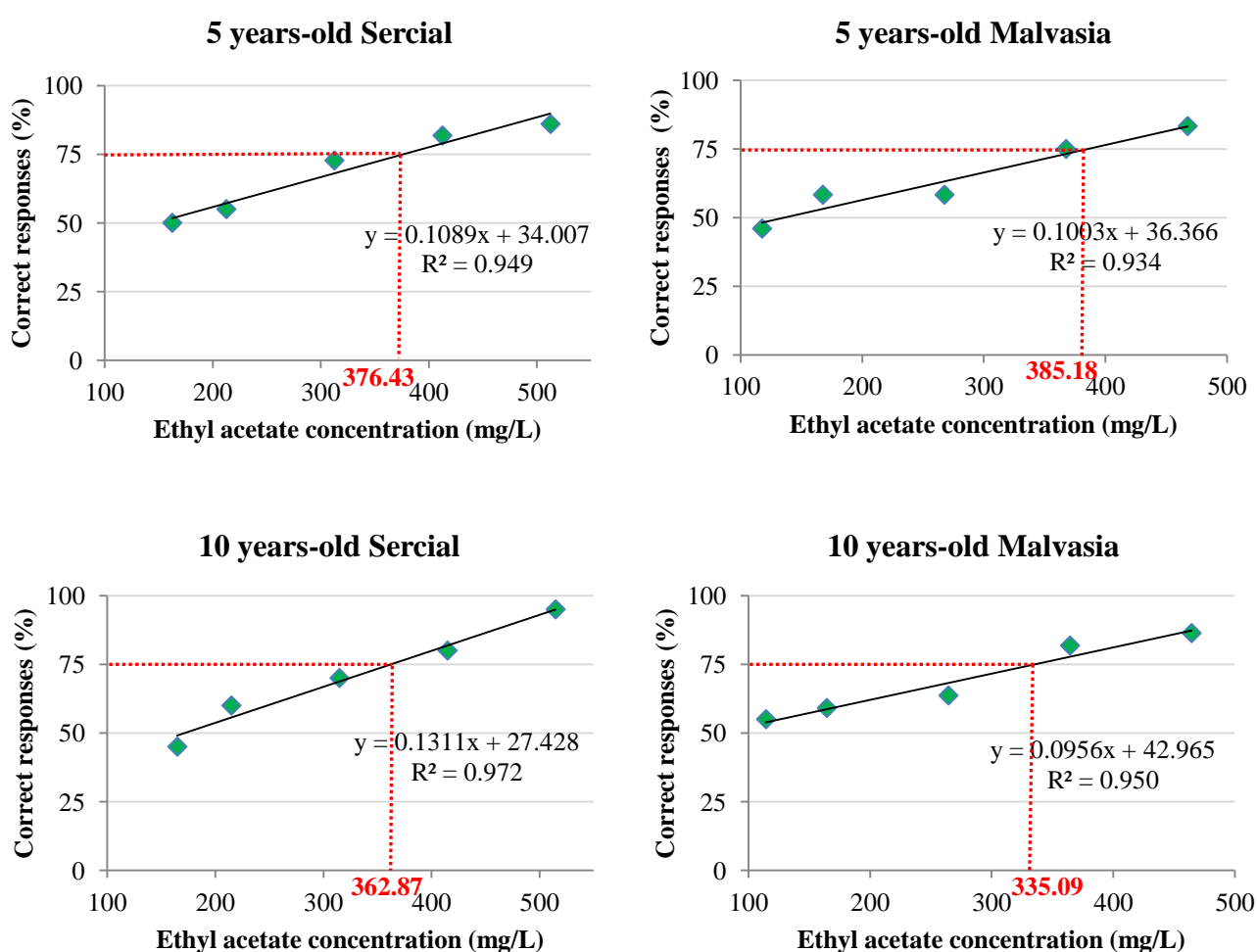


Fig. 16 - Olfactory perception threshold for ethyl acetate (untrained panel).

Ethyl acetate perception threshold for the untrained panel revealed similar results for all wines ages and styles, namely: 376.43 mg/L (5 years-old Sercial), 385.18 mg/L (5 years-old Malvasia), 362.87 mg/L (10 years-old Sercial) and 335.09 mg/L (10 years-old Malvasia). So, the

olfactory detection of ethyl acetate does not seem to be influenced by the Madeira wine style and age. And even though there is no limit legally established, these values were higher than those found for ethyl acetate sensory threshold in table wines (around 150 mg/L) (12, 60) and even for ice wines (198 mg/L) (63). This might be associated with the complexity of Madeira wines that can mask the ethyl acetate perception, since its threshold levels varies with the type of wine and intensity of the flavour (60, 67).

The ethyl acetate sensorial threshold tests obtained from the trained panel are described as followed (Fig. 17).

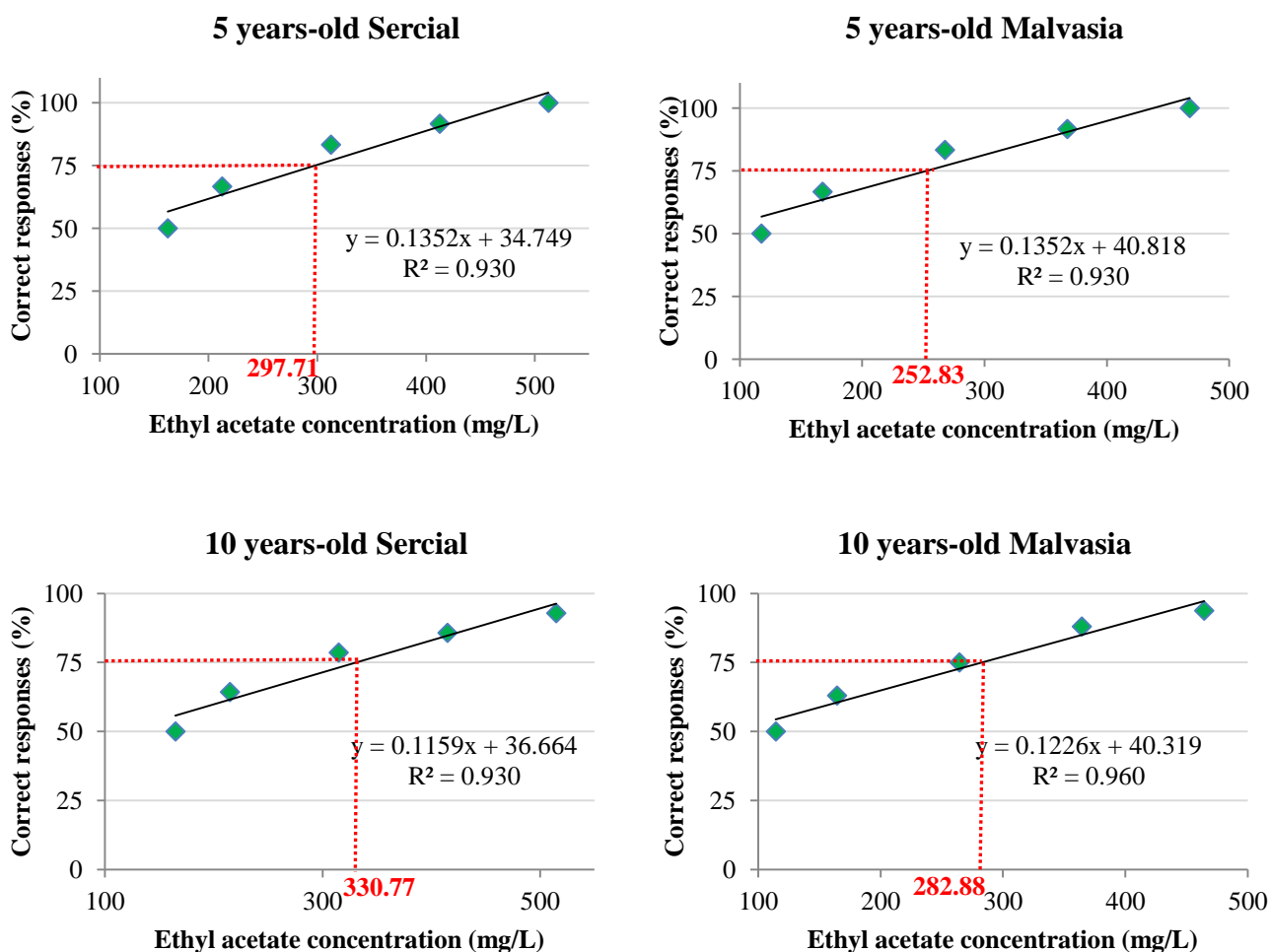


Fig. 17 - Olfactory perception threshold for ethyl acetate (trained panel).

According to Fig. 17, the olfactory perception threshold of ethyl acetate obtained by the trained panel was lower than those described by the untrained panel, namely: 297.71 mg/L (5 years-old Sercial), 252.83 mg/L (5 years-old Malvasia), 330.77 mg/L (10 years-old Sercial) and 282.88 mg/L (10 years-old Malvasia). Still, these results continue upper than those found in table wines (12, 60) and also in ice wine (63). Interestingly, in this case the sensorial threshold depends on the style (higher in Sercial) and wine age (higher in older wines) (60, 67). Although Malvasia has a more complex flavour, the characteristic acidity of Sercial can probably mask the ethyl acetate perception. Additionally, the increased complexity of Madeira wines acquired during the oak ageing seems to promote a more difficult olfactory detection of ethyl acetate, since its odour threshold presented higher values in 10 years-old Madeira wine samples.

6.3 Acetic acid evolution during vinification and ageing processes

Some studies were performed in order to evaluate the evolution of acetic acid during the vinification process of Madeira wine made from white varieties (Fig. 18).

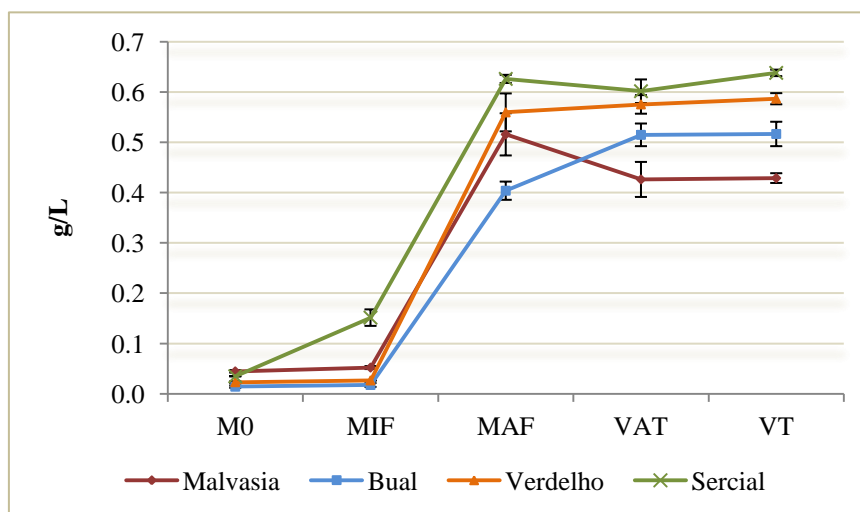
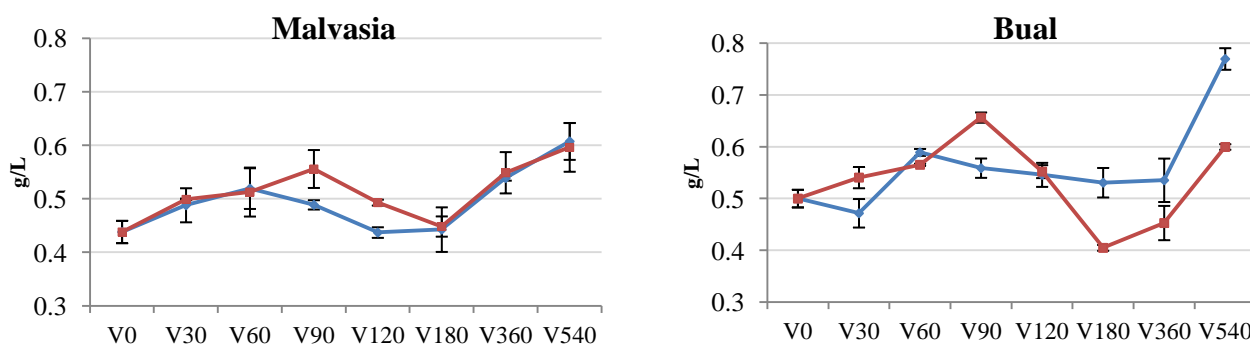


Fig. 18 – Acetic acid evolution during the vinification process.

In the initial stage (M0), the concentration of acetic acid is low in all wine samples. However, during maceration process (M0 to MIF) Sercial showed a slight increase on the acetic acid levels (0.15 g/L) probably due to the presence of greater amounts of acetic acid bacteria compared to the other varieties. These bacteria are commonly associated with grapes and must, and depending essentially on the presence of oxygen, can produce acetic acid (70). Clearly, and as expected, alcoholic fermentation (MIF to MAF) promoted a significant increase in the acetic acid

formation. Sercial revealed the higher amount (0.63 g/L) followed by Verdelho (0.56 g/L) and Malvasia (0.52 g/L). This fact is evidently associated with the fermentation time that is more extensive in Sercial, promoting a longer time for the action of the yeasts responsible for the formation of this acid. Still, even though Bual is a medium sweet wine, the lower concentration (0.40 g/L) might be associated with the initial sugar level in must (41, 54). The acetic acid formation in this stage was higher than that found in the literature for table wines (0.2 to 0.4 g/L) (62, 107). This can probably be related to the type of yeast involved in the Madeira wines fermentation, however, little is known about this. Additionally, wine fortification (MAF-VAT) did not seem to influence the levels of acetic acid in Sercial and Verdelho. On the other hand, the addition of ethanol promoted a slight increase in the acid levels of Bual likely due to its low pH that might favour the growth of some strains of acetic acid bacteria that consequently promote the acetic acid formation (62, 70). Still, in MAF stage a decreased was observed in Malvasia, which might be related with the acetic acid participation in other reactions such as the ethyl acetate formation (67). Finally, the treatments (VAT) performed in all wine styles did not affect the acetic acid levels.

Furthermore, the acetic acid evolution of all Madeira wine made from white varieties was evaluated during both ageing processes as described in the Fig. 19.



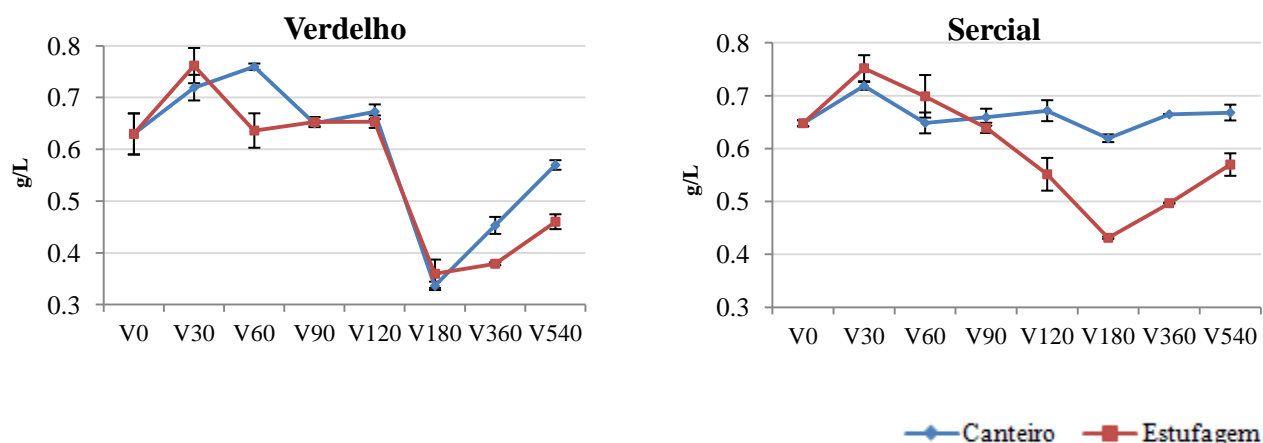


Fig. 19 – Acetic acid evolution: *estufagem* vs. *canteiro*.

The results indicated that the concentration of acetic acid either in *canteiro* or in *estufagem* was similar. So, and also as reported previously by Pereira V. (14), *estufagem* does not seem to increase acetic acid amounts when the wine is submitted at standard conditions (45°C during 4 months) conditions. All wine styles did not seem to have a clear tendency during ageing. In general, in the initial stage was observed a slight increase in acetic acid concentration followed by a significant decrease at 180 days of ageing, probably associated with the participation of acetic acid in other reactions, namely in the formation of ethyl acetate (70). Additionally, taking into account the initial wine and the wine after 540 days of ageing, was notorious the increase of the acetic acid concentration in Malvasia and Bual samples possibly due to the sugar content. Furthermore, after 540 days of ageing, *canteiro* generally showed higher concentration of this organic acid than *estufagem* in all Madeira wine styles (excepted Malvasia).

Madeira wine samples from oak casks at different ages were also evaluated in order to determine the evolution of acetic acid during oak ageing, as described in the Fig. 20.

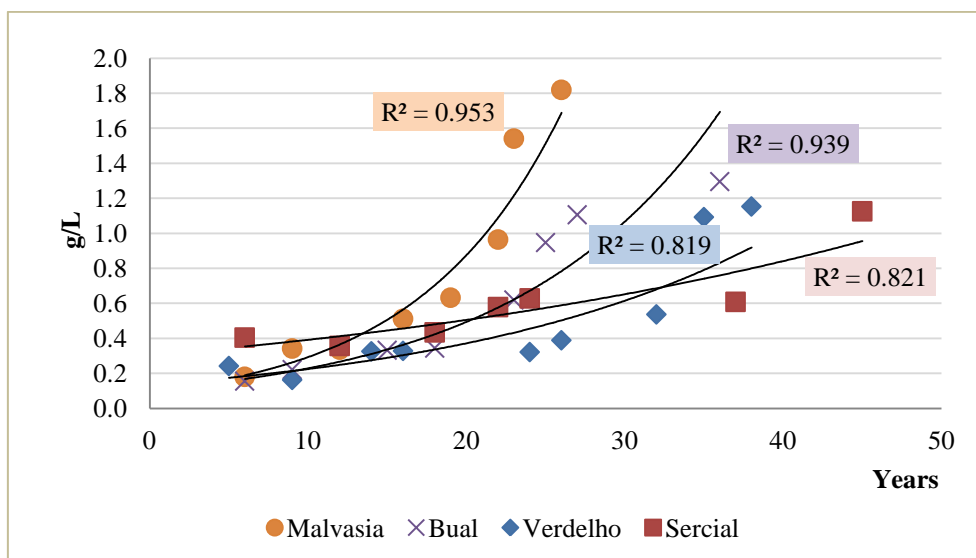


Fig. 20 – Acetic acid evolution with long oak ageing.

The results revealed an exponential increase of acetic levels with age for all Madeira wine made from white varieties. Interestingly, sweet wines (Malvasia, Bual) tended to have a more accentuated increase than the dry wines (Verdelho, Sercial). So, the rise verified in acetic acid amount during ageing in oak casks might be related not only with the chemical hydrolysis of hemicelluloses and the production of aldehydes from ethanol followed by ethanol oxidation (as mentioned above) (45, 75), but also seems to depend severely on the sugar content. According to Fig. 20 and also studies (8, 14, 32) the results indicates show that acetic levels tend to increase during ageing in oak casks reaching 1.80 g/L for 25 years-old Malvasia. However, the olfactory perception threshold for the trained panel (10 years-old Malvasia) is still above this value, namely 1.99 g/L as described in Fig. 15. So, even though there is an exponentially increasing of acetic acid during oak ageing, the Madeira wine quality might not be affected due to its complexity.

6.4 Ethyl acetate evolution during ageing

Ethyl acetate has also an important role on the wine volatile acidity, affecting its organoleptic properties. For that reason, it was essential to study its evolution during ageing. So, ethyl acetate evolution of all Madeira made from white varieties was evaluated for young Madeira wines (from 90 days up to 540 days of ageing), comparing both ageing processes (Fig. 21).

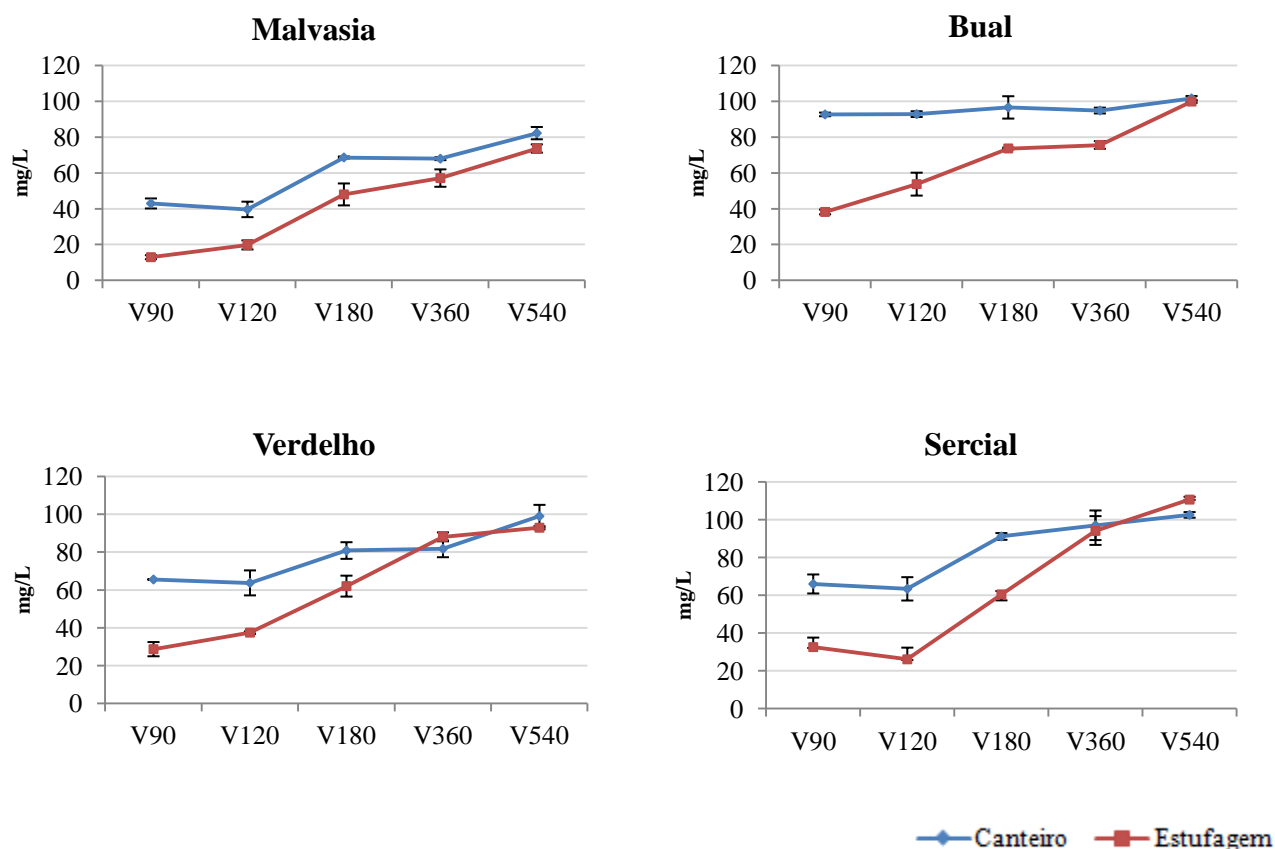


Fig. 21 – Ethyl acetate evolution: *estufagem* vs. *canteiro*.

As shown in the Fig. 21, the results revealed an increase in the ethyl acetate amount during the ageing of all wines in both processes, reaching maximum values of 110.60 mg/L for Sercial. *Canteiro* revealed greater concentration than *estufagem* on the initial stages for all wines, tending to approach over time. Notice that, in the stage of 180 days of ageing, a great increase occurred in the ester concentration. These results come to prove the acetic acid participation during ageing by non-enzymatic reaction between acetic acid and ethanol content, as mentioned before in Fig. 19 (12, 70).

Ethyl acetate evolution of Madeira wine made from white varieties with long ageing in oak casks was also evaluated as described in the Fig. 22.

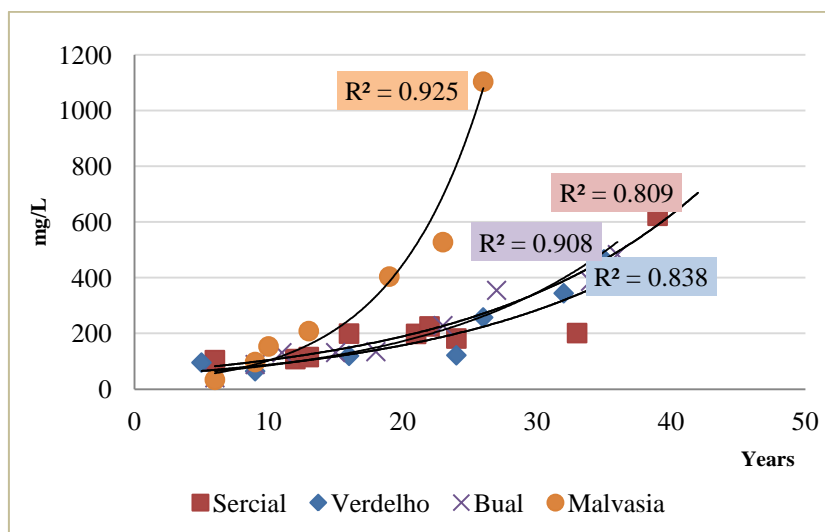


Fig. 22 – Ethyl acetate evolution with long oak ageing.

The results showed that ethyl acetate levels tended to raise with ageing in oak cask for all Madeira wine varieties, similarly to previous studies performed by Câmara (8). Clearly, and similar to what occurred during the evolution of acetic acid in oak casks (Fig. 20), the results revealed also an exponential increase of ethyl acetate levels with age, being more accentuated in sweet wines (Malvasia, Bual) than in dry wines (Verdelho, Sercial).

Notice that these results highlighted a linear relationship between acetic acid and ethyl acetate (Fig. 23). In other words, the increase verified in ethyl acetate amounts seems to depend directly on the acetic acid concentration, since this ester formation during ageing might be associated with the esterification of acetic acid in the presence of ethanol (12, 70).

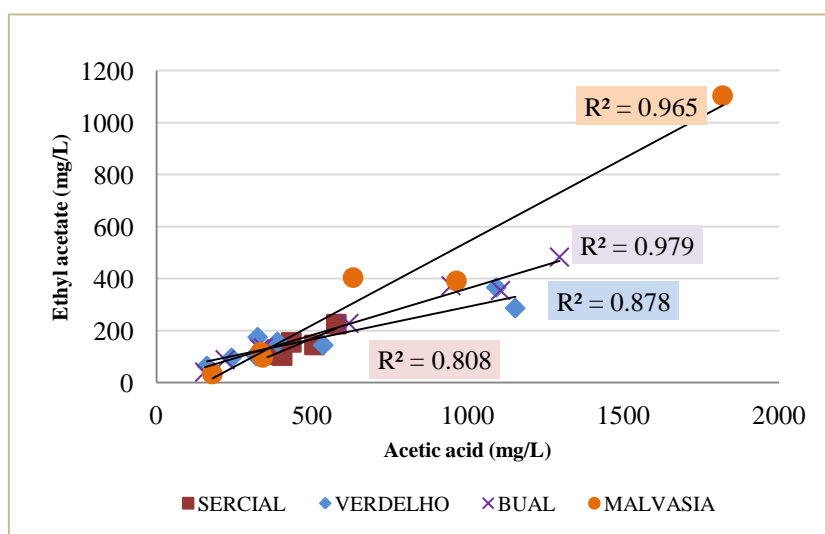
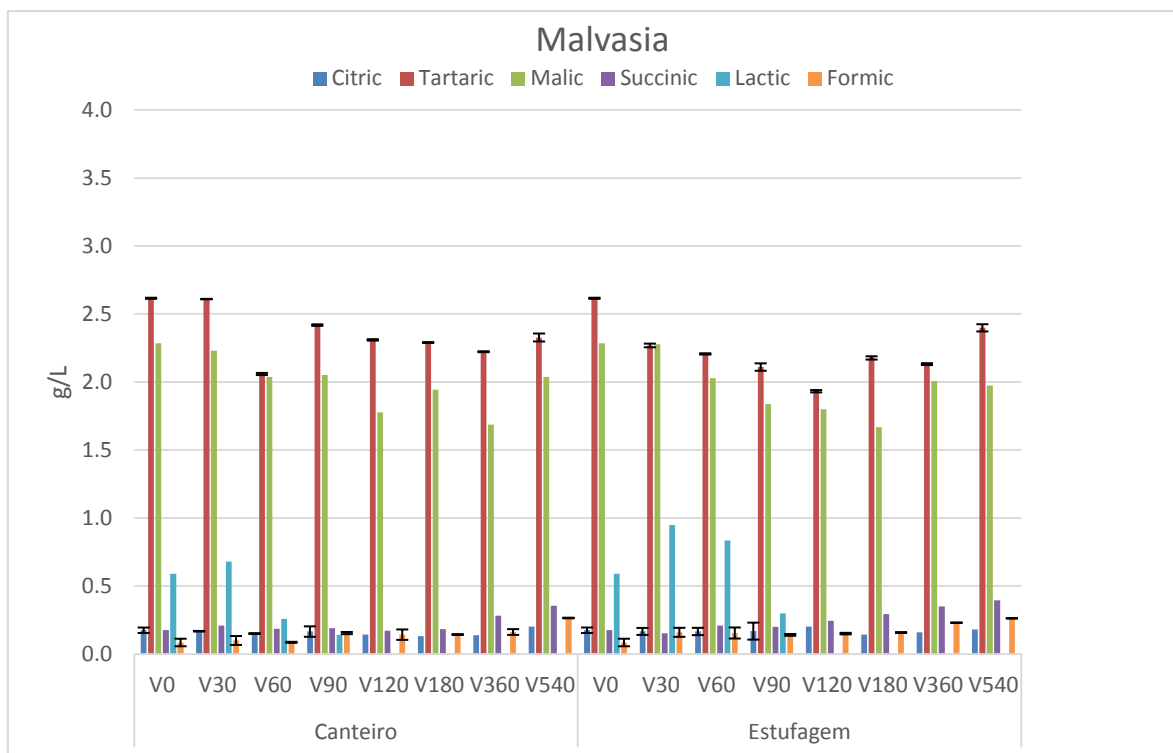


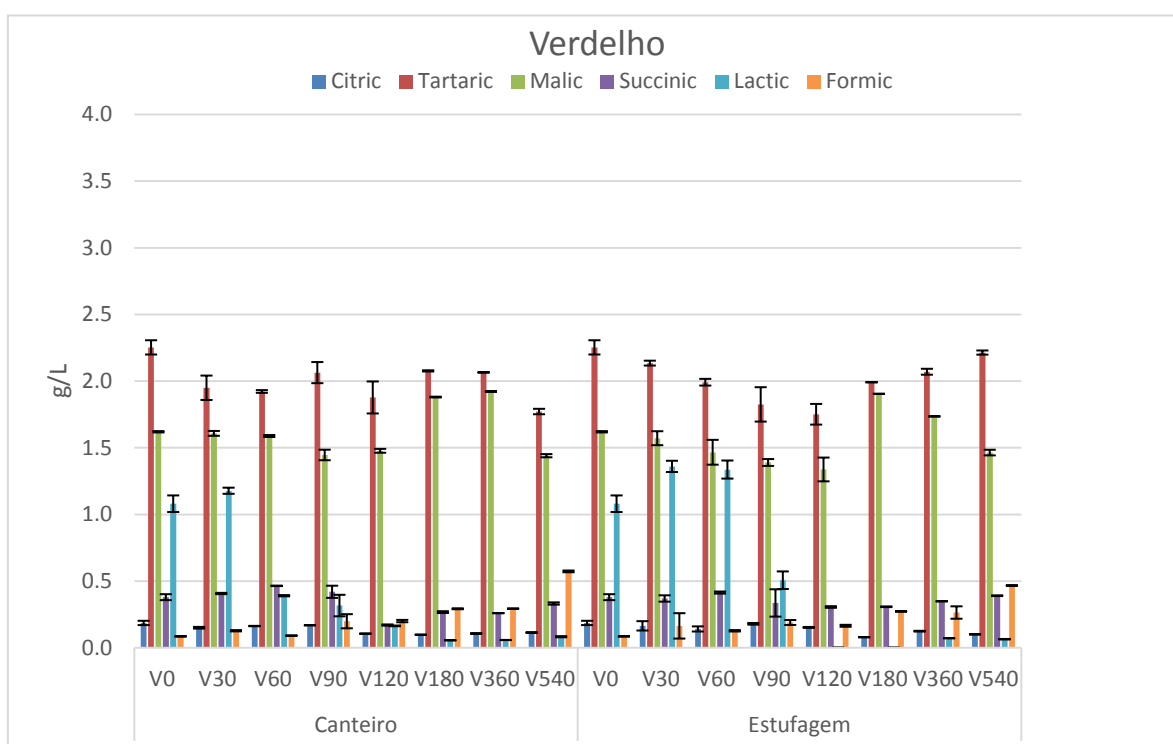
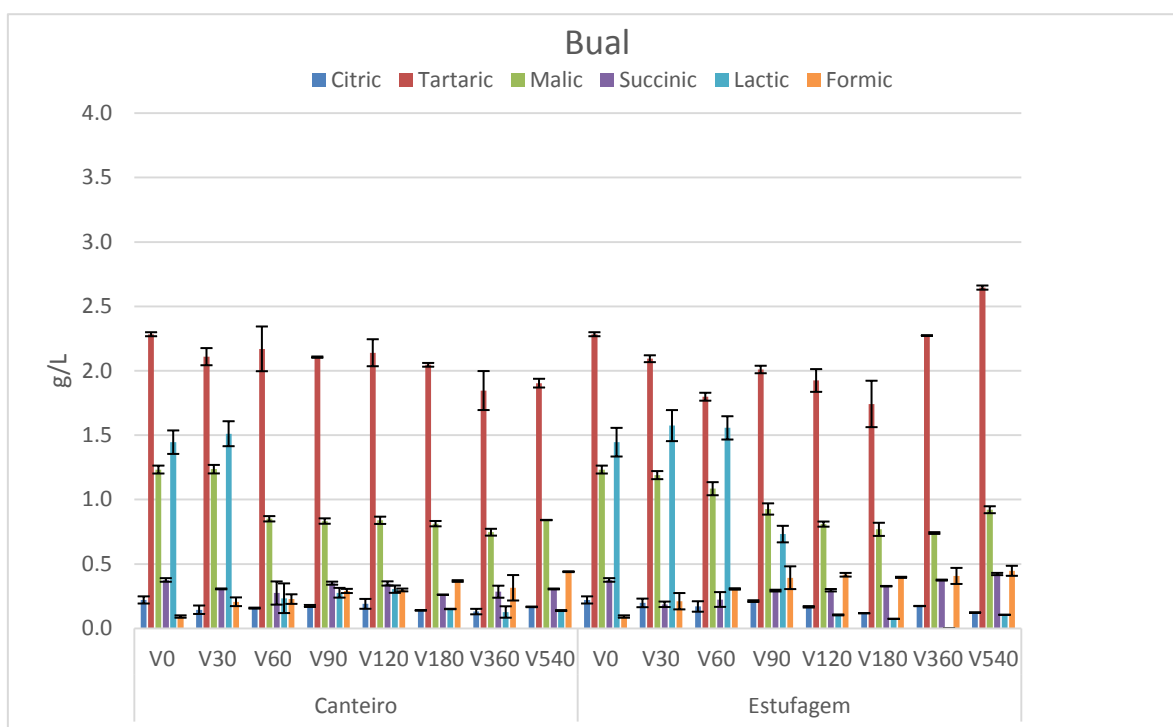
Fig. 23 – Linearity between acetic acid and ethyl acetate formation.

Although there are no legal limits for the ethyl acetate concentration in wines (70), during Madeira wine ageing the levels of ethyl acetate reached values above the olfactory perception threshold for table wines (approximately 150 mg/L (12, 60)) and ice wines ((240 mg/L (69)). The olfactory perception threshold of ethyl acetate for both panels were in average 3 fold higher than the values found for 10 years-old Madeira wines (Fig. 22). So, once the threshold levels of this ester vary with the type and the intensity of wine flavour (60, 67), the amount found for the Madeira wine white varieties might not have a negative impact in wine due to the aroma's complexity of Madeira wine acquired during oak ageing.

6.5 Organic acids determination during ageing process

The determination of organic acids in wines is an important parameter once it can contribute directly or indirectly for the final wine characteristics. In this sense, during ageing organic acids have an essential role in the wine aroma due to their participation in the volatiles formation, namely ethyl esters (7, 10, 14). Fig. 24 describe six organic acids that were assessed in young Madeira wine white varieties (Malvasia, Bual, Verdelho and Sercial) aged by both traditional ageing processes.





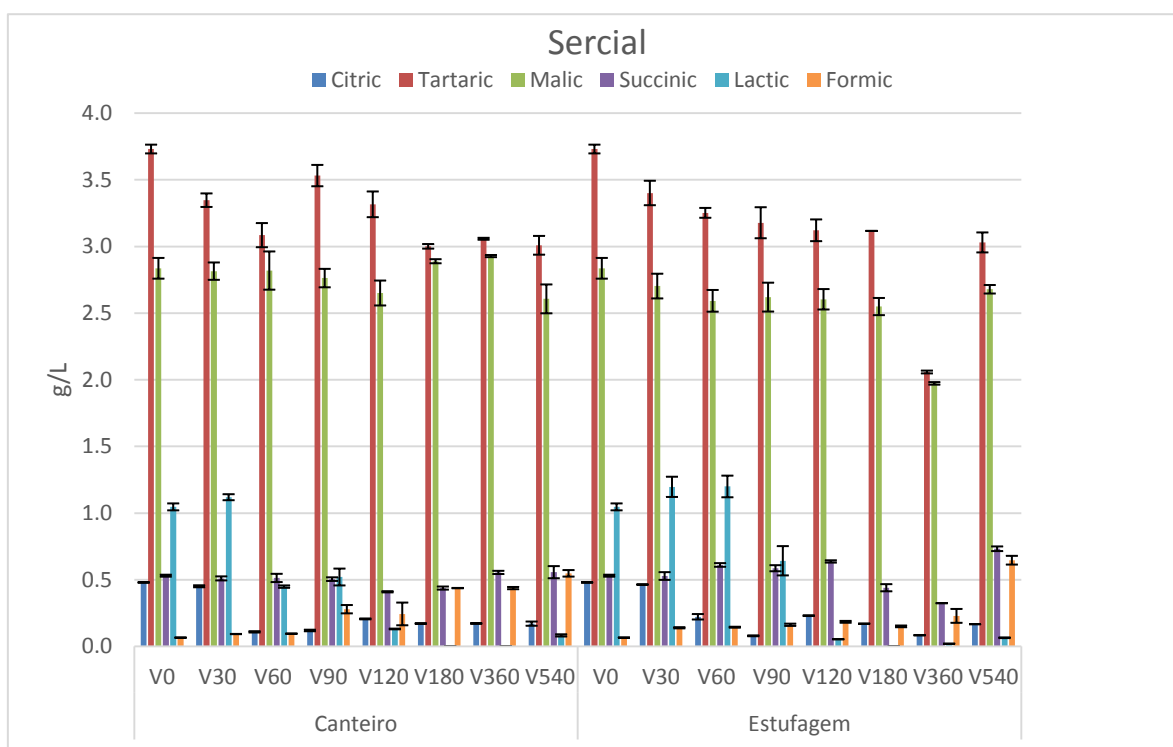


Fig. 24 – Organic acids levels found in the monitored Madeira wine samples.

The results showed that the concentration of organic acids present in these fortified wines varies depending on the wine style, suggesting that it is mainly dependent on the nature of the grapes varieties. So, Sercial revealed higher amount of organic acids which characterizes its typical acidity. On the other hand, Bual is the variety with less acidity. Similarly to previous studies of Madeira wine composition (7, 14, 32), tartaric and malic acids are those that have a greater contribution to wine acidity.

In monitored wines, the most abundant organic acid found was tartaric acid (ranging from 1.74 to 3.73 g/L). The maximum concentration of this acid found in wine samples was similar to red wines (1.09 – 3.80 g/L) (108-110), but it was high when comparing with other wines: white ((0.96-2.40g/L) (108-110)), Port wines ((0.82-2.75 g/L) (111, 112)) and even Madeira wines ((0.33-1.12 g/L) (14)). These results might be associated with the concentration of tartaric acid already present in the initial grapes used for the production of these wines (14). Notice that, tartaric acid concentration in all wine styles showed similar behaviour along time either in *canteiro* or *estufagem*. Once tartaric cannot be formed during the ageing processes, the oscillations verified were probably due to precipitations of tartrates salts during the storage of the samples.

Regarding to malic acid contents, 0.74 – 2.93 g/L, were in accordance with the results reported by Rudnitskaya (32) for Madeira wines. Malic acid levels also demonstrated similar behaviour during both ageing processes for all varieties. Notice that, the oscillations verified in some stages might be associated with precipitation during sample storage. Additionally, *estufagem* does not seem to influence malic content.

Even though, malolactic fermentation is not encouraged in Madeira wine vinification process, considerable levels of lactic acid (maximum concentration of 1.57 g/L) were found in these young wines. Similar results were reported before by Pereira V. (14), indicating that this fact might be derived from the yeasts action during alcoholic fermentation. As demonstrated in Fig. 24, lactic acid content is higher in the first stages (until 120 days of ageing), decreasing afterwards. Interestingly, even though Sercial undergone a longer period of fermentation, the levels of lactic acid were more pronounced in Bual samples.

Succinic acid is the predominant organic acid formed during alcoholic fermentation by the action of the yeasts (54) and for that reason the initial Sercial presented the highest levels (0.53 g/L) while Malvasia showed the lowest (0.18 g/L). *Estufagem* does not seem to influence the succinic content in all wine samples.

Formic acid contents (0.07 to 0.65 g/L) were higher than those found in previous studies of Madeira wine samples (14, 32). In all wine samples was verified an increase of formic acid concentrations during both ageing processes, which may be related with the sugar degradation during ageing (14).

Citric acid levels in the initial wines range from 0.18 to 0.48 g/L, being more evident in Sercial wines. These results are comparable with those found by Pereira V. (14) for Madeira wines (0.17 to 0.36 g/L) and also by Cunha (112) for Port wines (0.24 to 0.31 g/L). Still, once citric acid derived from grapes, it was observed a decreased in its level for all wine samples during both ageing processes.

Additionally, a study was conducted taking into account the organic acids evolution of old Madeira wines aged by *canteiro*. The results are presented in the Fig. 25.



Fig. 25 - Organic acids levels found in Madeira wine cask samples.

According to Fig. 25, the results indicated that contrarily to the previously found in young Madeira wine samples, the age tends to minimize the difference in terms of acidity existent between Madeira wine styles. It was not possible to define a clear tendency of each acid over ageing since

there are several factors involved such as: grape variety, year, grape region and winemaking process.

The most abundant organic acid found in almost wine samples was tartaric acid, ranging from 1.07 to 2.60 g/L, comparable with Port wines (111, 112) but higher than those observed in previous Madeira wine studies (14, 32). In addition, the results showed that tartaric content might tend to decrease with the increase of age. This fact can be related with the rising of alcohol content (>20%) during the ageing in casks of some Madeira wines (observed in the Table 5 (Appendix)), that promoted the tartrates salts precipitation (50).

Malic acid was the second more abundant organic acid found in these wine cask samples (ranged between 0.86 – 4.31 g/L) and did not show a clear tendency on its evolution with age. Interestingly, lactic acid levels showed an increase in the older wines of all grape varieties, reaching values of 2.17 g/L in the case of Malvasia. Its formation may be associated with the presence of the LAB (can occur in grape musts and in wineries) during the alcoholic fermentation when the must contains substantial fermentable sugar. Once these organisms might be resistant to high ethanol concentrations, they can still develop in wine after the fermentation by consuming nutrients that were not used (or consumed) by yeast (50, 113).

Succinic acid levels ranged from 0.20 to 0.74 g/L (comparable with the results found in the young Madeira wines) while formic acid showed higher content (0.30 to 1.08 g/L) than those found in younger wines. According to Fig. 25, formic acid seemed to increase during ageing reaching higher values in the sweetest wines. This fact came to reinforce that the formation of formic acid might be associate with the sugar degradation during ageing (14), as referred before. Finally, citric acid revealed lower amount (0.05 – 0.20 g/L) than those previously found in young Madeira wines samples and also in studies performed by Pereira V. (14) and Rudnitskaya (32). Citric acid is commonly found in grapes, and as expected, its concentration was tendency lower in older wines.

PART IV

CONCLUSIONS

FUTURE PERPECTIVES



7. CONCLUSIONS

The sensorial analysis revealed that the olfactory perception threshold of acetic acid for the untrained panel was in average 5.45 g/L and 6.22 g/L for 5 and 10 years-old, respectively. The trained panel showed a lower perception threshold, namely 4.54 g/L for 5 years-old Malvasia. However, after training this expert panel to recognize acetic acid odour, the olfactory threshold decreased significantly in average for 1.44 g/L (5 years-old) and 1.87 g/L (10 years-old). Nevertheless, the acetic acid perception limits were higher than the legal limits established for the volatile acidity of Madeira wines.

Additionally, the odour threshold of ethyl acetate was also higher (in both panels) than those found for table and ice wines. The threshold levels for the untrained panel were in average 380.81 mg/L (5 years-old) and 348.98 mg/L (10 years-old), while for the trained panel were 275.27 mg/L and 306.83 mg/L for 5 and 10 years-old, respectively. Interestingly, in a general perspective the results showed that the perception threshold of both compounds seems to depend essentially on the Madeira wine age and not on the sweetness degree. This fact may be related to the intense and complex flavour acquired during the maturation process that can easily mask the presence of acetic acid and ethyl acetate. So, for that reason the volatile acidity legal limits for Madeira wine can be reconsidered.

We also concluded that the concentration of acetic acid either in *canteiro* or *estufagem* was similar, occurring an exponential increase during ageing that greatly depends on the sugar content present in wines. Furthermore, the ethyl acetate amount is greater in *canteiro* than *estufagem* in the initial days of ageing, tending to approach over time. Also, a linear relationship between acetic acid and ethyl acetate was verified during oak ageing in all Madeira wine samples.

Finally, the organic acids present in young Madeira wines from different white varieties depend essentially on the nature of grapes varieties. However, with age this difference in terms of acidity between Madeira wine different styles tends to be minimized.

8. FUTURE PERSPECTIVES

As future work, we consider important:

- Promote sensorial tests of gustative perception threshold for acetic acid and ethyl acetate in order to determine the real values of volatile acidity perception in Madeira wines;
- Assess the role of other compounds that may contribute for volatile acidity, determining their evolution during vinification and ageing;
- Study the type of yeast and bacteria involved in the Madeira wine vinification process in order to determine its role on the acetic acid production.
- Study the reactions involved during Madeira wine oak ageing that can lead to the acetic acid formation.

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10. APPENDIX

Sensorial test



		Tese de mestrado - Andreia Miranda Determinação do limiar de percepção olfactiva da acidez volátil com Vinhos Madeira Concentração de ácido acético	
Local da Prova:	Data:	Hora:	Provedor:
<p>Leia atentamente as instruções que se seguem:</p> <p>1 - Em cada grupo, encontram-se amostras de vinho e/ou amostras de vinho com adição de uma determinada concentração de padrão de ácido acético glacial.</p> <p>1.1 - Das 3 amostras de cada linha do grupo, identifique qual a diferente.</p>			
Grupo I		1 2 3	4 5 6
Grupo II		1 2 3	4 5 6
Grupo III		1 2 3	4 5 6
Grupo IV		1 2 3	4 5 6
Grupo V		1 2 3	4 5 6
			

Table 5 – Basic oenological parameters of Madeira wines sample casks.

	Cask age	Alcohol (v/v)	Specific gravity (g/cm ³)	pH	Total acidity (g/L in tartaric acid)	Reducing sugar (g/L)
Malvasia	6	18.22	1.0078	3.47	5.00	62.07
	9	18.39	1.02741	3.52	6.13	110.58
	12	20.27	1.02968	3.46	7.94	117.13
	19	20.10	1.03974	3.49	7.76	143.22
	22	22.94	1.03600	3.63	9.88	130.08
	23	18.48	1.02524	3.48	6.20	105.46
	33	22.89	1.02084	3.62	8.81	90.42
Bual	6	17.91	1.01712	3.44	5.82	83.02
	9	19.58	1.02014	3.40	6.67	96.27
	15	17.79	1.02752	3.42	7.22	109.84
	18	19.54	1.02417	3.45	6.90	102.22
	23	20.82	1.03318	3.45	8.48	123.38
	25	22.28	1.01295	3.44	9.53	70.46
	27	21.21	1.01947	3.5	9.44	88.65
	36	22.51	1.03165	3.62	9.21	118.29
Verdelho	5	19.03	1.00753	3.49	5.99	66.54
	9	18.23	1.01625	3.44	6.06	82.4
	14	20.35	1.01143	3.34	9.07	70.26
	16	20.09	1.01091	3.43	7.11	69.77
	24	19.33	1.00778	3.44	6.01	62.07
	26	20.12	1.03387	3.46	7.86	128.91
	32	18.89	1.0119	3.40	7.63	68.61
	35	22.66	1.01617	3.50	9.79	80.62
	38	20.57	1.01578	3.59	8.85	76.14
Sercial	6	19.92	1.00225	3.56	5.40	54.17
	12	18.76	0.98704	3.42	6.98	7.61
	18	20.83	0.99072	3.27	9.33	18.95
	22	21.36	0.99876	3.30	9.91	34.36
	24	20.13	0.99814	3.29	9.52	35.97
	37	19.35	0.99809	3.50	6.84	34.51
	42	21.59	0.98733	3.43	7.10	10.44
	45	20.34	1.00247	3.49	7.52	45.3