Fernal

Madeira Wine Vinification Process
Analysis and control

MASTER DISSERTATION

Pedro Marcelo Silva Rodrigues
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
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February | 2016

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AGRADECIMENTOS

Quero começar por agradecer à minha família e em especial à minha mãe, Idalina Rodrigues, ao meu pai, Paulo Rodrigues, à minha avó, Filomena Andrade e aos meus irmãos, Alexandre Rodrigues e André Rodrigues pela paciência e compreensão. Obrigado por tudo. Um enorme obrigado por toda a dedicação e apoio. O meu agradecimento torna-se difícil de expressar por palavras. E porque a vida não tem valor sem ser partilhada, um grande obrigado aos meus familiares e amigos mais próximos.

Por outro lado, quero agradecer às pessoas que de forma mais direta contribuíram para a conclusão desta etapa: quero expressar a minha gratidão ao meu orientador principal, Professor Doutor José Carlos Marques, por me ter dado a oportunidade de trabalhar sob a sua orientação e com isso engrandecer a minha formação pessoal e profissional. Queria também estender a minha gratidão à minha coorientadora, Doutora Cristina Pereira, pela partilha de conhecimento e pelo apoio constante no trabalho a ser desenvolvido, desde o primeiro momento em que tive contacto com o laboratório.

Aos meus colegas de laboratório: Maria João Carvalho, Andreia Fátima Miranda, João Micael Leça, Isabel Freitas pela amizade, pela partilha constante de conhecimento, pelo companheirismo e pela ajuda que tem fornecido. À Carolina Vieira pela simpatia e apoio durante o decurso do presente estudo.

Também quero agradecer à empresa J. Faria, em particular ao Sr. Luís pela oportunidade do projeto em questão e à empresa P.E. Gonçalves pelo fornecimento das amostras analisadas neste projeto e pela disponibilidade na partilha de informação essencial para a compreensão dos resultados.

Por fim, gostaria de agradecer à Universidade da Madeira e a todas as pessoas que tive a oportunidade de conhecer ao longo da vida académica (1º ciclo em bioquímica e 2º ciclo em Bioquímica Aplicada e que de alguma forma contribuíram para alcançar este objetivo.

RESUMO

O presente estudo é realizado em parceria com a empresa J. Faria e Filhos e tem por objetivo fazer uma análise exaustiva dos seus vinhos durante a fase de vinificação e identificar possíveis pontos de melhoria no respetivo processo de produção.

Foram analisados três vinhos distintos produzidos na vindima de 2014. O processo de vinificação foi acompanhado durante 4 semanas, tendo sido registadas as quantidades de uvas recebidas e a avaliação do respetivo estado fitossanitário, os tempos e temperaturas de fermentação e a evolução das densidades até ao momento da fortificação. A caraterização dos vinhos foi realizada numa primeira fase em termos da evolução de parâmetros enológicos, nomeadamente a evolução da densidade, acidez volátil e total, teor alcoólico, pH e índice total de polifenóis, e numa segunda fase procedeu-se à análise da evolução de ácidos orgânicos, dos principais açúcares presentes no vinho e da evolução do perfil volátil. Após análise dos resultados do perfil volátil, optou-se por desenvolver e validar uma metodologia para quantificação dos ácidos gordos voláteis, nomeadamente por SMPE-GC-MS. Resumidamente, obteve-se os seguintes desempenhos para a metodologia desenvolvida: boa linearidade (R^2=0.999) e sensibilidade (LOD =0.026-0.068 mg/L), boa precisão, a qual foi avaliada em termos de repetibilidade e reprodutibilidade (RSD<8,5%) e uma boa recuperação (103,11-119,46%).

Os resultados demonstraram a necessidade de melhor controlar as temperaturas de fermentação, para manter um melhor equilíbrio na proporção em que aparecem alguns compostos voláteis, nomeadamente esteres e álcoois superiores e minimizar as concentrações de outros compostos (ex. ácidos decanóico, octanóico e hexanóico), que quando acima do seu limiar de perceção olfativa, não são benéficos para o aroma global do vinho. No que diz respeito ao momento escolhido para parar a fermentação, considera-se que pode também haver ligeiras alterações as quais podem igualmente ser benéficas para manter a tipicidade do bouquet destes Vinhos Madeira.

Palavras-chave: Vinho Madeira, processo de vinificação, ácidos orgânicos, glucose e frutose, perfil volátil, ácidos gordos voláteis.

SUMMARY

The present study was done in collaboration with *J. Faria e Filhos* company, a Madeira wine producer, and its main goal was to fully characterize three wines produced during 2014 harvest and identify possible improving points in the winemaking process.

The winemaking process was followed during 4 weeks, being registered the amounts of grapes received, the fermentation temperatures, the time at which fermentation was stopped and evolution of must densities until the fortification time. The characterization of musts and wines was done in terms of density, total and volatile acidity, alcohol content, pH, total of polyphenol, organic acids composition, sugars concentration and the volatile profile. Also, it was developed and validated an analytical methodology to quantify the volatile fatty acids, namely using SPME-GC-MS. Briefly, the following key features were obtained for the latter methodology: linearity (R^2=0.999) e high sensitivity (LOD =0.026-0.068 mg/L), suitable precision (repeatability and reproducibility lower than 8,5%) and good recoveries (103,11-119,46%).

The results reveal that fermentation temperatures should be controlled in a more strictly manner, in order to ensure a better balance in proportion of some volatile compounds, namely the esters and higher alcohols and to minimize the concentration of some volatiles, namely hexanoic, octanoic and decanoic acids, that when above their odours threshold are not positive for the wine aroma. Also, regarding the moment to stop the fermentation, it was verified that it can be introduced changes which can also be benefit to guarantee the tipicity of Madeira wine bouquet.

Keywords: Madeira wine, vinification process, organic acids, glucose and frutose, volatile profile, volatile fatty acids

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List of abbreviations

CV – Coefficient of variation.

GC-MS – Gas chromatography - mass spectrometry.

GC-O – Gas chromatography – olfactometry.

He – Helium.

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.

LAB – Lactic acid bacteria.

LOD – Limit of detection.

LOQ – Limit of quantification.

SD – Standard deviation.

NaCl – Sodium chloride.

NaOH – Sodium hydroxide.

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

R^2 – Correlation coefficient.

SO_2 – Sulphur dioxide.

TIC – Total ion count.

UK – United Kingdom.

USA – United States of America.

UV – Ultraviolet

Vis – Visible.

TN – *Tinta Negra.*

T – Tonality.

TP – Total polyphenols.

VFA – Volatile Fatty Acid

tR – Retention times.

% 420 – Yellow percentage.

mg/L – Milligram per litre.

min – Minutes.

L – Litres.

GAE – Gallic acid equivalent.

g/L – Gram per litre.

% 620 – Blue percentage.

°C - Degree Celsius.

$CaCO_3$ – Calcium carbonate.

CI – Colour intensy.

Da – Dalton.

PART I – General Introduction

1. Scope and Goal

In the last decade, we have been witnessing to some shift of paradigm regarding the vinification process of Madeira wine, which has been mainly conducted based on the empirical knowledge. Notwithstanding this valuable know-how, the increasing demands in wine commercialization calls for studies which positively contribute for improving the winemaking process control, in more consistent and informed manner, in order to guarantee the requirements of different markets and ensure the high quality level expected for these wines.

The present work looks for contributing in this sense. The study is carried out in collaboration with a local producer which aims an exhaustively characterization of its wines during the vinification process, as well as the identification of possible improving points. Accordingly, the study was outlined to analyse different lots of Madeira wine produced in its facility, at different stages of vinification process and using different analytical procedures which allow a comprehensive chemical characterization of wines to further identify how vinification practices and product quality control can be improved. All wines analyzed in this study were produced from *Tinta Negra* grape variety.

The Madeira wine producer involved in this work was J Faria & Filhos. This is one of the eight producers of Madeira wine. This company produces all styles of Madeira wine, namely sweet, medium sweet, medium dry and dry wines, essentially with 3 years old and some lots with 10 years old, all produced from *Tinta Negra* grape variety(1).

2. Madeira Wine

Over the centuries, the Madeira wine has been worldwide recognized and the hallmark image of Madeira Island. Madeira wine is classified as fortified wine, it has an alcoholic content between 17 up to 22%, and it is commonly served as an aperitif or dessert wine, due to its intense flavour and complexity (2).

The history of Madeira wine starts soon after the island's colonisation, at the 15th century, followed by a period of high levels of exportation. In the eighteen century, for-

tification and *Estufagem* practices were introduced in the vinification process of Madeira wines, which mark its identity even today. The 19th century was a period of ups and downs, it began with high levels of exportation, but in the middle of the century, there was the devastation of the vineyards by powdery mildew and then by Phylloxera. From this time, *Tinta Negra* (TN) grape variety (Fig.1) became the predominant grape variety in Madeira island (2, 3).

Figure 1: TN grapes

This grape variety represents around 85 – 90% of the Madeira Island production. It has a remarkable versatility in terms of its use in the production of Madeira wine. It is used to produce dry, medium dry, sweet and medium sweet wines, according to the sugar content in the wine (4). TN bunches are medium to large, having medium to small berries, with a very thin and soft skin, and an intense dark red colour when ripe. This grape variety is essentially produced in the south of the island, namely at *Funchal*, *Câmara de Lobos* and in the north in *São Vincente*. Its musts can achieve an alcoholic potential of 9% – 12% (3).

2.1. WINE GROWING REGION: THE SOILS AND CLIMATE

The unique features of Madeira wine have also influence from the soil and climate of the island. The soils in Madeira are from volcanic origin, rich in organic matter and minerals, such as magnesium and iron. This contributes, in some extent, for the Madeira wine's acidity, which is a key factor for balancing the wine complexity.

The climate in Madeira island is sub-tropical, with hot humid summers and mild winters. The annual average temperature varies from 12ºC up to 28ºC. Around 75% of the rainfall occurs mostly in the autumn and winter and 20% in the spring. Madeira island is characterized by different microclimates throughout the winegrowing areas, due to the variations in altitude, temperature and humidity (4).

The vineyards usually grown in terrace steps called “poios”, spread over steep hillsides. There are two systems for conducting the vines. In the traditional system called *Latada*, the vines grown horizontally at about two meters above ground sup-

ported by trellises. In the *Espaldeira* system, introduced more recently, the vines grow vertically in rows on wires. In both cases, and due to the characteristics of the terrain, the mechanisation is almost impossible and thus the whole cultivation cycle is usually made by man hand, increasing the costs of the entire process (5).

2.2. Winemaking process

2.2.1. Harvest

The harvest usually begins in mid-August and ends in October. During this period, the grapes are visually inspected (appearance, mildew, and other defects) and evaluated in terms of potential alcohol (to ensure a minimum of 9%). After the official order, grapes are carried to producer in containers with capacity of about 50 kg, to be processed as soon as possible (6).

2.2.2. Destemmed and Crushing

At wineries, grapes are weighed and the probable degree of alcohol is verified using a refractometer. Then, the stems are removed. At this phase, two different procedures can occur. In the first case, called *bica aberta* the masses of crushing phase are pressed and the must is separated to ferment without grape solids. In the second procedure, called *curtimenta*, the fermentation occurs with grape solids and the pressing only takes place after reaching the desired degree of sweetness, at the end of fermentation.

The must is highly vulnerable to microbial contamination, thus an aqueous solution with 5% of potassium metabisulphite ($K_2S_2O_5$) is usually added to inhibit the growth of the natural microbial flora (bacteria) (2, 7).

2.2.3. Fermentation

Alcoholic fermentation is the anaerobic transformation of sugars, mainly glucose and fructose, into ethanol and carbon dioxide. At the same time that this reaction proceeds, a lot of other biochemical, chemical and physicochemical processes takes place. Besides ethanol, several other compounds are produced throughout alcoholic fermentation such as higher alcohols, esters, glycerol, organic acids, carbonyl compounds and other compounds (2).

A common practice in wine fermentation process is the inoculation of selected yeast for the fermentation occurs. However, in Madeira winemaking, fermentation starts spontaneously, owing to the presence of indigenous yeasts present in grapes or yeast from cellar equipment and tanks (8).

In general, as the fermentation advances the activity of yeasts leads to an increase of the temperature. The raise of the fermentation temperature affects not only the speed of the fermentation but also the quality of the final wine. It is recognized that low temperatures and consequently slower fermentations avoid the loss of volatile aromas, thus these conditions are the required to improve the wine quality (2).In the case of Madeira wine production, the temperature of fermentation is usually controlled, in order to ensure values bellow 25 ºC.

2.2.4. Fortification

According to the style of wine to be produced, the winemaker stop the fermentation by the addition of natural grape spirit, containing 96% (v/v) of ethanol. This step is only done after being attained 4% of alcohol exclusively derived from alcoholic fermentation, which will ensure the presence of fermentative aromas in the final wine. After fortification, the wine normally contains alcohol content between 17 and 18% (v/v). Together with the advantage of halting the alcoholic fermentation on the desired point to produce a specific style of wine, fortification can also prevent the metabolism of some bacteria prejudicial to wine, such as the lactic acid which could produce too much acetic acid (9).

2.2.5. Clarification

At this phase, the goal is to ensure that particles in suspension settle to the bottom of the storage tank. This can be achieved only by the gravity force, the wine stays resting during one or two months, or by adding fining agents (such as bentonite) to speed up and ensure the precipitation of all suspended particles (7).

2.2.6. Aging of Madeira Wines

The Madeira wine ageing process can be conducted according to one of the following procedures: *Estufagem* or the *Canteiro*. In *Estufagem*, the wines are stored in stainless steel tanks and the wine is heated at 45º C for three months. Completed this procedure, the wine is cooled and then stored in casks or in stained steel tanks for at least of 90 days or until the winemaker decide that the wine is ready to be bottled (2).

The wines selected to age according *Canteiro* procedure are stored in oak casks, normally on the top of the warehouse, where the temperatures are much higher, during a period of 3 years. This is an oxidative aging process, during which the wine develops intense and complex aromas (3, 10).

3. Brief background on grape and wine compounds

3.1. Grapes constitution

Like we all know grapes are constituted by several compounds distributed in different sections of the grape. One of the main components of grape is water; which constitutes around 70 – 80% of total weight. The grapes contain 15–25% w/w of reducing sugars, glucose and fructose. These fermentable sugars provide ethanol in the wine, through yeast metabolism, and they are also the precursors for numerous aroma compounds. There are a small quantity of nitrogenous compounds in grapes that achieve values of 0.3–1.0% and have a particular significance in wine as flavour precursors (11).

The fraction of phenolic compounds that most contribute to wine colour are localised in the epidermal cells of the skin. Additionally, the skin also contains several volatile compounds that contribute to the wine aroma, especially terpenes responsible for the so-called 'aromatic fruity – grape' flavour (11). The flesh of the grapes is constituted by fermentable sugar like glucose and fructose and non-volatile acids like malic, tartaric and citric, which are represented in Figure 2.

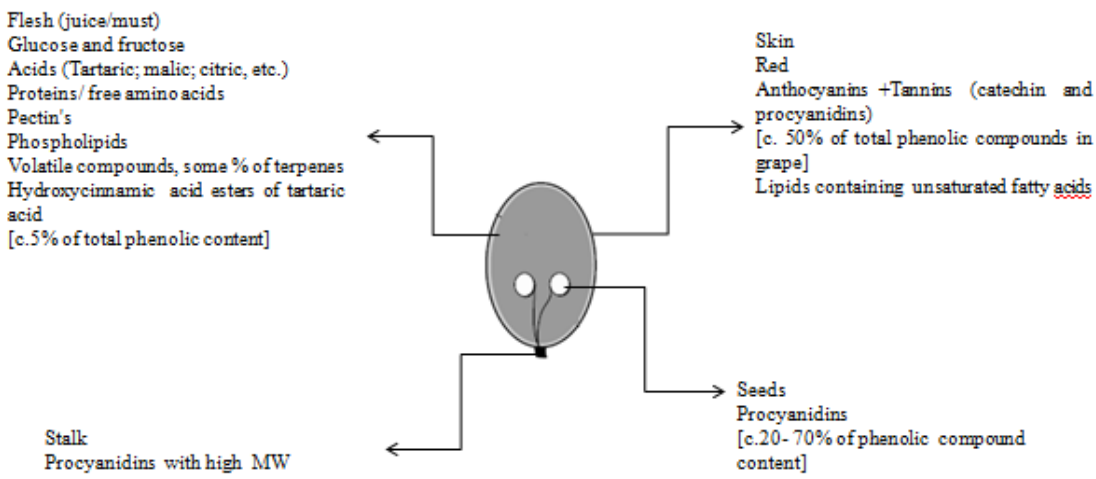

Figure 2: Localization of the diverse constituents of grapes. (Adapted from (1))

3.2. Organic acids in wines

Organic acids are characterized for their acidic properties. These compounds normally have an ionization capacity, releasing a hydrogen ion in an aqueous medium(2).The concentration of organic acids has a significant importance in wines. Some of these compounds are present in the grapes, such as malic, citric and tartaric acids. Others like lactic, acetic and succinic acids have two possible formation pathways, either from fermentation or from bacterial activity. It seems they affect the stability, the colour and the flavour of wines(12).Nowadays some authors associate the concentration of organic acids with the region, climate and ripening stage of the grapes. Wines produced in warmer countries are known to have more tartaric acid, while malic acid prevails in wines produced in colder countries (13).

3.2.1. Tartaric acid

Tartaric acid is known to be the strongest acidic compound in wines. Due to this fact, it has a great influence on wine pH. This acid has an important role, decreasing the pH of must and acting as a preservative against the development of detrimental bacteria (2, 14).

The content of Tartaric acid in wine normally ranges between 2-5 g/L and keeps wine pH with values between 3.0 to 3.5. Red wines have a higher concentration of this compound than white wines. Consequently, due to its significance in wine acidity, numerous wine features are affected, such as colour, flavour and chemical and microbiological stability(15).

3.2.2. Malic acid

The malic acid is the second most important acid in grapes and is found in all living organisms. In fact, the juice of unripe grapes, just before colour change, contains concentrations of 25 g/L. In the two weeks following the first signs of colour change, the malic acid content drops by half, particularly due to dilution as the grapes grow bigger. At maturity, musts from northerly regions contain values between 4 to 6.5 g/L of malic acid content, comparing with the southerly regions, where the concentrations only vary between 1 to 2 g/L (16-18). In the process of cellular respiration, malic acid is used as substrate and is affected by temperature. It is involved in two respiratory metabolic cycles: the tricarboxylic acid (TCA) cycle and the glyoxylic acid cycle (GAC). This process is slower in colder climates than in warmer regions. Normally, malic acid concentrations in grapes can reaches to 15–16 g/L at harvest time (17).This acid tends to contribute to half of total acidity of grapes and wines. Its concentration decrease as the maturation process takes place, particularly during hot periods at the end of the season.

Consequently, malic acid concentration is one of the principal indicator for the determination of harvest date (7).

3.2.3. Acetic acid

In wines, volatile acidity is mostly caused by acetic acid. Its presence at high concentrations gives an off-flavour (vinegar odour) and a disagreeable sensation in the mouth. For that reason, volatile acidity is one of the most important analytical parameters in oenology. Acetic acid can have various formation pathways: it can be produced by yeast activity, lactic acid bacteria and acetic acid bacteria. Normally, *Saccharomyces cerevisiae* produces small quantities of acetic acid during alcoholic fermentation, reaching concentrations of about 0.1–0.3 g/L. However, when stuck or sluggish fermentations occur, large amounts of this acid can be generated(19).At normal wine levels (0.3g/L), acetic acid can be a desirable flavorant, adding complexity to taste and odour. If its content is greater than 0.3g/L, acetic acid progressively imparts wine with a sour taste and aroma(20).

3.2.4. Lactic acid

During the fermentation stage, a small content of lactic acid is formed by yeasts. When wine has a higher concentration of lactic acid, it is associated to the malolactic bacteria metabolic activity. It is produced from an enzyme that decarboxylates malic acid to lactic acid, designated as malolactic fermentation. The presence of the stereoisomer L-lactic acid in wines is typically an indicator of this kind of fermentation (7).

3.2.5. Succinic acid

Succinic acid exists in small amounts in grapes, and it is one of the non-volatile organic acids produced during the must fermentation stage. Its concentration in wines can alter depending on the yeast strain. Succinic acid is resistant to bacterial attack and it is mostly stable in wines. This acid can also influence in wine quality, as there are a number of factors that can influence its concentration, such as temperature, pH, Sulphur dioxide (SO_2) concentration, nutrient content (14, 15).

3.2.6. Citric acid

Citric acid is mostly found in citrus fruits, like lemons and oranges. Its presence in the ripening phase is limited. Its content in wine tends to vary between 0.5 to 1 g/L. Although appearing in small quantities, this acid has a significant influence in wine flavour. During the must fermentation, this compound decreases as yeast activity promotes its transformation into acetic acid (15).

3.3. Volatile compounds in wines

During the fermentation process there are two stages of wine aroma: in the initial stage grape aromas are predominant, and during the secondary stage the fermentation aromas become more relevant. The main primary wine aromas, or varietal aromas, are composed mostly by monoterpenes. These are secondary products of plant metabolism and are dispersed among the skin and pulp. Varietal aromas give fruity and flowery attributes to the wines (2, 21-25).The secondary aromas are the final products of the biochemical reactions that occur during alcoholic fermentation, namely aliphatic alcohols, esters, carboxylic acids, aldehydes, glycerol and diols, among others. The concentration of these compounds depends on many factors, such as fermentation conditions and yeast strains (21, 22, 25).The volatile fraction of wine consists of more than eight hundred different compounds belonging to various chemical classes (26).

3.3.1. Terpenic compounds

Terpenes belong to the secondary metabolism components of plants. They are mainly located in the skin of grapes and are mostly linked to sugars. They contribute to the varietal aroma of grapes and wines(27).Monoterpenes (terpenic compounds with 10 carbon atoms) can appear in the form of simple hydrocarbon compounds (limonene, myrcene, etc.), aldehydes (linalal, geranial, etc.), alcohols (linalol, geraniol, etc.), carboxylic acids (linalic and geranic acid, etc.), and esters (linalyl acetate, etc.).About forty different terpenic compounds have been identified in grapes. Their olfactory perception thresholds are relatively low. Two of the most odoriferous terpenes are citronellol and linalol (24, 28).

3.3.3. Esters

Esters are produced by the reaction between acetyl coenzyme A (acetyl-CoA) and an alcohol, mediated by *alcohol-acylcoAtransferases*. Mainly there are two types of esters in wines: the esters of higher alcohols and the esters of fatty acids. The first group mentioned is formed by the reaction between a higher alcohol and acetyl-coA, giving off various odours such as glue and banana. The second group of esters mentioned are produced by the reaction between ethanol and acyl-coA. These compounds are known to give fruity aromas to wines. All esters, with the exception of ethyl acetate, give a pleasant odour and contribute positively to wine aroma (8, 29).The ethyl acetates of fatty acids are produced during the fermentation stage, under anaerobic conditions. They are hydrolysed during the *Estufagem* stage and tend to decrease its concentration. Total concentrations of ethyl acetates of a few mg/L gives wine a pleasant odour of wax and honey.

The most predominant ester in wine is ethyl acetate, formed during fermentation in a small amount. During the aging process in oak barrels, its concentration grows to larger amounts as consequence of the action of aerobic acetic bacteria. Ethyl acetate is traceable at concentrations around 200 times lower than acetic acid. The olfactory acuity threshold of ethyl acetate is about 160 mg/L in wine. However, it is probable that at low concentrations (50–80 mg/L) ethyl acetate contributes to a wine's olfactory complexity(29).

3.3.4. Higher alcohols

Higher alcohols are derived from the metabolism of amino acids. This reaction happens when keto acids are decarboxylated and reduced. Another way to produce these compounds is through the Ehrlich reaction, directly from sugars (8, 29, 30).

Normally they are below their odour threshold. Some of these alcohols occur during alcoholic fermentation and can reach concentrations between 150–550 mg/L. The presence of higher alcohol and their corresponding esters play an important role on the complexity of wine aroma. Isobutyl and amyl alcohols are two of the most relevant higher alcohols. Several factors can influence the formation of these compounds, such as yeast strain, oxygenation, temperature and the presence of matter in suspension, among others (8, 29).

3.3.5. Carboxylic acids

Carboxylic acids are organic compounds catalysed by a multienzymatic complex called fatty acid synthetase. This enzyme uses as substrates acetyl-coA and malonyl-coA to produce palmitic acid. These compounds can also be biosynthesized by bacterial activity. Therefore, propanoic, 2-methylpropanoic and 3-methylbutanoic acids are products of protein metabolism by yeast. The reaction of fatty acids with a substantial amount of ethanol leads to the chemical formation of esters (31).

Medium chain fatty acids like hexanoic, octanoic and decanoic acids can occur by the catabolism of long-chain fatty acids. Possibly the yeasts use this catabolic reaction in the beginning of the alcoholic fermentation. They are described as goaty and cheese aromas (31, 32).

The fermentation conditions (temperature, oxygen, pH and sources of nitrogen) and the yeast strain are essential for the development of these volatile fatty acids (VFA).The VFA below the perception threshold contribute for wine complexity, while above these limits they have a negative impact in aroma(31).

3.3.6. Lactones

Lactones are produced by an internal esterification reaction between an acid function and an alcohol function in the same molecule. One example is γ-butyrolactone present in wine at concentrations of some mg/L. Another example is sotolon from a condensation reaction, between α-keto butyric acid and ethanal. During the process of aging, the oak releases lactones into wine, such as *cis* and *trans* isomers of 3- methyl-γ –octalactone, identified as 'oak lactones'. These compounds have a coconut odour. In wine the concentration founded are on the order of a few tens of mg/L, considerably higher than the perception threshold (29).

3.4. Phenols

The group of polyphenols are constituted by two groups of compounds: the flavonoids and non-flavonoids. The first group mentioned is categorized by two phenol structures related by an oxygen-containing carbon-ring structure (a pyran) and comprise flavanols like for example: flavan-3-ols, catechins, anthocyanins responsible for the red colour and the mouth-feel. This group constitutes the major polyphenols in red wines, with concentrations around 1,000 to 1,800 mg/L. The second group contains phenolic compounds like hydroxybenzoic and hydroxycinnamic acids, characterized by a simpler structure. It is known that non-flavonoids increase the stabilization of red wines, regularly associated to intra- and intermolecular reactions between anthocyanins (33-38).Anthocyanins are one of the most important chemical families responsible for the colour of red wine. On the other hand, when they react with colourless phenolic compounds, also present in the skin (eg. catechin, epicatechin, quercetin and phenolic acids), they influence wine colour, leading to some loss of red colour.

4. The control of vinification process

The control of vinification process starts when grapes arrive to wineries. At this time, grapes are firstly evaluated in terms of sanitary conditions (odours from grapes

deterioration, contamination with fungi or incomplete grape maturation) and weight. After that, grapes are analysed in terms of probable alcohol content and then accepted to produce Madeira wine if a minimum of 9.5 degrees is obtained. As described in section 2.2.2, after this phase, the grapes are destemmed and the producers decide to proceed with the fermentation maintaining or not the contact between skin and must. To promote the contact between skins and must is very important, since this practice ensure the extraction of compounds presented in skins which are important to wine colour. Also, the contact between the grapes and skins during the fermentation contributes for wine astringency and herbaceous character of wines (2, 21-25).

The temperature is one of the most significant parameter to be controlled during fermentation. These parameter has an impact in the metabolism of yeast and in the production of volatile compounds. Acetates, ethyl esters, higher alcohols, aldehydes and ketone, for example, are affected by temperature during fermentation (39).

The main volatile compounds in wine are ethyl esters of fatty acids and acetates. There are many factors that influence the formation of these compounds, being the most significant the fermentation temperature, which once controlled leads to the production of compounds which contribute positively to wine aroma (40).

Other important compounds in wine aroma are higher alcohols, which development is dependent on the fermentation temperature. A temperature increase results in the rise of the total higher alcohols concentration (29, 41, 42).

Up to this time, there are few articles regarding the vinification process of Madeira wines. Most of the studies have been done to analyzed the ageing process, namely the *Estufagem*. Recently, studies demonstrated that the optimal ageing time and temperature is around 4 month at 45ºC. According to Pereira (2011) during this period occurs the production of two furanic compounds, furfural (F) and 5-hydroxymethylfurfural (HMF), resulting from degradation of carbohydrates, normally associated to non-enzymatic browning reactions, characterized by Maillard reactions (MR), sugar degradation in acid medium and caramelization.

Regarding the vinification process, recently Carvalho (43) report that the greatest variations in colour evaluated by CIELab methodology occurs during the vinification, especially for medium dry and dry wines produced from TN grape variety.

The wines produced from TN were also characterized by Perestrelo (2006) in terms of volatile composition. The wines were analyzed one years after the harvest time.

They suggest that the higher alcohols (aliphatic and aromatic) constitute the largest group of the volatile composition and identify that ethyl esters and fatty acids constitute vital groups for wine sensory properties (44).

Regarding the vinification process, as stated above there are not many articles reporting studies about the evaluation of important parameters to be controlled in the production of Madeira wines. On the other side, this topic has been explored for table wines (42, 45-50).

In 2002, Torija and co-workers (48), studied the effect of fermentation temperature, between 15ºC and 35ºC,using *saccharomyces cerevisiae*. Furthermore, they also studied the effects of fermentation temperature on the fatty acid composition of yeast cells and the consequent formation of volatile compounds (47). They reported that the content of VFA was dependent on the strain used in the fermentation and also have a great influence from temperature. In 2007, Molina *and co-workers* (44) studied the impact of fermentation temperature (between 15 and 28°C) on yeast development and the production of aroma compounds. They also concluded that fermentation temperature plays an important role in the wine´s aromatic profile.

PART II – EXPERIMENTAL

MATERIAL AND METHODS

5.1. Samples

The three wines analysed in this study were produced as dry wines, that is, the fermentation was stopped by the addition of natural grape spirit when the must specific gravity reached 990g /dm^3. Samples were collected at different stages of fermentation, namely at the first day of fermentation, after 24, 48, 60 and 72 hours after the beginning of fermentation and a day and a week after the fortification. The label used to identify each sample is presented in table 1. The three wines analysed are identified according to tank in which they are produced, namely P2, P3 and T13 which correspond to tanks of fermentation with 60000, 40000 and 20000L of capacity. The amount of grapes produced in each case was about 56300, 38900 and 19300Kg, respectively. In the particular case of wine produced in P2, it was also followed during the ageing process and analysed before (4 months after VAT2 point) and at the end of *Estufagem* process All samples were collected in triplicate, stored at -26^{0}C until be analysed and when necessary filtered (0.45 μm) before the analysis. In the particular case of musts, 10 mL of a solution of Calcium Chloride with the concentration of 800g/L were added, to prevent that the fermentation proceed.

Table 1: Label of samples

M0	Initial must
M24H	Must after 24 hours
M48H	Must after 48 hours
M60H	Must after 60 hours
M72H	Must after 72 hours
VAT 1	First fortification
VAT 2	One week after of fortification
VAE	Wine before *Estufagem* process
VE	Wine after *Estufagem* process

5.2. Vinification procedure analyzed

The wines were produced at P. E. Gonçalves unit, in *Câmara de Lobos.* When the grapes arrived at this unit, they followed the normal procedures. They were inspected by a technician of IVBAM in terms of sanitary conditions and probable alco-

hol content. Then, the fermentation process begins, namely with the crushing phase. After crushing the must is collect in a tank with 500 L of capacity. After this step, the must obtained is transferred to a new tank of fermentation (P2, P3 e T13) in which the fermentation occurs. The density is an important factor for controlling the fermentation process. The proportion of ethanol to be added depends largely on the density of the wine. For a wine with a density value of 990 g / L, it is added about 10% of ethanol in relation to the total weight of grapes in the tanks. When ethanol is added, the homogenization is performed using a pump during one hour. When the fortification is completed, three analysis are carried out, namely the volatile acidity (by titration), the alcoholic content and the analysis of baumé degree (which measures the amount of residual sugar in the wine).

For different wines analyzed, P2, P3 and T13, the alcoholic degrees obtained were: 18.2%, 17.78 and 18.25%, respectively.

At the end, the decanting phase takes place. The wine is left standing during several weeks. At this stage, the wine is submitted to corrections in terms of the final alcohol content. Then, the wine is submitted to *Estufagem*, during 3 months. The recommended temperature for *Estufagem* is about 45 ºC, however, in this study these values are not always attained during the three months.

6. Chemical characterization

In this section, it will be described the analytical procedures selected to characterize the wines analyzed in this study, namely by firstly indicating the chemical used and then describing the methodology followed. It was considered the analysis of oenological parameters, the total of polyphenols, the organic acids, the screening of volatile compounds, and finally the quantification of a specific family of volatile compounds, the volatile organic acid. The latter analysis was considered after analyzed the results of volatiles compounds, as it will be explained in Results and Discussion chapter.

6.1. Routine quality control analysis

The analysis of alcohol content (v/v), density, total acidity, pH and reducing sugars (g/L) 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). The equipment was previously calibrated from reference values (values obtained from procedures suggested by ISO standards) and the following correlation coefficients (R^2) were obtained: 0.987 for alcohol; 0.999 for density; 0.982 for total acidity; 0.842 for pH and 0.998 for reducing sugars.

6.2. Total phenols (TP) procedure

6.2.1 Chemicals and reagents

The Gallic acid and Folin-Ciocalteu reagent were supplied from Fluka Biochemika AG (Buchs, Switzerland) while sodium carbonates from Panreac Química S.A. (Barcelona, Spain). All chemical have the purity higher than 98%.

6.2.2 Methodology

The samples were tested for total polyphenols (TP, expressed in gallic acid equivalents, mg/L), using the Folin-Ciocalteu's method adopted from the International Organization of Vine and Wine. Briefly, the method consider the following steps: 50 µL of wine/calibration standards were diluted in 2 mL of distilled water, 250 µL of Folin–Ciocalteu reagent and 1 mL of 20% (w/v) sodium carbonate aqueous solution, bringing the volume to 5 mL with distilled water and then the sample was mixed. Then, after 30 min, the absorbance at 750 nm was measured. The concentration of the TP was calculated according to the following gallic acid calibration curve: A_{750} = 0.001 GAE (mg/L) + 0.045 (R^2 = 0.999), set for the range of 100 to 800 mg/L. The spectrophotometric studies were performed using the equipment Shimadzu (UV/VIS spectroscopy).

6.3. Organic acid and reducing sugars methodology

6.3.1 Chemicals and reagents

The organic acids standards used were obtained from the following suppliers: *L*-malic, *L*-tartaric and succinic from Merck (Darmstadt, Germany) all with purity of 99,5%; acetic and lactic from Panreac Química S.A., with purity ≥ 85% (Barcelona, Spain) and citric from Fluka BioChemika AG, with the purity of 99,5%. Ultra-pure water was from Simplicity UV apparatus from Millipore (Milford, MA, USA) and was used for the preparation of standards and mobile phases. Sulfuric acid was supplied from Riedel-de-Haën, with the purity of 99,5%. The eluents were filtered with a membrane filters from Pall (0.20 mm, Ann Arbor, MI, USA).

6.3.2 Methodology

The analysis of organic acids was carried out using the Waters alliance liquid chromatography (Milford, MA, USA) through an auto injector (Waters 2695, separations module) and photodiode array detector. The column used is an Hi-plex H (300 x 7,7 mm; 8 µm; Agilent technologies; USA).The chromatographic separations of organic acids were carried out using an isocratic elution with the mobile phase: 0.0025M of sulphuric acid. The flow rate was set at 0.6 ml/min, the column thermostated at 65 ºC and injection volume was set to 10 µl. Samples were injected in duplicate, after being filtered through membrane filters Acrodisc® CR PTFE from Waters (0.45 µm).

The organic acids were detected at 210 nm. Chromatographic peaks were recognized by the comparison of elution order, retention times, the spectral UV–Vis with those of standards and spiking samples with pure compounds. The quantification was accomplished using a standard calibration method. The coefficient of variation (% CV) between samples replicates was in an average 0-10 %. The calibration curves range between 50-5000 mg/L for lactic acid, 100-5000 mg/L for acetic acid, malic and tartaric acids; 20 -1000 mg/L for citric and succinic acids; 100-1000 mg/L for formic acids.

6.4. Volatile compounds methodology

6.4.1. Chemicals and reagents

The following chemicals were used: 3-octanol standard from Acros Organics with the purity of 99,5% (Loughborough, UK); NaCl from Panreac Química S.A. with the purity of 99,5%.Type 1 ultra-pure water from Simplicity UV apparatus from Millipore (Milford, MA, USA) were used.

6.4.2. Methodology

7.5 µl of internal standard (IS, 3- Octanol, 250 mg/L) were added to 15 mL of each sample, before diluting 5 mL of this solution into 5 mL of ultra-pure water, in a 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.

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

The compounds were desorbed for 5 min at 240 ºC inserting the fiber into GC injection port of 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 used was a DB-WAXetr, 30 m x 0.250 mm and 0.50 µm of film thickness from Agilent (Folson, CA, USA). The carrier gas was He at 1 ml/ min. The transfer line and ion source temperatures were both retained at 230ºc. The oven temperature program starts at 40º c for 2 min the increased up to 230 ºCc at 4 ºCc/min and finally kept at 230 ºC for 15 min. The samples were analysed in a total ion count (TIC), recording the mass range 30- 400 m/z. The coefficient of variation (% CV) between samples replicates was in an average 0-20 %.

6.5. Volatile Fatty acids methodology

6.5.1. Chemicals and reagents

The following acids compounds were studied: Isobutyric, butyric, hexanoic and decanoic acid supplied from Aldrich chemistry (Steinheim, Germany); Valeric acid, isovaleric acid, Nonanoic acid and Dodecanoic acid obtained from Acros Organics (New Jersey, USA);, Octanoic acid was obtained from SAFC (St. Louis, MO, USA);).

The synthetic wine was prepared using a solution of 6 g/L of tartaric acid from Merck with the purity of 99,5% (Darmstadt, Germany); 18% ethanol from Sigma-Aldrich with the purity of 99,5% (St. Louis, MO, USA) and pH adjusted to 3,50 with NaOH 1M from Panreac Química S.A. with the purity of 98% (Barcelona, Spain) and 3-octanol standard from Acros Organics with purity of 97% (Loughborough, UK).

6.5.2. Chromatographic and apparatus conditions

The extraction conditions used to perform this analysis was the following: use DVB/CAR/PDMS fiber, 10 ml of samples in 20 ml vial and 40 min of extraction at 40ºC. In this case, the pre-incubation time and agitation were not revealed as being important for extraction.

These conditions were selected according to a Design of Experiments procedure. The presentation and discussion of this optmization procedure are outside the scope of this thesis, therefore the results regarding the optimization procedure were not presented.

In this analysis, 2 µl of standard (IS, 3- Octanol, 500 mg/L) were added to 10 mL of each sample. The HS-SPME / GC- MS systems was the same described in section 6.4.2. The column used in this case was a DB-WAX 60m x 0.250 x with 0.50 µm film Thickness from Agilent (Folson, CA, USA). The carrier gas was He at 1 mL/ min. The transfer line and ion source temperatures were both maintained at 240ºC. The oven temperature program starts at 100º C for 3 min the increased up to 240 ºc at 5 ºC/min and finally kept at 240 C for 20 min. The mass spectrometer operated in electron impact (EI) mode at 70 Ev. The standard and samples were performed in SIM (selective ion moni-

toring) mode. The characteristic ions m/z to identify and quantify each one of the VFA are described in table 2.

Table 2: Retention times (min) and quantification ions for the different volatile Fatty Acid and standard

Compounds	Retention time (min)	Q Ions (m/z)	Ion
3- Octanol (PI)	12,24	41+55+59+83+101	**59**
Isobutiric acid	16,83	41+43+73+88	**43**
Butyric acid	18,29	45+60+73	**60**
Isovaleric acid	19,24	43+60+87	**60**
Valeric acid	20.81	60+73	**60**
Hexanoic acid	23,19	60+73	**60**
Octanoic acid	27,6	60+73+101+115	**60**
Nonanoic acid	29,67	60+73+129	**60**
Decanoic acid	31,66	60+73+129	**60**
Dodecanoic acid	36,43	60+73+129+157	**60**

6.5.3. Validation

The described SPME/GC-MS methodology for determination of VFA (volatile fatty acids) in fortified wines was validated in terms of linearity, sensitivity, selectivity, precision and accuracy. The standards solutions were prepared by spiking synthetic wine at six concentration levels: 0.2; 1; 2; 8; 12 and 16 mg/L.

The calibration curves were determined by plotting the analyte peaks areas ratio (VFAs/IS area), from the six increasing standard solutions, against the corresponding VFA concentrations.

Linearity (R^2) was determined based on the linear regression results. To analyse the sensitivity, the limit of detection (LOD) and the limit of quantification (LOQ) where determined using the following equations: LOQ=3.3 α/b and LOQ= 10 α/b, being α the y-intercept standard deviation and b the slope.

The selectivity was determinate using the analysis of several fortified wines, to ensure the absence of chromatographic interferences, at the retention times of VFA.

The intra and inter-day analysis of 2 standard solutions and 1 fortified wine where used to estimate the precision. The repeatability (intra-day analysis) was verified by10 successive replicate determinations and the reproducibility of inter-day was obtained by the analyses of the same samples on 3 different days. Those two parameters were expressed as relative standard deviation (% RSD).

The accuracy of the method was determinate through a recovery study, spiking samples in triplicate, with known amounts of VFA at three representative concentration levels, within the calibration range. The recovery was obtained by comparing mean values of 3 replicates with theoretical concentration of each one. Carry over was also analysed running a blank sample after extracting the standards solutions with the highest content of VFA.

PART III –Results and Discussion

7. Results and discussion

The current study aims to analyze the vinification process of a specific producer of Madeira wine, in order to identify possible improving points in winemaking process which guarantee the high quality patterns that are expected for Madeira wines.

The study comprises the follow up of the vinification process of three different wines and then characterizes the wines in terms of key parameters and chemical compounds. The study includes the analysis of routine quality control parameters (density, alcohol content, total acidity and pH), the total polyphenolic content, the organic acid compounds, the main sugars presented in wine, the screening of volatiles compounds and the quantification of volatile fatty acids. Regarding process parameters, it was evaluated the times and temperatures of fermentation, the moment at which the fermentation is stopped and the absence of maceration.

The presentation of results is organized as follow: first, a brief reference to density, brix degree, alcohol content, total acidity and pH evaluation. Then, it is presented the results of colour and total of polyphenols evolution, followed by the presentation of the wine evolution in terms of profile organic acids, sugars and volatile compounds. To conclude, and according to the analysis of volatile profile, it was presented the results of volatile fatty acids quantification, including the results of methodology validation.

7.1. Basic parameters to assure the quality of wines

In Fig. 3 it is represented the results of temperature evolution during the vinification process, together with density, total acidity and pH results.

The values of temperature are above the desired values, namely 25-28 °C (20), which can affect the quality of the wine produced. This can lead to a considerable loss of volatile compounds, namely the acetates, ethyl esters, higher alcohols, aldehydes and ketones and originate the formation of undesirable compounds (47, 51).

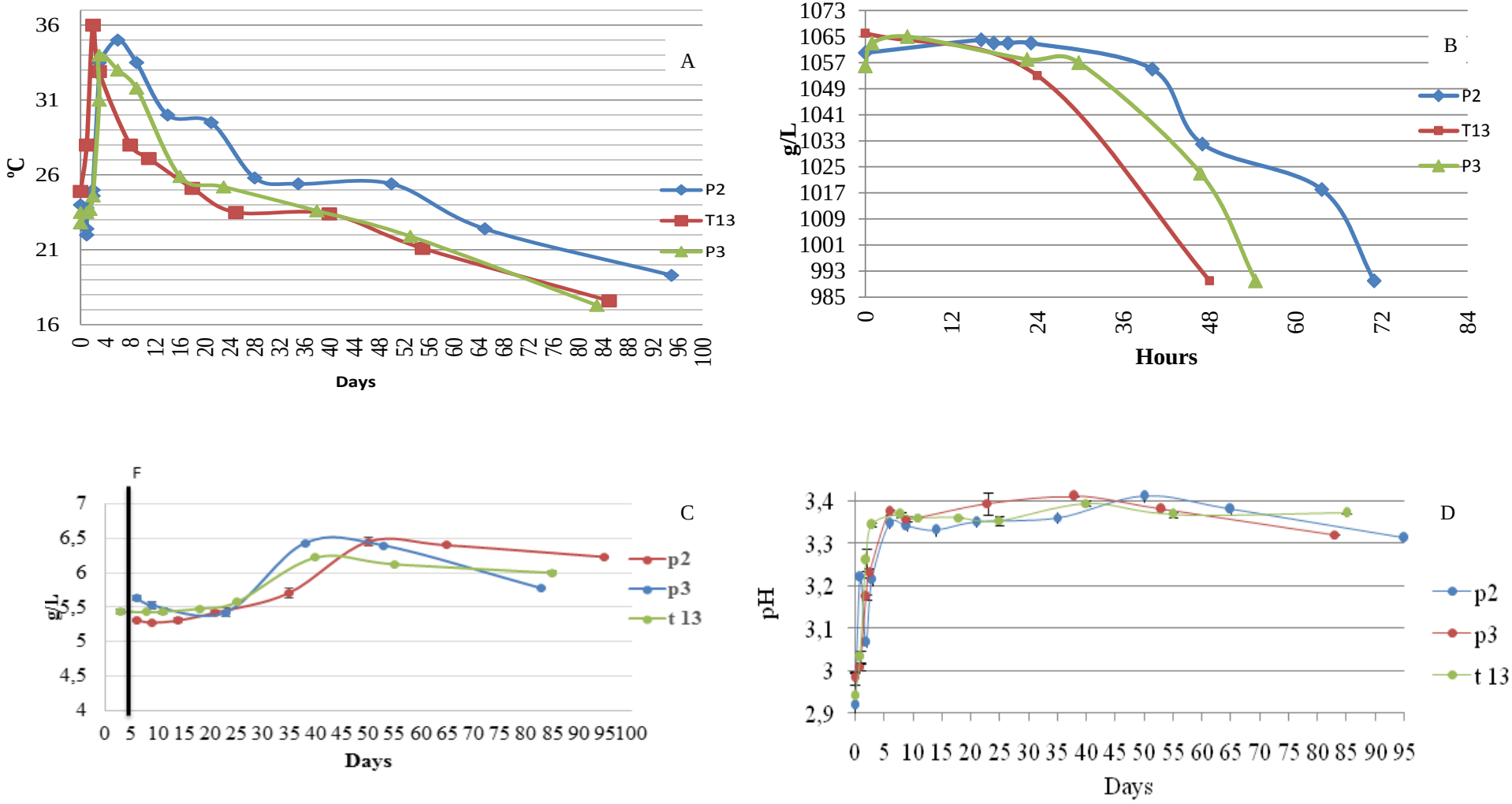

Figure 3: A- Evolution of temperature in samples; B - Evolution of density in samples C- Evolution of total acidity and D-Evolution of pH in samples.

The T13 wine is that which registers higher temperatures during the fermentation, which justify the shortest fermentation period (48h). The discussion along the following sections will consider this fact, in order to evaluate the possible impact of temperature in fermentation process. The fermentation at temperatures above 30 ° C results in alterations of volatile profile (2).

Regarding the total acidity (Fig 3-C), the values are comprised within the range of acceptable values and previously observed for Madeira wines (2). The slightly increase observed is in accordance with the trend of organic acids evolution (discussed in the following sections). In general, the concentrations of individual organic acids analyzed increases which contribute in some extend to the total wine acidity (which depends from all types of acids present in musts or wines, including organic acids and inorganic acids). In general, volatile acidity lies within the limits for a young wine (below 0.3 g/L expressed in terms of acetic acid).

The density value chosen to stop the fermentation is around 990 g/mL, that is, the value adequate to produce a dry wine. The slight increase observed in the initial density of musts produced in P2 and P3 is related with the fact of continually introducing grapes in the fermentation tanks during this period. This is due to the impossibility of performing the crushing phase at one time. Regarding the pH, it was observed a normal raising of pH, attaining values around 3.4, which are the expected values for these wines.(2)

All samples studied are classified in the category of extra dry, presenting of values degrees brix in the range of 8.5 to 9 and the correspondents values of Baumé degree is around 0 degrees. Regarding the the volatile acidity parameter, it was observed values lower than 0.3 g/L.

7.2. Residual sugar content

This section present the evolution of the main sugar compounds presented in wines, namely glucose and fructose. Simultaneously, it is present the results of the percentage of ethanol.

The values of glucose and fructose are in accordance with the expected, since they often occur in similar proportions and in general both totalize about 150 to 250 mg/L (2, 52).

The differences on the initial values of the three wines are probably related with the different locations from which the grapes were harvested or the grape maturity state. In spite of be the sugar at higher concentration, glucose is consumed faster. This fact is due to glucose be preferentially fermented by the great majority of yeasts than fructose. The absence of both these sugars after fermentation should also be discussed. Glucose and mainly the fructose are involved in many of the reactions related to Madeira wine bouquet (2), therefore proceed with the fermentation until these compounds are fully consumed can limit the appearance of those typical flavours. Generally the residual sugar content of dry wine is around 1.5 up to a maximum of 4 g/L (undetectable concentrations on the palate), therefore it is suggested that in future these minimum are verified (7, 53).

Regarding the percentage of ethanol, in all cases it was observed that a minimum of 4% were obtained from the fermentation (the limit imposed). After the fortification, the values vary from 19 up to 20%, which are in accordance with the limits established for Madeira wine.

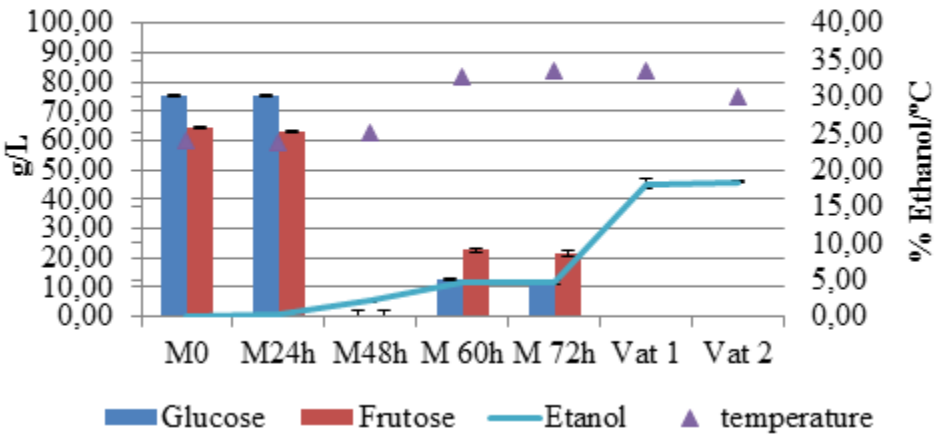

Figure 4: Evolution of residual Sugars and ethanol during the process of vinification, in the P2 sampling point.

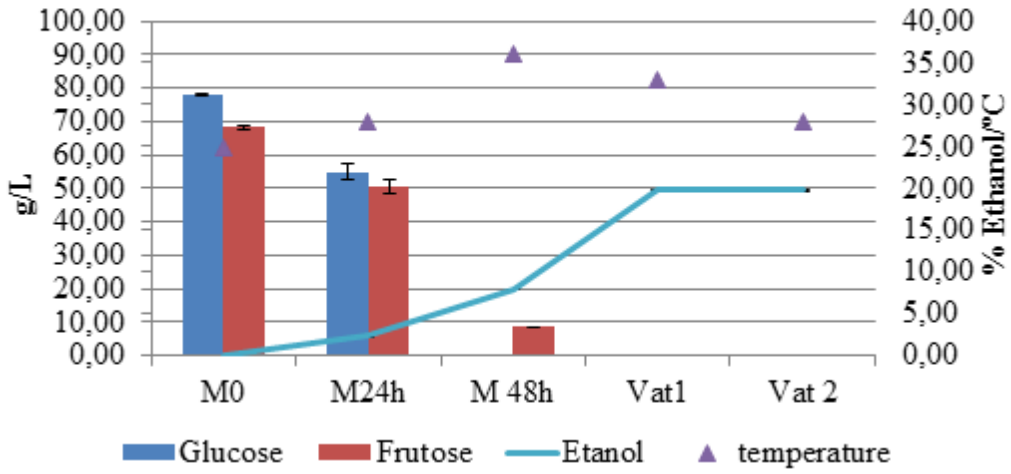

Figure 5: Evolution of residual Sugars and ethanol during the process of vinification, in the T13 sampling point.

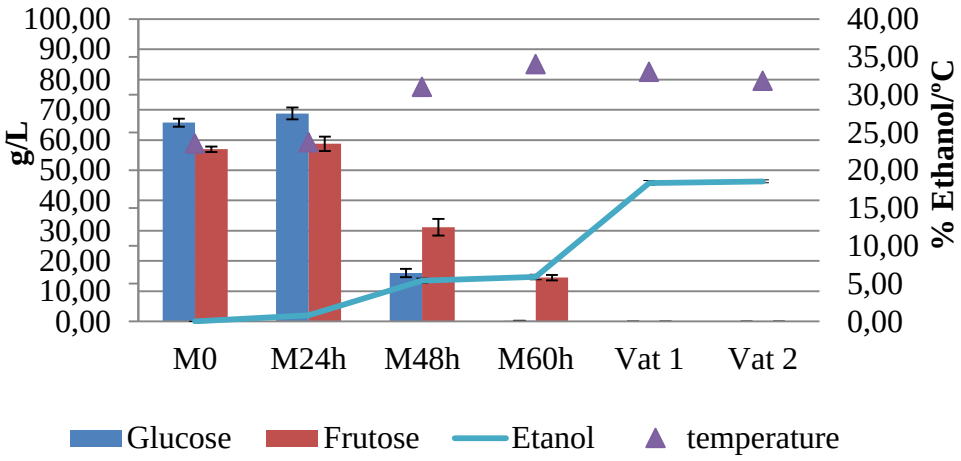

Figure 6: Evolution of residual Sugars and ethanol during the process of vinification, in the P3 sampling point.

7.3. Colour evolution using Glories Method

This section is related to the evaluation of wine colour, which is considered one of the most important organoleptic characteristics to determine wine quality. This parameter can reveal important information such as wine defects and the type and state of conservation. For the routine analysis of colour, most wineries use the method described by Glories, which measures the absorbance at three wavelengths (420, 520 and 620 nm) to determine the contribution of yellow, red and blue colours, respectively. These measurements can also be used to calculate colour intensity (CI) and hue (T) (54, 55).

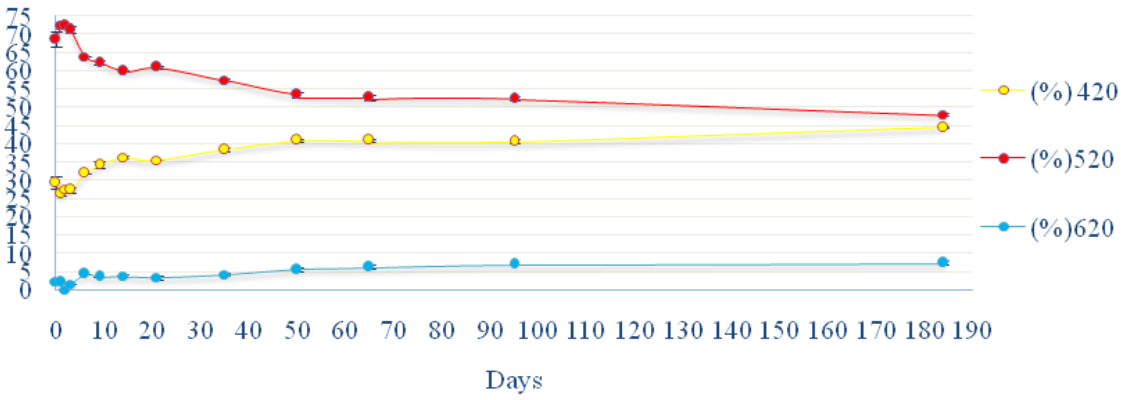

Figure 7: Evolution of glories parameters, %420. %520 and %620 of Madeira wine in the P2 sampling place

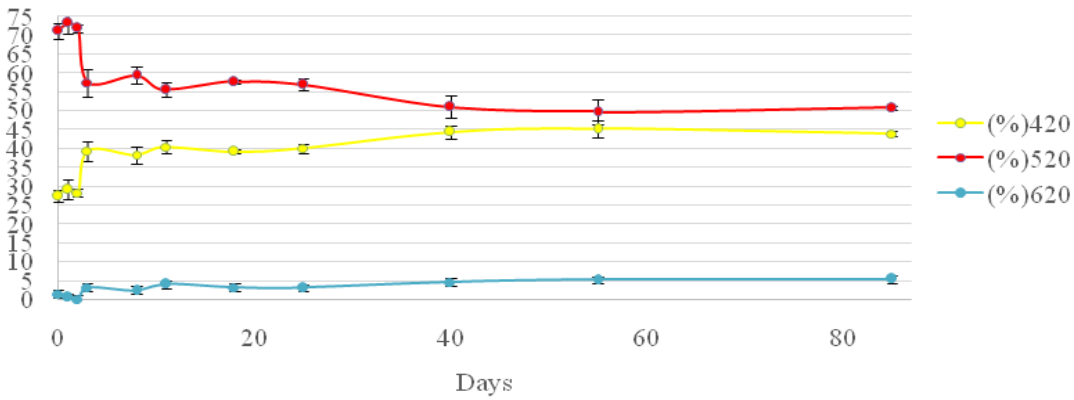

Figure 8: Evolution of glories parameters, %420. %520 and % 620 of Madeira wine in the P3 sampling place

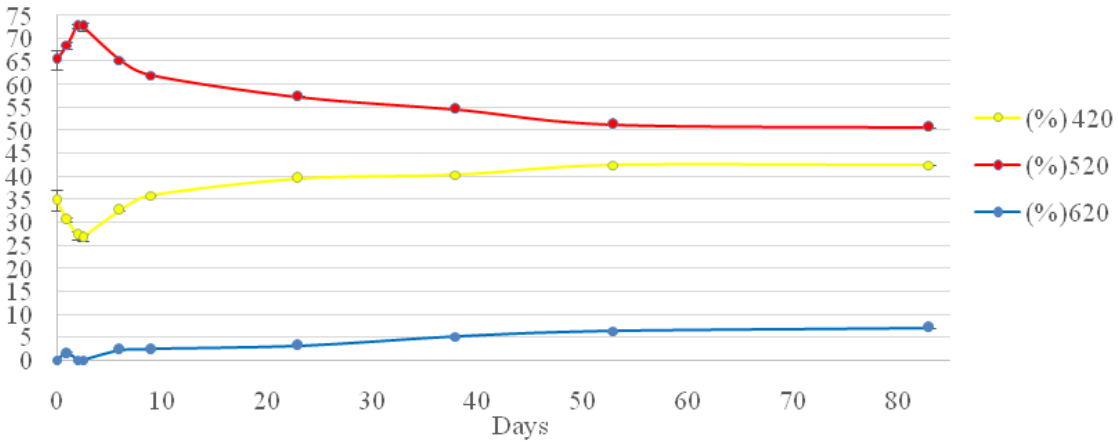

Figure 9: Evolution of glories parameters, %420. %520 and %620 of Madeira wine in the T13 sampling place

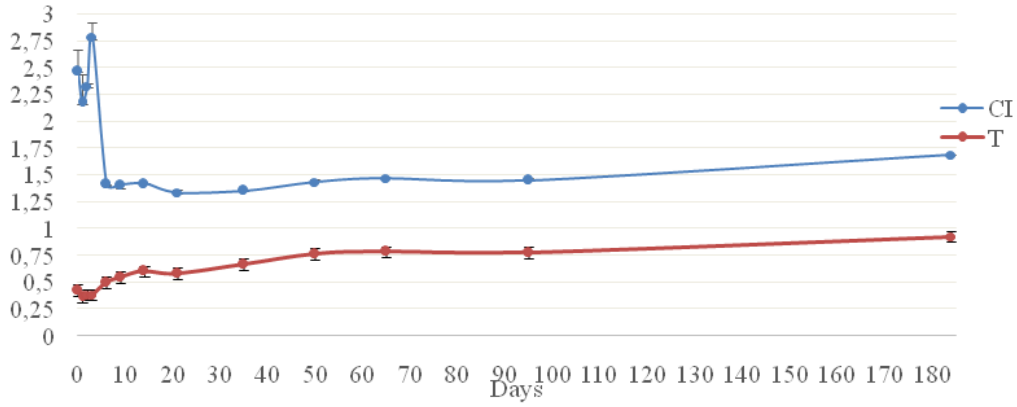

Figure 10: Evolution of glories parameters, CI and T of Madeira wine in sampling place T13

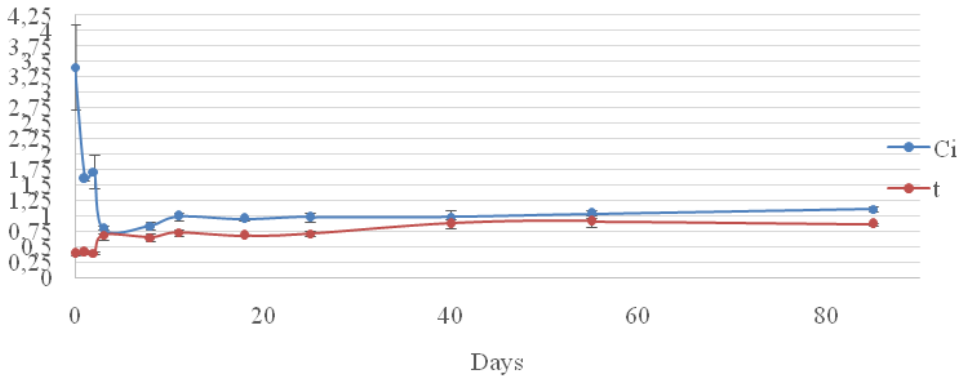

Figure 11: Evolution of glories parameters, CI and T of Madeira wine in P2 sampling place

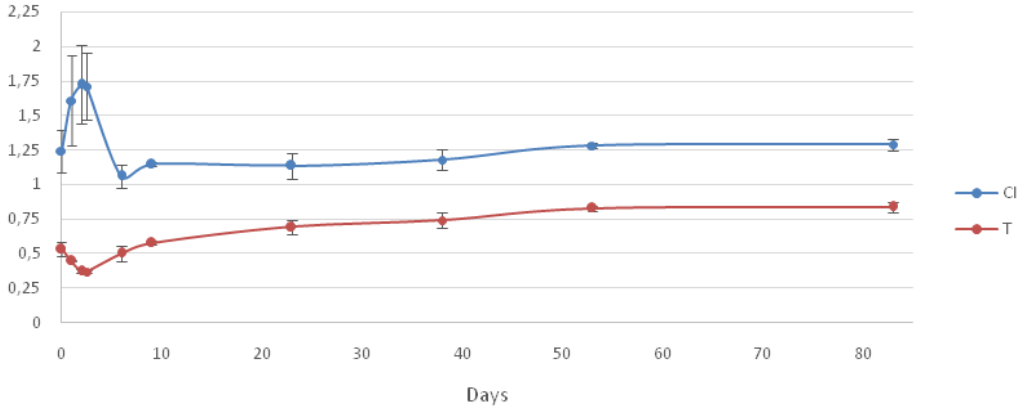

Figure 12: Evolution of glories parameters, CI and T of Madeira wine in P3 sampling place

In Fig. 7 to 9 are represented the results for colour evolution during the vinification and *Estufagem* processes of all samples (P2, P3 and T13). The results reveal that all samples, have a similar behaviour characterized by a decrease of the red colour and an increase of the yellow colour. Some authors have also described an increase of the yellow colour after the *Estufagem* process; these variations are mostly the consequence of co-pigmentation and co-polymerization reactions of anthocyanins with other phenolic compounds during the vinification process. Temperature also influences the synthesis and stability of anthocyanins, causing a decrease in their concentration at high temperatures. At the end of the *Estufagem* process, P2 demonstrated a percentage of red colour superior to the yellow colour (2, 35, 56, 57).When comparing the results of CI and T, in Fig. 10 to 12, with the values previously described by Pereira (2, 33) for the same parameters, great differences can be observed. In fact, the values described by this author

for CI and T are nearly twice higher than the values obtained in all three samples studied.

7.4. TP profile

Normally, during the wine ageing process the total content phenolic compounds (TP) tends to decrease. The results obtained for this parameter, shown in Figure 13 are consistent with those described by Pereira (33), who reported that the process of aging affected the total content of phenols, due to hydrolysis, oxidation and complexation reactions. These compounds can also be degraded and polymerized throughout enzymatic and non-enzymatic reactions during this period (58, 59).

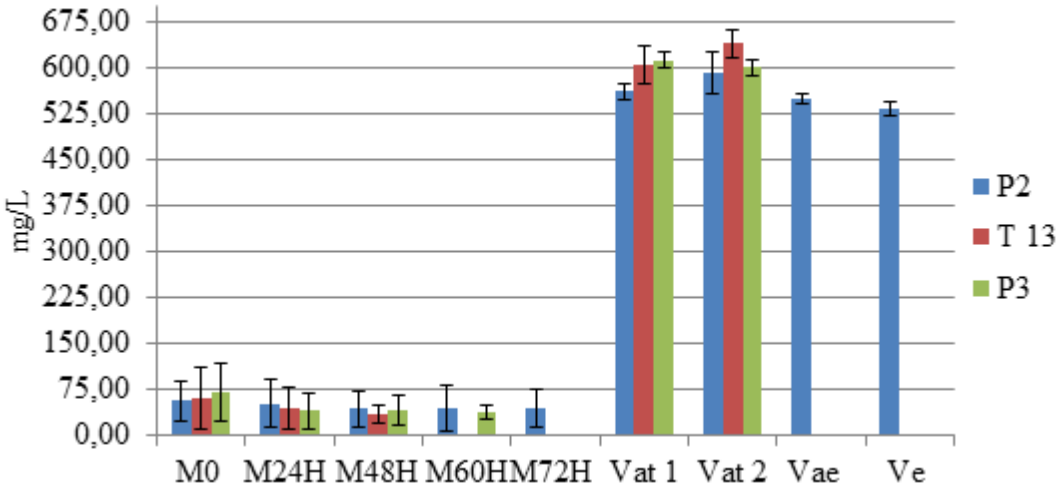

Figure 13: Total polyphenols (TP) content over the process in the various sampling points

During the fermentation period, in all P2 wines from M0 to M72H, the TP concentrations determined were around 56.472 to 44.061 mg (GAE)/L. This represents a decrease with time of fermentation, which is then followed by an increased after the first fortification. In T13 wines, from M0 to M48H, the values for TP vary from

60.572 to 34.911 mg (GAE)/L and in P3 wines, from M0 to M60H, the values for TP decrease from 69.103 to 36.667 mg (GAE)/L (Fig.13).

Still during the vinification process, TP concentrations are found to vary from 591.463 at Vat 1 to 533.315 mg (GAE)/L at Ve, in P2. In T13 wines, the concentrations of TP decreased from 639.167 to 603.759 mg (GAE)/L and in P3 wines from 612.315 to 599.648 mg (GAE)/L, both in the first two fortifications (Vat 1 and Vat 2) respectively (Fig.13). Several factors can influence TP, such as environmental factors, wine processing techniques and vinification and *Estufagem* conditions (58, 59).

7.5. Organic acids evolution

The concentration of organic acids is important in wines. Some of these compounds are present in the grapes, such as malic, citric and tartaric acids. Others like lactic, acetic and succinic acids have two possible formation pathways, either from fermentation or from bacterial activity. These compounds affect the stability, the colour and the flavour of wines (12).

In figure 17, it is presented a typical chromatogram obtained for the M0 samples according to methodology described in section 6.3. The results of organic acid evolution during the vinification process were then analyzed and the results presented is figure 14, 15 and 16. Also, it should be noted that the concentration of tartaric acid is not considered in the discussion, because this analysis was not possible to be performed before samples be stored at -26ºC.

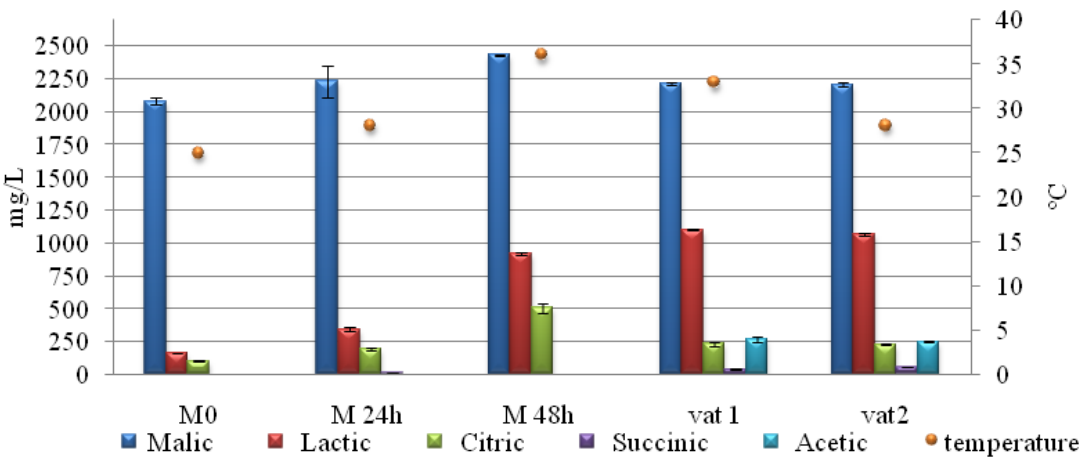

Figure 14: Organic acids during the vinification process in T13 sampling place.

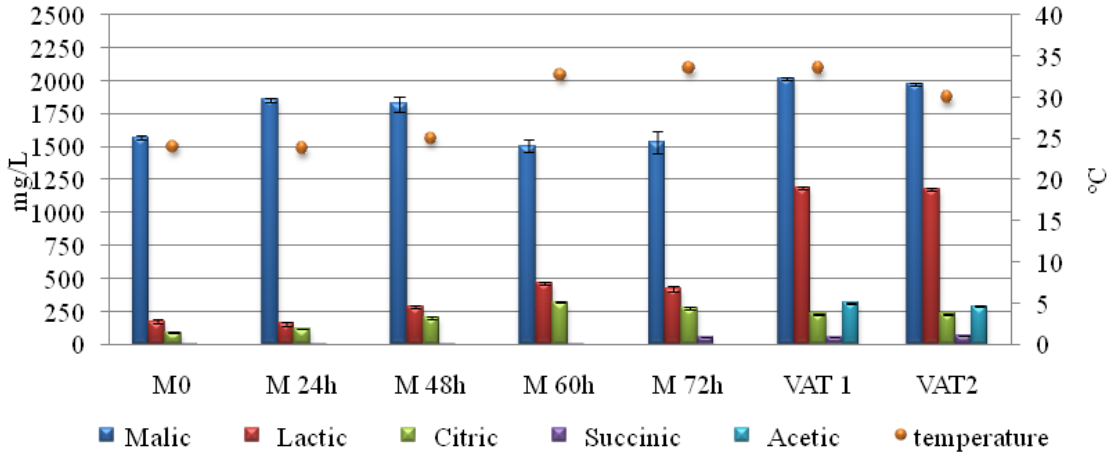

Figure 15: Organic acids during the vinification process in P2 sampling place.

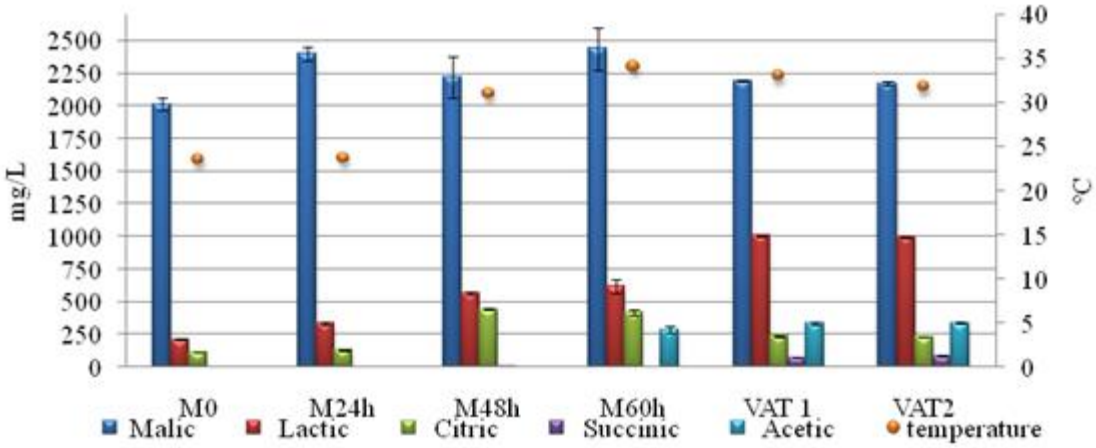

Figure 16: Organic acids during the vinification process in P3 sampling place.

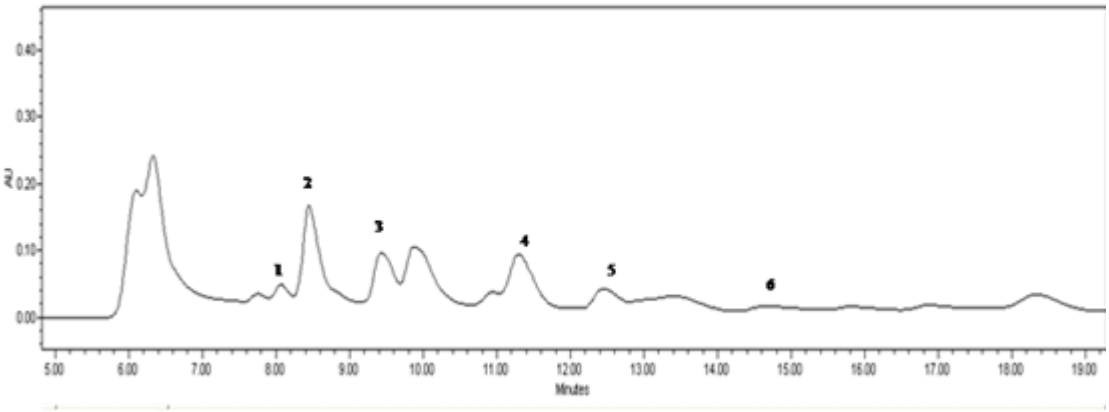

Figure 17: Typical chromatogram of Madeira wines produced from TN during the fermentation.

Peak identification: (1) citric acid, :(2) tartaric acid, :(3) Malic acid, :(4)Succinic acid, :(5) lactic acid, :(6)acetic acid.

Regarding the concentration of malic acid, P2 was the must with lower value. The differences between the three musts can be related with the different localizations where the grapes grown, since its presence in must is essentially due to their presence in grapes (16-18). The concentration founded, in must and wines, varies from 1750 mg/L up to 2400mg/L. At the end of vinification process, the values range from 2000 uo to 2200mg/L, values that are in according with previous values reported for Madeira wines (0.33-1.12g/L) and even Port wines (0.82-2.75 g/L) (2).

Malolactic fermentation is not encouraged in Madeira wine vinification process, however it is usually to find significant amounts of lactic acid in Madeira wines. When the concentration of lactic acid varies between 800 to 2600 mg/L, it is usually related to the conversion of malic acid into lactic acid through the process of malolactic fermentation (9, 18, 69, 70). In the wines analyzed, the concentration of latic acid increase up to 1100 mg/L, suggestion that this secondary fermentation could have occurred. This evolution was not accompanied by a decrease of malic since due to fermentation tank be refilled with fresh must.

Succinic acid is essentially formed during alcoholic fermentation by the action of the yeasts. Indeed, the values observed increase by reaching low values compared with those reported in literature, around values of 2 g/L.

Citric acid is also presented at small concentration, lower that the values described in literatures that normally is values around 0.5 to 1 g/L in wines (15, 16). In this case, the type of grape variety can be associated to the values observed.

Comparing the results discussed above with those reported by Pereira (60) for aged Madeira wines, it was verified that the values of lactic, malic, acetic, succinic and citric are half of the values presented in her article and thesis.

During the vinification process it was observed that acetic acid concentration increase in the three wines analyzed, but attaining small concentrations (a maximum of 250 mg/L). These values are in accordance in the lower volatiles acidities observed during the fermentation. Comparing these results with those obtained by Miranda (15), it is clear that the values founded in the wines study will not compromise the wine quality in terms of total acidity.

7.6. Volatile profile of wines

This section presents the results of the volatile profile of the three dry wines studied. It was identified about fifty volatiles compounds, which are then organized in six chemical families to facilitate the data analysis. It was considered the following families: esters, higher alcohols, fatty acids, terpenes, carbonyl compounds and a small group comprising volatiles compounds which do not belong to one of the previous families. An example of a chromatogram is presented in Figure 21.

In figures 18-20 it is present an estimate of the concentration of the different chemical family analyzed during the vinifcation process, which correspond to the sum of the all volatiles classified as belonging to those families. The amount of each volatile compound was estimated regarding the added amount of IS. As can be observed in figures 18-20, the three wines have different profiles in terms of volatile composition, which can be related with the different temperatures and times of fermentation. Esters are the chemical family presented in higher concentration. These compounds are secondary aromas and usually contribute with positive aromas, essentially with fruity notes. Higher alcohols are the second family in terms of intensity. These are also secondary aromas and in general are produced during the fermentation. Fatty acid represents also a significant portion of the volatile profile analyzed. The high concentrations that some compounds of this family appear in this analysis, namely the hexanoic and octanoic acid, lead us to develop a methodology to quantify this volatile family in a more precisely manner.

Regarding the P2 wine it seems to be an increased trend in terms of the concentration of all families of volatiles compounds until the end of fermentation. The same behaviour was not observed for the remaining wines. Differences were also evident after the fortification step. While T13 presents a significant increase in terms of all volatile families, P2 shows an opposite behaviour.

At this phase, it can be suggested that fermentation temperatures should be maintained at lower values, since at lower temperatures the esters family grows up to values higher as described by literature (44).

The higher alcohol found was hexan-1-ol that contributed with “herbaceous” and “vegetal” odour. The average concentration of hexan-1-ol in TN wines studied (0.1±0.01 mg/L), is lower than the perception threshold thus its sensorial contribution is expected. Regarding esters, the ethyl esters of C6 and C8 fatty acids were those found in higher concentrations, which are responsible for the “fruity” and “wine-like” aroma. The average concentration of hexanoate in TN wines studied (0.25±0.02 mg/L) and the average concentration octanoate in TN wines studied (1.17±0.11 mg/L).

These compounds make a positive contribution to the general quality of wine being responsible for their “fruity” and “floral” sensory properties.

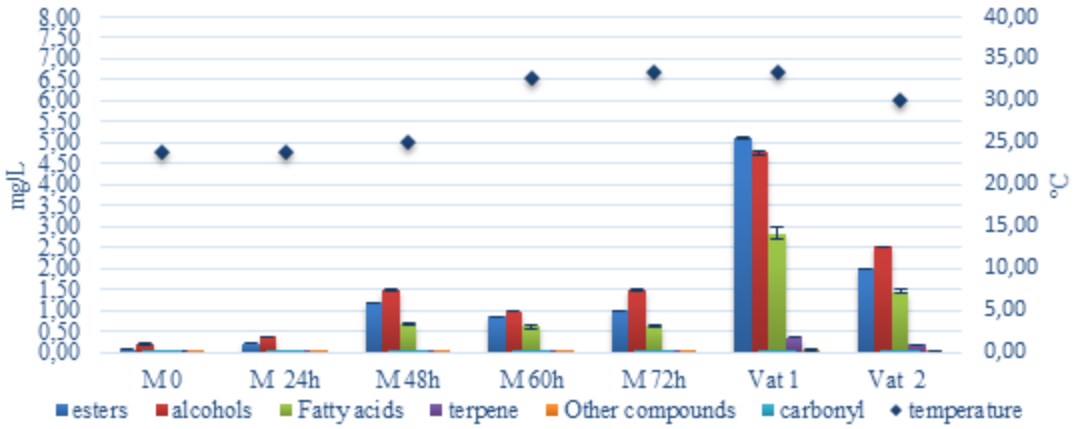

Figure 18: Volatile profile evaluation of wine samples in sampling place P2

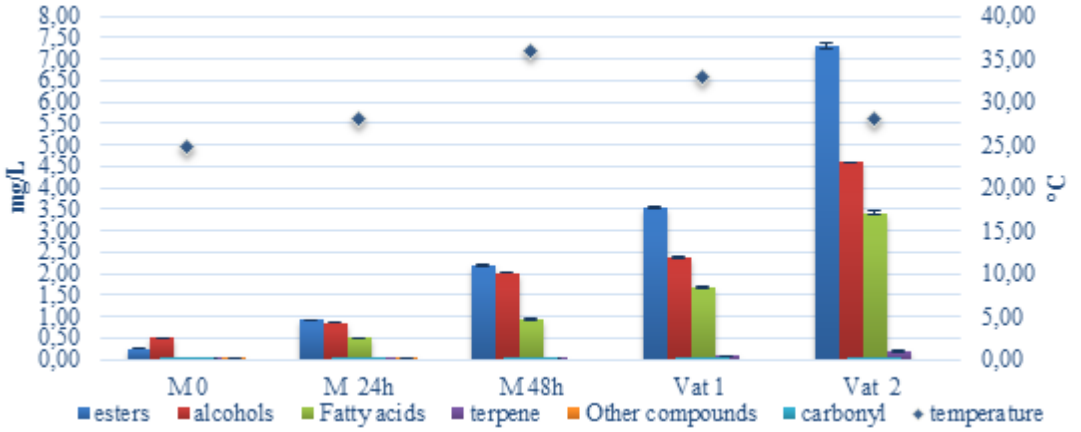

Figure 19: Volatile profile evolution of wine samples in sampling place t13

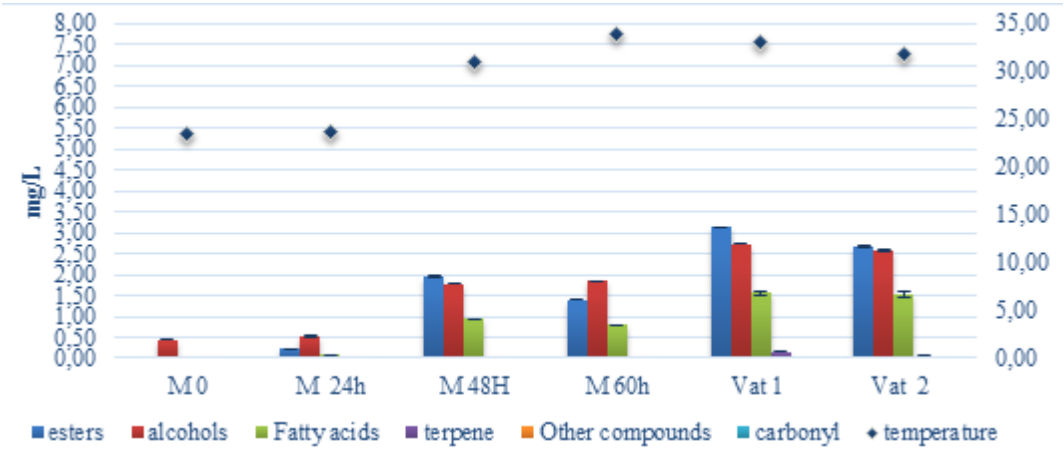

Figure 20: Volatile profile evolution of wine samples in sampling place P3

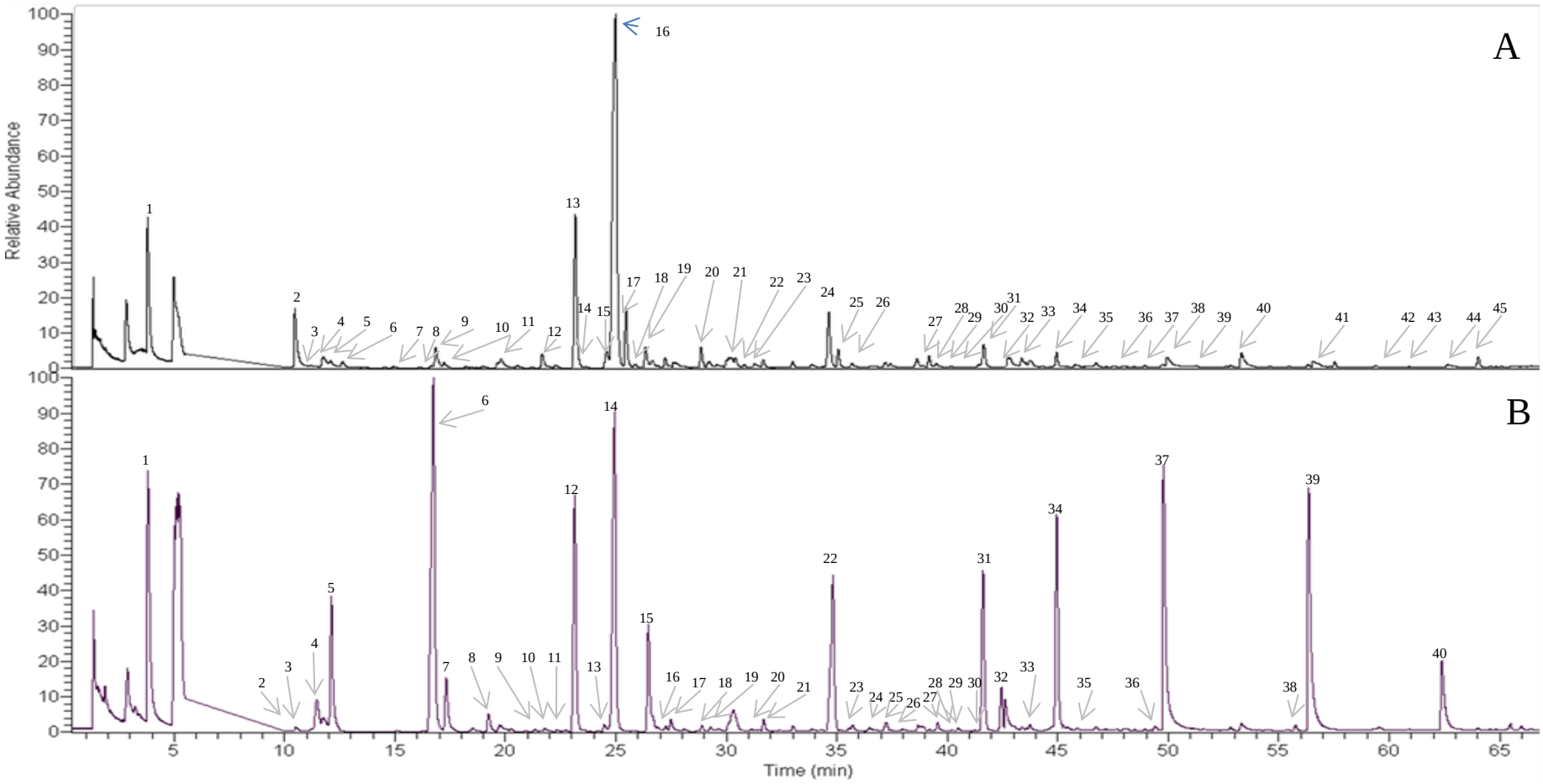

Figure 21: Typical chromatogram of the volatile profile of must at two different stages of fermentation process (M0 and M60).

A- (1) ethyl acetate; (2) hexanal;(3) geranic oxide;(4) 2-methyl-1- propanol ;(5)3-methyl-1- butanol acetate;(6) 1-chloro heptane;(7) 1 butanol;(8)1.2-dimethyl -benzene heptanal;(9) e- 2- hex-enal;(10) 1chloro- octane;(11) 3-octanone;(12) hexyl ester acetic acid; (13) 1-octen-3-one;(14) 1-hexanol;(15) 3-hexen-1-ol;(16) 3-octanol;(17) 2-hexen-1-ol; (18) 2-octenal; (19) e 1-octen-3)-ol;(20) 2-ethyl 1- hexanol ;(21) decanal ;(22) benzaldehyde;(23) linalool;(24) 1- octanol ;(25) 4-(1,1-dimethylethyl –cyclohexanone;(26) (e)-2-decenal;(27) 1-nonanol;(28) 2-undecenal;(29) 1-decanol;(30) 2-hidroxy-benzoic acid;(31) methyl ester;(32) 2,4-decadienal ; (33) α-Damascenone; (34) hexanoic acid; (35) pentyl ester benzenethanol (36) 1-dodecanol (37) isopropyl Myristate (38)octanoic acid; (39) nonoic acid; (40) 2-(dodecyloxy) ethanol ;(41) decanoic cid ; (42) diethyl Phthalate ;(43) 1-hexadecanol ; (44) butil ester hexadecanoic acid; (45) dodecanoic acid. B - (1 ethyl acetate;(2) hexanal; (3) geranic oxide; (4) 2-methyl-1-propanol; (5) 3-methyl-1-butanol acetate; (6) iso amyl alcohol; (7) ethyl ester hexanoic acid; (8) hexyl ester acetic acid; (9) 2-heptanol (10) 6- methyl -5-hepten- 2- one;(11) ethyl ester 2-hydroxy propanoic acid; (12) 1-hexanol; (13) 3-hexen-1-ol;(14) 3-octanol;(15) ethyl ester octanoic acid;(16) 1-octen-3-ol;(17) acetic acid ; (18) 2-ethyl 1- hexanal ;(19)Decanal;(20) benzaldehyde;(21) 1-octanol;(22)ethyl ester decanoic acid;(23)3-methylbutil ester octanoic acid;(24) 1- nonanol;(25) 3-(methythio)-1-Propanol;(26)1-decano ;(27) á-citronellol;(28) 2-hidroxy-benzoic acid methyl ester;(29)ethyl ester benzene acetic acid;(30) 2-phenylethyl ester acetic acid;(31)ethyl ester dodecanoic acid;(32) hexanoic acid;(33) ben-zenethanol;(34) 1-dodecanol;(35) nerolidol;(36) ethyl ester tetradecanoic acid;(37) octanoic acid;(38) ethyl ester hexadecanoic acid;(39)decano ic acid ;(40) dodecanoic acid

In figure 22, it is presented the volatile profile for P2 wine before and after *Estufagem* process. No significant differences was observed in any of volatile families analyzed. This is not in accordance with the expected evolution for *Estufagem* process. One of the reasons that probably contribute for this fact is related with *Estufagem* temperatures, which do not reached the 45ºC during the three months. The second reason can be related to absense of sugars (frutose and glucose) in wines, whcih are the main substracts for the chemical reactions observed during the *Estufagem* ageing process (Maillard reaction and sugar degradation in acid medium). Also, the suggestion previously presented, the 4 months of *Estufagem* instead of the 3 months can be a good option for the process under studied.

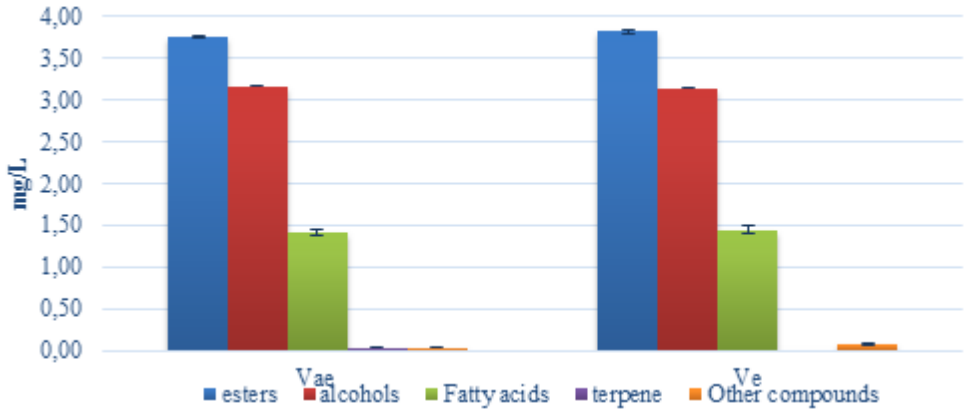

Figure 22: Volatile profile comparison of wine samples, before and after *Estufagem* process, in P2 sampling place.

7.7. Fatty acids Evaluation

This methodology was created, due the fact these compounds (fatty acids), they influence the bouquet of aromas produced in the Madeira wine. Outside of limited threshold, they have a negative influence for wine. This method is more specific for this kind of compounds, since they affect the general aroma of the wine. Juan (61) studied various grape varieties of Madeira wine and he makes a hierarchical list and in that list he ranked the compounds that influence the aroma of Madeira wine using a GC-O methodology. And at least 2 of the analysed compounds are in that rank and with values around 60 to 84 % identified by butyric acid and Isobutiric acid. Therefore

this method is specific to fatty acids a family, since these compounds affect the complexity of the wine, and they are important to form corresponds ester.

7.7.1. Method validation

Figure 23 represents a typical chromatogram of a fortified wine sample, analysed by the present method. It shows that the application of the developed SPME/GC-MS methodology, to measure VFA in fortified wines, had good selectivity, since peaks had good resolution. Selectivity was confirmed by de absence of interferences in VFA retention times in the selected samples.

A good correlation coefficient (R2 =0.995-0.999) was verified for all compounds, confirming the linearity of the method (table 3).

The sensitivity of the methodology was evaluated using the LOD and LOQ determinations. The LOD and LOQ were low (0.026-0.068mg/L; 0.080-0.206 mg/L), being close or even lower to those found in literature (31, 62-64), conferring to the methodology a great sensitivity (Table 3).

The precision (repeatability and reproducibility) was analysed by the variation of intra- and inter-day repetition of the method. The methodology revealed a good repeatability (4.8-8.4%) and reproducibility (5.3-8.5%) (Table 3).

The method developed and optimized for the determination of VFA by SPME extraction with GC–MS detection revealed to be fast, simple and sensitive.

The applicability of the methodology was verified by the determination of VFAs in 11 fortified wines samples. The results are presented in Table 4 and the concentrations obtained proved that the calibrations cover the range of interest.

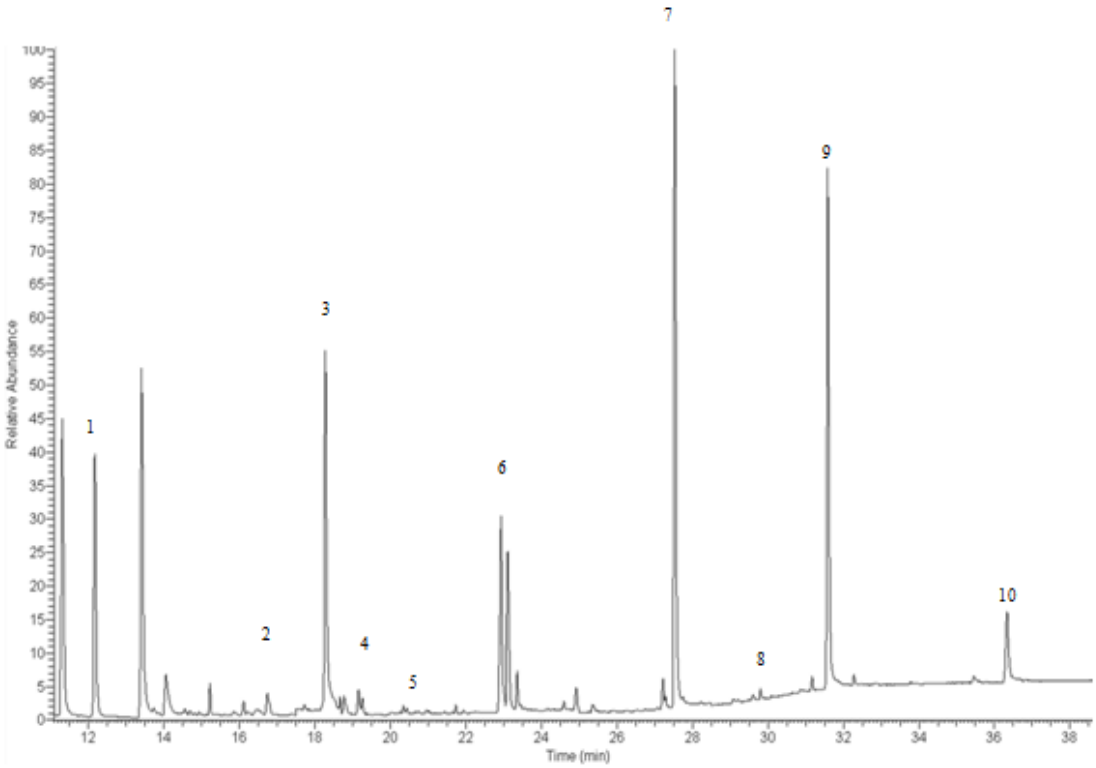

Figure 23: Typical chromatograms of TN sample. For peak identification:1- 3-octanol;2-isobutiric acid; 3-butyric acid, 4-isovaleric acid, 5 valeric acid, 6-hexanoic acid,7- octanoic acid, 8-nonanoic acid, 9- decanoic acid and 10- Dodecanoic acid.

Table 3: Retention times, identification and quantification ions, linearity range and other validation parameters of volatile organic acids methodology proposed (ions in bold were used for the quantification)

Compounds	tR (min)	Identification ions	Linear range	Slope	Intercept	R^2	LOD	LOQ	Recovery (%)	Repeatability (highest value)	Reproducibility (highest value)
Dodecanoic acid	36.43	**60**+73+129+157	0.206-20.552	2.516	-0.328	0.995	0.067	0.202	119.46 ± 3.43	6.6%	7.3%
Decanoic acid	31.66	**60**+73+129	0.200-16.080	2.280	-0.345	0.999	0.067	0.202	105.85 ± 5.97	6.2%	7.7%
Nonanoic acid	29.67	**60**+73+129	0.201-16.083	2.197	-0.337	0.999	0.053	0.160	105.61 ± 3.35	4.8%	6.3%
Octanoic acid	27.6	**60**+73+101+115	0.209-16.730	1.907	-0.202	0.999	0.052	0.157	108.48 ± 2.19	4.8%	5.4%
Hexanoic acid	23.19	**60**+73	0.204-12.264	1.319	-0.227	0.999	0.044	0.132	105.25 ± 2.01	5.6%	5.3%
Valeric acid	20.81	**60**+73	0.202-12.110	0.652	-0.051	0.999	0.026	0.080	116.13 ± 1.71	6.0%	7.6%
Isovaleric acid	19.24	43+**60**+87	0.214-17.146	0.534	-0.007	0.999	0.068	0.206	112.20 ± 7.54	5.4%	8.5%
Butyric acid	18.29	45+**60**+73	0.208-12.480	0.270	-0.007	0.999	0.052	0.156	106.32 ± 16.03	8.4%	5.6%
Isobutiric acid	16.83	41+**43**+73+88	0.203-16.269	0.024	0.187	0.999	0.053	0.162	103.11 ± 5.96	7.6%	7.8%

Table 4: Volatile fatty acids of different fortified wines studied (average ± standard deviation), Ions in bold above the threshold

Compounds	**mg/L**										
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
Dodecanoic acid	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Decanoic acid	0.214±0.005	0.232±0.001	0.31±0.043	0.217±0.001	0.221±0.014	0.223±0.001	0.248±0.008	0.241±0.013	n.q.	n.q.	0.338±0.010
Nonanoic acid	0.171±0.001	0.175±0.008	0.18±0.007	0.172±0.003	0.175±0.014	0.172±0.002	0.168±0.005	0.172±0.007	0.167±0.005	0.162±0.002	0.17±0.009
Octanoic acid	**0.775±0.001**	**0.737±0.009**	**0.540±0.036**	**0.697±0.014**	**1,020±0.144**	**0.632±0.005**	**0.658±0.033**	**0.651±0.054**	0.369±0.019	0.342±0.010	**1,121±0.058**
Hexanoic acid	**1,207±0.040**	**0.960±0.039**	**0.920±0.007**	**0.986±0.024**	**1,116±0.109**	**0.879±0.028**	**0.872±0.010**	**0.692±0.054**	**0.795±0.042**	**0.522±0.014**	**0.783±0.035**
Valeric acid	0.153±0.050	0.142±0.025	0.144±0.004	0.134±0.002	0.099±0.002	0.107±0.001	0.106±0.001	0.098±0.003	0.085±0.001	0.095±0.001	0.086±0.001
Isovaleric acid	2,315±0.001	1,951±0.001	1,697±0.086	1,873±0.018	1,477±0.100	1,233±0.044	0.358±0.008	0.469±0.016	0.623±0.038	0.574±0.014	0.989±0.062
Butyric acid	**4,582±0.039**	**3,861±0.354**	**3,357±0.169**	**3,708±0.036**	2,923±0.198	2,441±0.087	0.708±0.016	0.927±0.031	1,232±0.075	1,136±0.028	1,957±0.123
Isobutiric acid	**13,861±0.287**	**4,822±0.078**	**5,195±0.035**	**7,965±0.404**	**2,032±0.067**	**1,972±0.017**	0.020±0.005	0.479±0.019	4,311±0.382	1,144±0.052	3,851±0.306

7.7.2. SAMPLE ANALYSIS

The results obtained for the previously described sampling during fermentation and *Estufagem* of *TN* wines are presented on table 5 and describe the evolution of VFA formed during these periods. Of the nine VFA quantified, five of them, namely decanoic, octanoic, hexanoic, isovaleric and butyric acids, are above the limit of odour threshold in some points (6161, 63, 65, 66). These five compounds contribute with unpleasent notes such as vinegar, fatty, animal, spicy, cheese, rancid, pungent, green and butter (Table 5), altering the typical wine aroma. Comparing the results presented in Table 5 with those obtained by Perestrelo (2006), it is noticeable that the concentration of butyric acid is above the concentrations described by this author. The concentrations of decanoic, octanoic, hexanoic and isovaleric acids, on the other hand, are lower when compared to those by Perestrelo (2006).

Comparing the results presented in Table 5 with those obtained by Cacho (2006), it is noticeable that the concentrations of isovaleric, isobutiric and butyric acids are lower the concentrations described by this author.

All fermentation places (P2, P3 and T13) demonstrate an increase of these compounds during the fermentation process. These results can be justified by the effect of higher temperatures (higher than 30ºC) as described in Figure 3-B in the chapter 7.1.

Table 5: Volatile fatty acids of 3 different lots studied during the vinification process (average ± standard deviation)

Compounds	Oth (mg/L)	P2 (mg/L)									P3 (mg/L)						T13 (mg/L)					Common descriptors [a]
		M0	**M24**	**M48**	**M60**	**M72**	**VAT 1**	**VAT 2**	**VAE 1**	**VE**	**M0**	**M24**	**M48**	**M60**	**VAT 1**	**VAT 2**	**M0**	**M24**	**M48**	**VAT 1**	**VAT 2**	
Dodecanoic acid	6,1[1]	n.q.	n.q.	0.253 ±0.021	0.269 ±0.009	0.255 ±0.006	0.398 ±0.095	0.241 ±0.031	0.207 ±0.011	0.218 ±0.020	n.q	n.q	0.218 ±0.006	0.226 ±0.023	0.205 ±0.006	0.207 ±0.006	n.q	0.234 ±0.013	0.302 ±0.037	0.236 ±0.015	0.230 ±0.011	Dry, metallic, laurel oil flavour
Decanoic acid	1,0[1]	n.q.	n.q.	0.881 ±0.067	**1.183 ±0.106**	**1.042 ±0.131**	**1.133 ±0.145**	**1.201 ±0.102**	**1.042 ±0.114**	0.966 ±0.143	0.207 ±0.015	0.269 ±0.020	0.939 ±0.041	**1.076 ±0.171**	0.779 ±0.057	0.976 ±0.067	n.q	0.779 ±0.101	**1.197 ±0.148**	**0.961 ±0.049**	**1.215 ±0.090**	vinegar, animal and fatty
Nonanoic acid	3[1]	0.166±0.005	0.170 ±0.001	0.166 ±0.002	0.171 ±0.005	0.169 ±0.011	0.200 ±0.023	0.173 ±0.005	0.170 ±0.011	0.171 ±0.011	0.167 ±0.005	0.168 ±0.006	0.171 ±0.007	0.171 ±0.007	0.171 ±0.006	0.170 ±0.002	0.184 ±0.005	0.165 ±0.002	0.166 ±0.003	0.175 ±0.003	0.178 ±0.015	Green
Octanoic acid	0.5[1]	0.146±0.008	0.159 ±0.005	**1.339 ±0.200**	**2.307 ±0.152**	**1.870 ±0.257**	**1.591 ±0.204**	**1.920 ±0.175**	**1.758 ±0.237**	**1.657 ±0.129**	0.192 ±0.010	0.279 ±0.017	**1.987 ±0.244**	**2.329 ±0.302**	**2.026 ±0.217**	**2.242 ±0.187**	0.153 ±0.009	**1.231 ±0.116**	**2.340 ±0.239**	**2.070 ±0.190**	**2.359 ±0.182**	animal, spicy and cheese
Hexanoic acid	0.42[1]	0.379±0.050	**0.504 ±0.039**	**0.972 ±0.139**	**1.207 ±0.071**	**0.906 ±0.035**	**0.859 ±0.067**	**0.907 ±0.056**	**0.842 ±0.081**	**0.837 ±0.022**	**0.577 ±0.062**	0.374 ±0.015	**1.028 ±0.135**	**1.296 ±0.142**	**1.107 ±0.097**	**1.146 ±0.073**	**0.533 ±0.036**	**0.830 ±0.101**	**1.460 ±0.135**	**1.167 ±0.105**	**1.181 ±0.049**	rancid, purgent and green
Valeric acid		0.109±0.009	0.122 ±0.004	0.101 ±0.006	0.099 ±0.004	0.091 ±0.002	0.110 ±0.013	0.088 ±0.003	0.082 ±0.001	0.087 ±0.004	0.135 ±0.009	0.091 ±0.001	0.087 ±0.002	0.086 ±0.002	0.089 ±0.003	0.085 ±0.002	0.139 ±0.012	0.095 ±0.002	0.098 ±0.116	0.090 ±0.003	0.087 ±0.002	Buttery
Isovaleric acid	0.003[1]	n.q.	n.q.	**0.216 ±0.022**	**0.355 ±0.023**	**0.287 ±0.004**	**0.515 ±0.145**	**0.440 ±0.029**	**0.412 ±0.045**	**0.377 ±0.019**	**0.406 ±0.048**	**0.264 ±0.026**	**0.470 ±0.086**	**0.670 ±0.051**	**0.595 ±0.040**	**0.627 ±0.025**	**0.139 ±0.013**	**0.246 ±0.029**	**0.410 ±0.054**	**0.398 ±0.037**	**0.395 ±0.013**	Sweaty, Cheese
Butyric acid	0.173[1]	n.q.	**0.359 ±0.039**	**0.427 ±0.043**	**0.702 ±0.046**	**0.567 ±0.009**	**0.977 ±0.215**	**0.869 ±0.058**	**0.815 ±0.089**	**0.745 ±0.038**	**0.803 ±0.096**	**0.521 ±0.050**	**0.866 ±0.138**	**1.326 ±0.101**	**1.191 ±0.070**	**1.239 ±0.049**	**0.275 ±0.027**	**0.485 ±0.058**	**0.771 ±0.128**	**0.788 ±0.072**	**0.781 ±0.024**	rancid, butter, cheese
Isobutiric acid	2,3[1]	0.285±0.0378	0.320 ±0.039	0.679 ±0.053	1.057 ±0.069	0.920 ±0.060	1.567 ±0.375	1..169±0.112	1.426 ±0.144	1.783 ±0.109	0.412 ±0.050	0.457 ±0.045	0.971 ±0.123	1.530 ±0.128	1.204 ±0.081	1.192 ±0.048	0.178 ±0.021	0.570 ±0.067	1.275 ±0.109	1.242 ±0.219	1.211 ±0.057	Sweaty, Pungent, Sour, Cheesy, Beefy

[1] According to FC 196(16) 682–693 and JSFA 80(00)1659-1667

PART IV – Conclusions

8. CONCLUSIONS

According to the results previously presented, there are some parameters related with the control of vinifcation process that should be highlighted: the temperature of fermentation and the moment at which fermentation is interrupted.

Notwithstanding the wines were produced following the same principles, significant differences were observed, which indicates the needs for more strictly procedures to control the parameters of the process.

The results show the importance of control these factors in wine composition, namely: in the composition of volatile profile, organic acids, sugar consumption, colour evolution and content of VFAs.

In the particular case of VFA, it was found that five of the nine compounds were above the limit of odour threshold. These five compounds are: decanoic; octanoic; hexanoic, isovaleric, butyric acids. These five compounds contribute with unpleasant notes such as vinegar, fatty, animal, spicy, cheese, rancid, pungent, green and butter, not contributing in a positive way for the typical wine aroma.

The method developed and optimized for VFA in fortified wines is fast, simple and sensitive. This analytical method it was validated, achieving good results in conditions of linearity, sensitivity, selectivity, precision and accuracy.

10. References

1. Elliott T. The Wines of Madeira: An Indispensable Guide to the Wines, Grapes and Producers: BPR Publishers; 2010 d. 102-21 p.

2. Pereira VNG. Effect of the estufagem process on the chemical constituents of Madeira wines: Universidade da Madeira; 2011.

3. IVBAM.Vinho Madeira. Instituto do Vinho, Bordado e do Artesanato da Madeira. Consulted at: 21-01-2015, URL: http://www.vinhomadeira.pt 2009

4. Elliot T. Wines of Madeira: An indispensable guide to the wines, grapes and producers. Hampshire: : Trevor Elliott Publishing.; 2010 a. 28-31 p.

5. Nogueira JM, Nascimento AM. Analytical characterization of madeira wine. Journal of Agricultural and Food Chemistry. 1999;47(2):566-75.

6. Elliot T. Wines of Madeira: An indispensable guide to the wines, grapes and producers. Hampshire: : Trevor Elliott Publishing.; 2010 b. 34-6 p.

7. Jackson RS. Wine science: principles and applications: Academic press; 2008.

8. Zamora F. Biochemistry of Alcoholic Fermentation. In: Moreno-Arribas MV, Polo MC, editors. Wine Chemistry and Biochemistry: Springer New York; 2009. p. 27-57.

9. Elliot T. Wines of Madeira: An indispensable guide to the wines, grapes and producers. Hampshire: : Trevor Elliott Publishing.; 2010 c. 54-5 p.

10. Perestrelo R, Albuquerque F, Rocha SM, Câmara JS. Chapter 7 - Distinctive Characteristics of Madeira Wine Regarding Its Traditional Winemaking and Modern Analytical Methodologies. Advances in Food and Nutrition Research. 63: Academic Press; 2011. p. 207-49.

11. Bakker J, Clarke RJ. Grape Varieties and Growing Regions. Wine Flavour Chemistry: Wiley-Blackwell; 2011. p. 53-88.

12. Zotou A, Loukou Z, Karava O. Method Development for the Determination of Seven Organic Acids in Wines by Reversed-Phase High Performance Liquid Chromatography. Chromatographia. 2004;60(1-2):39-44.

13. Zeravik J, Fohlerova Z, Milovanovic M, Kubesa O, Zeisbergerova M, Lacina K, et al. Various instrumental approaches for determination of organic acids in wines. Food Chemistry. 2016;194:432-40.

14. Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D. Organic Acids in Wine. Handbook of Enology: John Wiley & Sons, Ltd; 2006. p. 1-49.

15. Miranda AFS. Olfactory perception threshold assessment of volatile acidity in Madeira wines. 2015.

16. Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D. Handbook of Enology The Chemistry of Wine Stabilization and Treatments 2nd Edition: John Wiley & Sons Ltd; 2006.

17. Su J, Wang T, Wang Y, Li Y-Y, Li H. The use of lactic acid-producing, malic acid-producing, or malic acid-degrading yeast strains for acidity adjustment in the wine industry. Applied Microbiology and Biotechnology. 2014;98(6):2395-413.

18. Palma M, Barroso CG. Ultrasound-assisted extraction and determination of tartaric and malic acids from grapes and winemaking by-products. Analytica Chimica Acta. 2002;458(1):119-30.

19. Moreno-Arribas MV, Polo MC. Wine Chemistry and Biochemistry. Springer Science+Business Media2009.

20. Jackson RS. Wine Science Principles and Applications

Elsevier; 2008.

21. Pisarnitskii AF. Formation of Wine Aroma: Tones and Imperfections Caused by Minor Components (Review). Applied Biochemistry and Microbiology. 2001;37(6):552-60.

22. Styger G, Prior B, Bauer F. Wine flavor and aroma. Journal Industrial Microbiology and Biotechnology. 2011;38(9):1145-59.

23. Selli S, Canbas A, Cabaroglu T, Erten H, Günata Z. Aroma components of cv. Muscat of Bornova wines and influence of skin contact treatment. Food Chemistry. 2006;94(3):319-26.

24. Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D. Varietal Aroma. Handbook of Enology: John Wiley & Sons, Ltd; 2006. p. 205-30.

25. Bakker J, Clarke RJ. Volatile Components. Wine Flavour Chemistry: Wiley-Blackwell; 2011. p. 155-238.

26. Rodrigues F, Caldeira M, Câmara JS. Development of a dynamic headspace solid-phase microextraction procedure coupled to GC–qMSD for evaluation the chemical profile in alcoholic beverages. Analytica Chimica Acta. 2008;609(1):82-104.

27. Mateo J, Jiménez M. Monoterpenes in grape juice and wines. Journal of Chromatography A. 2000;881(1):557-67.

28. Câmara JS, Herbert P, Marques JC, Alves MA. Varietal flavour compounds of four grape varieties producing Madeira wines. Analytica Chimica Acta. 2004;513(1):203-7.

29. Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D. Alcohols and Other Volatile Compounds. Handbook of Enology: John Wiley & Sons, Ltd; 2006. p. 51-64.

30. Hazelwood LA, Daran J-M, van Maris AJA, Pronk JT, Dickinson JR. The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on Saccharomyces cerevisiae Metabolism. Applied and Environmental Microbiology. 2008;74(8):2259-66.

31. Pérez Olivero SJ, Pérez Trujillo JP. A new method for the determination of short-chain fatty acids from the aliphatic series in wines by headspace solid-phase microextraction–gas chromatography–ion trap mass spectrometry. Analytica Chimica Acta. 2011;696(1–2):59-66.

32. Ancín C, Ayestarán B, García A, Garrido J. Evolution of fatty acid contents in Garnacha and Viura musts during fermentation and the aging of wine. Zeitschrift für Lebensmitteluntersuchung und-Forschung A. 1998;206(2):143-7.

33. Pereira V, Albuquerque F, Cacho J, Marques JC. Polyphenols, antioxidant potential and color of fortified wines during accelerated ageing: The madeira wine case study. Molecules. 2013;18(3):2997-3017.

34. Mazza G, Fukumoto L, Delaquis P, Girard B, Ewert B. Anthocyanins, Phenolics, and Color of Cabernet Franc, Merlot, and Pinot Noir Wines from British Columbia. Journal of Agricultural and Food Chemistry. 1999;47(10):4009-17.

35. He F, Liang N-N, Mu L, Pan Q-H, Wang J, Reeves MJ, et al. Anthocyanins and their variation in red wines I. Monomeric anthocyanins and their color expression. Molecules. 2012;17(2):1571-601.

36. Roubelakis-Angelakis KA. Grapevine molecular physiology & biotechnology: Springer Science & Business Media; 2009.

37. Liang Z, Wu B, Fan P, Yang C, Duan W, Zheng X, et al. Anthocyanin composition and content in grape berry skin in Vitis germplasm. Food Chemistry. 2008;111(4):837-44.

38. Gómez-Míguez M, Heredia FJ. Effect of the Maceration Technique on the Relationships between Anthocyanin Composition and Objective Color of Syrah Wines. Journal of Agricultural and Food Chemistry. 2004;52(16):5117-23.

39. Torija MJ, Roze N, Poblet M, Guillamo JM, Mas A. Effects of fermentation temperature on the strain population of Saccharomyces cerevisiae. International Journal of Food Microbiology. 2002;80:47– 53.

40. Saerens SMG, Delvaux F, Verstrepen KJ, Van Dijck P, Thevelein JM, Delvaux FR. Parameters Affecting Ethyl Ester Production by Saccharomyces cerevisiae during Fermentation. Applied and Environmental Microbiology. 2008;74(2):454-61.

41. Mallouchosa A, Komaitisa M, Koutinas A, Kanellaki M. Wine fermentations by immobilized and free cells at different temperatures. Effect of immobilization and temperature on volatile by-products. Food Chemistry 2003;80:109 – 13.

42. Rollero S, Bloem A, Camarasa C, Sanchez I, Ortiz-Julien A, Sablayrolles J-M, et al. Combined effects of nutrients and temperature on the production of fermentative aromas by Saccharomyces cerevisiae during wine fermentation. Applied Microbiology and Biotechnology. 2015;99(5):2291-304.

43. Carvalho MJ, Pereira V, Pereira AC, Pinto JL, Marques JC. Evaluation of Wine Colour Under Accelerated and Oak-Cask Ageing Using CIELab and Chemometric Approaches. Food Bioprocess Tech. 2015;8(11):2309-18.

44. Molina A, Swiegers J, Varela C, Pretorius I, Agosin E. Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. Applied Microbiology and Biotechnology. 2007;77(3):675-87.

45. Beltran G, Novo M, Guillamón JM, Mas A, Rozès N. Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. International Journal of Food Microbiology. 2008;121(2):169-77.

46. Mallouchos A, Komaitis M, Koutinas A, Kanellaki M. Wine fermentations by immobilized and free cells at different temperatures. Effect of immobilization and temperature on volatile by-products. Food Chemistry. 2003;80(1):109-13.

47. Torija MaJ, Beltran G, Novo M, Poblet M, Guillamón JM, Mas A, et al. Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile compounds in wine. International Journal of Food Microbiology. 2003;85(1–2):127-36.

48. Torija MJ, Rozès N, Poblet M, Guillamón JM, Mas A. Effects of fermentation temperature on the strain population of Saccharomyces cerevisiae. International Journal of Food Microbiology. 2003;80(1):47-53.

49. Kourkoutas Y, Kanellaki M, Koutinas AA, Tzia C. Effect of fermentation conditions and immobilization supports on the wine making. Journal of Food Engineering. 2005;69(1):115-23.

50. Arroyo-López FN, Orlić S, Querol A, Barrio E. Effects of temperature, pH and sugar concentration on the growth parameters of Saccharomyces cerevisiae, S. kudriavzevii and their interspecific hybrid. International Journal of Food Microbiology. 2009;131(2–3):120-7.

51. Molina AM, Swiegers JH, Varela C, Pretorius IS, Agosin E. Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compound. Applied Microbiology and Biotechnology. 2007;77:675–87.

52. Pérez-Magariño S, González-San José ML. Polyphenols and colour variability of red wines made from grapes harvested at different ripeness grade. Food Chemistry. 2006;96(2):197-208.

53. Alexandre H, Charpentier C. Biochemical aspects of stuck and sluggish fermentation in grape must. Journal Industrial Microbiology and Biotechnology. 1998;20(1):20-7.

54. Pérez-Magariño S, González-Sanjosé ML. Application of absorbance values used in wineries for estimating CIELAB parameters in red wines. Food Chemistry. 2003;81(2):301-6.

55. Rasines-Perea Z, Prieto-Perea N, Romera-Fernández M, Berrueta LA, Gallo B. Fast determination of anthocyanins in red grape musts by Fourier transform mid-infrared spectroscopy and partial least squares regression. European Food Research Technology. 2015;240(5):897-908.

56. Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D. Phenolic Compounds. Handbook of Enology: John Wiley & Sons, Ltd; 2006. p. 141-203.

57. Bimpilas A, Panagopoulou M, Tsimogiannis D, Oreopoulou V. Anthocyanin copigmentation and color of wine: The effect of naturally obtained hydroxycinnamic acids as cofactors. Food Chemistry. 2016;197, Part A:39-46.

58. Paixão N, Perestrelo R, Marques JC, Câmara JS. Relationship between antioxidant capacity and total phenolic content of red, rosé and white wines. Food Chemistry. 2007;105(1):204-14.

59. Oliveira CM, Barros AS, Silva Ferreira AC, Silva AMS. Influence of the temperature and oxygen exposure in red Port wine: A kinetic approach. Food Research International. 2015;75:337-47.

60. Pereira V, Camara JS, Cacho J, Marques JC. HPLC-DAD methodology for the quantification of organic acids, furans and polyphenols by direct injection of wine samples. Journal of separation science. 2010;33(9):1204-15.

61. Campo E, Ferreira V, Escudero A, Marqués JC, Cacho J. Quantitative gas chromatography–olfactometry and chemical quantitative study of the aroma of four Madeira wines. Analytica Chimica Acta. 2006;563(1–2):180-7.

62. Takahashi K, Goto-Yamamoto N. Simple method for the simultaneous quantification of medium-chain fatty acids and ethyl hexanoate in alcoholic beverages by gas chromatography-flame ionization detector: Development of a direct injection method. Journal of Chromatography A. 2011;1218(43):7850-6.

63. Romero I, García-González DL, Aparicio-Ruiz R, Morales MT. Validation of SPME–GCMS method for the analysis of virgin olive oil volatiles responsible for sensory defects. Talanta. 2015;134:394-401.

64. Ábalos M, Bayona JM, Pawliszyn J. Development of a headspace solid-phase microextraction procedure for the determination of free volatile fatty acids in waste waters. Journal of Chromatography A. 2000;873(1):107-15.

65. Ferreira V, López R, Cacho JF. Quantitative determination of the odorants of young red wines from different grape varieties. Journal of the Science of Food and Agriculture. 2000;80(11):1659-67.

66. Lukic I, Radeka S, Grozaj N, Staver M, Persuric D. Changes in physico-chemical and volatile aroma compound composition of Gewurztraminer wine as a result of late and ice harvest. Food Chemistry. 2016;196:1048-57.