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Micro-oxygenation in Madeira Wine

MASTER DISSERTATION

Ana Isabel Camacho de Freitas
MASTER IN APPLIED BIOCHEMISTRY

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Resumo

A micro-oxigenação consiste na adição deliberada e controlada de pequenas quantidades de oxigénio durante o processo de vinificação, com o intuito de melhorar as características organoléticas do vinho. Tendo em conta o carácter oxidativo do processo de envelhecimento do vinho Madeira, avaliou-se o impacto de três tratamentos de micro-oxigenação (t1- 66 mg/L por mês, antes da estufagem; t2 - 66 mg/L por mês, durante a estufagem; t3 – oxigénio dissolvido acima de 7 mg/L, durante a estufagem) no conteúdo de oxigénio dissolvido, parâmetros enológicos básicos, cor, polifenóis, furanos, composição volátil e características sensoriais. Em geral, a adição de um fluxo padrão de oxigénio não teve um impacto notável na cor, na composição polifenólica, nos furanos ou no perfil volátil, favorecendo, no entanto, o desenvolvimento de sotolon (até 97%) e de acetais heterocíclicos (até 95% para o *trans*-dioxolano). Já as condições de sobre-oxigenação (t3) diminuíram consideravelmente a intensidade da cor (cerca de 41%) e o conteúdo polifenólico (até 29% durante a estufagem). Adicionalmente, este tratamento desacelerou a formação de furanos e de sotolon até cerca de 36%, comparativamente ao controlo. Para tal, foi desenvolvida uma metodologia para a determinação simultânea de sotolon e acetais heterocíclicos (*cis*- e *trans*-dioxano e *cis*- e *trans*-dioxolano), tendo por base a miniaturização da extração líquido-líquido e análise por GC-MS/SIM. Após otimização, o procedimento de extração foi validado relativamente ao sotolon, demonstrando ter boa sensibilidade (LOD e LOQ de 2,3 e 6,8 μg/L, respetivamente), linearidade (R^2 de 0,999), precisão (desvios padrão para repetibilidade e reprodutibilidade inferiores a 8% e 10%, respetivamente) e exatidão (recuperação média de 105,4%), recorrendo a uma abordagem ecológica, rápida e de baixo custo. Relativamente aos acetais heterocíclicos, a metodologia foi avaliada em termos de precisão, com desvios padrão inferiores a 13% para a repetibilidade e a 17% para a reprodutibilidade.

Palavras-chave: Envelhecimento oxidativo, oxigénio dissolvido, cor, polifenóis, composição volátil

Summary

Micro-oxygenation is the deliberate and controlled addition of small amounts of oxygen during the winemaking process, with the intent of improving the wine's organoleptic characteristics. Given the oxidative nature of Madeira wine aging, the impact of three micro-oxygenation treatments (t1- 66 mg/L per month before *estufagem*; t2 - 66 mg/L per month during *estufagem*; t3 – DO above 7 mg/L during *estufagem*) on the dissolved oxygen content, basic oenological characteristics, color, polyphenolics, furans, volatiles and sensorial characteristics were verified. In general, standard oxygen flow addition did not have a noticeable impact on wine color, polyphenolic composition, furans or total volatile composition, but favored the development of sotolon (up to 97%) and heterocyclic acetals (up to 95% for *trans*-dioxolane). Over-oxygenation conditions (t3) considerably decreased color intensity (about 41%) and polyphenols (up to 29% during *estufagem*). Additionally, the t3 treatment slowed the development of furans and sotolon up to about 36%, comparing to the control. To accomplish this analysis, a methodology for the simultaneous evaluation of sotolon and heterocyclic acetals (*cis*- and *trans*-dioxane and *cis*- and *trans*- dioxolane) was developed, based on a miniaturized liquid-liquid extraction followed by GC-MS/SIM analysis. The optimized extraction procedure was fully validated for the analysis of sotolon, showing good sensitivity (LOD and LOQ of 2.3 and 6.8 μg/L, respectively), linearity (R^2 of 0.999), precision (standard deviation for repeatability and reproducibility lower than 8% and 10%, respectively) and accuracy (average recovery of 105.4%), using an eco-friendly, fast and low-cost approach. In regards to the heterocyclic acetals, the methodology was assessed in terms of precision, with standard deviations lower than 13% for repeatability and lower than 17% for reproducibility.

Key-words: Oxidative aging, dissolved oxygen, color, polyphenols, volatile composition

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List of abbreviations

ABV – Alcohol by volume

AF – Alcoholic fermentation

CI – Color intensity

DoE – Design of experiments

DO – Dissolved oxygen

ET0 – Beginning of *estufagem* and before t2 treatment

ET2M – During *estufagem* and before t3 treatment

ET4M – End of *estufagem* and t3 treatment

EU – European Union

GC-MS – Gas chromatography-mass spectrometry

HMF – 5-hydroxymethylfurfural

HS-SPME – Headspace solid-phase micro-extraction

HPLC – High performance liquid chromatography

HPLC-PDA – High performance liquid chromatography - photodiode array detection

IS – Internal standard

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

KI – Kovats Index

LAB – Lactic acid bacteria

LLE – Liquid-liquid extraction

LOD – Limit of detection

LOQ – Limit of quantification

MEPS/UHPLC – Micro-extraction by packed sorbent/ ultra-high performance liquid chromatography

MLF – Malolactic fermentation

MOX – Micro-oxygenation

MOX1 – wine submitted to micro-oxygenation treatment t1

MOX2 – wine submitted to micro-oxygenation treatment t2

NTU – Nephelometric Turbidity Units

PPO – Polyphenol oxidase

QuEChERS – "Quick, Easy, Cheap, Effective, Rugged, and Safe"

R^2 – Correlation coefficient

ROS – Reactive oxygen species

RP-HPLC – Reversed-phase high performance liquid chromatography

RS – Reducing sugars

RSD – Relative standard deviation

SD – Standard deviation

SIM – Selective ion monitoring

T0 – before *estufagem* and t1 treatment

T1- micro-oxygenation treatment 1

T2- micro-oxygenation treatment 2

T3- micro-oxygenation treatment 3

TA – Total acidity

TIC – Total ion count

To – Tonality

TN – Tinta Negra

UV-vis – Ultraviolet-visible

VA – Volatile acidity

ρ – Volumetric mass density

PART I - GENERAL INTRODUCTION

1. MICRO-OXYGENATION

The importance of the role of oxygen (O_2) in the biochemical and microbiological transformations that occur throughout the winemaking process, and that consequently affect the sensory characteristics of wine, has long been recognized. In this regard, the impact of oxygen can assume a positive or negative role, depending on the nature and duration of exposure (1). An excess of oxygen is known to promote the oxidation of phenolic and volatile compounds causing browning, development of aldehyde and oxidized aromas, disappearance of fresh and fruity aromas and may even potentiate undesirable bacterial growth (2, 3) – all of these phenomena can lead to great losses in table wine quality. On the other hand, oxygen is also essential for the occurrence of polymerization and condensation reactions between tannins and anthocyanins which, among other factors, result in the formation of new polymeric pigments, which are known to give higher color intensity and stability to wine, but also lower astringency (1).

The exposure of wine to oxygen by contact with air is inevitable. During alcoholic fermentation, oxygen has long been recognized as beneficial to the fermentation process, since it promotes the synthesis of sterols in the cellular wall of yeast (2). During aging, particularly in oak barrels, the permeation of oxygen through the wood pores ensures the continuous addition of small amounts of oxygen, which in turn is considered essential for obtaining a higher quality wine (4). At this stage of the winemaking process, adequate amounts of oxygen promote polymerization and condensation reactions of polyphenols, producing stable forms of anthocyanins with an intense coloration and a greater resistance to degradation, to discoloration by sulfur dioxide (SO_2) and to pH changes. However, an excess of oxygen during the aging process may also lead to the formation of high molecular weight polymers (1). The precipitation of these polymers leaves the wine dry and with low color intensity.

Aging in oak barrels usually precedes an aging period in bottle, desirably in low oxygen conditions (5). The winemaking process can then be seen as a careful balance between the stages when the exposure to adequate amounts of oxygen is essential for the development of a high quality wine, and the stages during which the exposure to oxygen can irreversibly spoil the wine. Thus, it became necessary to develop a technique by which the exposure of must or wine to oxygen could be controlled – micro-oxygenation.

Most studies about the impact of oxygen addition are focused on red table wines, even though some studies have recently targeted Port wines (6, 7).

1.1. History and definition

Micro-oxygenation (MOX) is a relatively new technique that has been gaining increasing acceptance by wine producers and has already become a common practice in many wineries. This technique was developed in the early 1990s in the region of Madiran, in South West France (8). In this region it is typically produced a red wine from the Tannat, Cabernet Sauvignon and Cabernet Franc grape varieties. This combination results in a very tannic wine with high astringency that requires extended periods of aging in barrel and in bottle until it can be commercialized. This led Patrick Ducournau, after a period of initial studies, to create a device capable of delivering small quantities of oxygen in order to recreate the diffusion of this gas that naturally occurs during aging in oak barrels. The technique was patented in 1993 and in 1995 Ducournau and Lemaire founded the company OenoDev, in order to provide education and equipment for micro-oxygenation purposes (1, 3). At this time several studies were developed on the subject, mostly by French universities, and in 1996 the European Commission authorized its use in Europe (9). In 1998, a partnership between OenoDev and the american company Vinovation was realized in an attempt to expand the application of the technique (5). Since then, micro-oxygenation has quickly expanded worldwide, particularly to the United States of America, Italy, Australia, New Zealand and Chile, and nowadays it is a relatively common oenological practice.

In general, micro-oxygenation is defined as the deliberate and controlled addition of small amounts of oxygen during various stages of the winemaking process, with the goal to positively change the organoleptic characteristics of the final product (2, 10). This technique differs from traditional winemaking processes such as racking, in which the wine is transferred to different barrels by successive decantations, or aging in oak casks, which allows the permeation of oxygen through the wood pores. Although both processes involve the deliberate addition of oxygen to wine, its quantification is not possible. The terms aeration and micro-aeration are also sometimes used instead of micro-oxygenation. However, these refer to the addition of air to wine, unlike MOX which strictly involves the addition of oxygen (5). Micro-oxygenation also differs from hyper-oxidation or

hyper-oxygenation, which in turn consists of the deliberate oxidation of must in the production of white wines (5). This oxidation process is specific for white wines, thus it cannot be considered a form of micro-oxygenation. Another point of contention regarding the definition of micro-oxygenation relates to the timing, dosage and duration of the treatment. In this sense, some authors distinguish between micro- and macro-oxygenation. In general, the term macro-oxygenation is used for short-term treatments with high doses of oxygen, wherein the treatment is usually performed during the final stages of alcoholic fermentation. Complementarily, the designation micro-oxygenation is reserved for long term treatments, typically after fermentation, with much lower doses of oxygen (2, 5, 11, 12). However, the use of these terms in the literature is not consistent and the two are sometimes interchangeable. For that reason, throughout this dissertation only the expression micro-oxygenation will be used.

1.2. Applications, benefits and risks

Micro-oxygenation was developed with the clear objective of promoting positive oxidation reactions, in order to improve structure and stabilize the color of wine (9). These characteristics are usually associated to barrel aging, which allows oxygen to slowly diffuse through the wood pores. It was established that it was this slow oxidation that allowed beneficial reactions to occur, imparting favorable characteristics to wine. This is probably why micro-oxygenation is often seen as a possible alternative to barrel aging, in a shorter period of time and with lower monetary costs (10). In fact, it has been suggested that the effects of barrel aging can be replicated in stainless steel tanks by the application of micro-oxygenation coupled with oak substitutes, such as oak chips or staves. However, most wine makers still prefer to regard micro-oxygenation as a complementary technique to improve wine characteristics, rather than a way to substitute or speed-up the barrel aging process.

Nowadays, improved flavor and color stabilization still remain the most advertised benefits to be gained from micro-oxygenation (1, 2). As it will be further explained in Section 1.7., the addition of moderate quantities of oxygen promotes the polymerization reactions of tannins, resulting in a wine with softer tannins and decreased astringency. Oxygen is also involved in reactions between anthocyanins and tannins that

result in the production of colored pigments that are more resistant to pH changes, sulphur dioxide bleaching and degradation (2, 13).

In addition to these two effects, several other possible benefits have been suggested for micro-oxygenation. Several authors have often proposed that oxygen addition can improve aroma and flavor, by lessening vegetative and herbaceous aromas. Parish (1) stated that micro-oxygenation could decrease the reductive character and vegetative aromas and increase oxidative stability, protecting wine against future adverse oxidation, and that micro-oxygenation can be used in the early stages of alcoholic fermentation, as a preventive treatment against slow or stuck fermentations (2).

However, as is often the case, micro-oxygenation also presents several recognized risks. These usually concern the duration of the treatment, as there are no clear indicators to help determine if a wine is being under or over micro-oxygenated. The consequences of over-oxygenating a wine, in particular, are a little more serious, as they are irreversible. Extending the treatment beyond its optimal can result in a dry wine with tannic character, oxidized aroma and loss of color intensity (5). Another concern is the possibility of the development of aerobic microorganisms, such as acetic acid bacteria or *Brettanomyces* (10). The first can cause an increase in volatile acidity and, in more extreme cases, complete spoilage of the wine. The second is responsible for the appearance of foul-smelling volatile phenols (5).

Some authors also raise questions regarding the possible impact of micro-oxygenation on other wine characteristics, in particular on the anti-oxidant activity and on some important wine volatile compounds (14).

1.3. Parameters that influence micro-oxygenation

Micro-oxygenation is a sensitive technique and several factors can affect its impact on wine. Several of these factors are directly related to the wine itself. **Turbidity** (2), for instance, can impact micro-oxygenation due to the high capacity that lees have to consume oxygen. To a lesser extent, the presence of metal ions, especially iron and copper, can also influence micro-oxygenation, since they act as catalysts in oxidation reactions (5).

During maturation, oxygen is mainly consumed in condensation and polymerization reactions of polyphenols. Therefore, the initial **phenolic content** of grapes, in particular the concentrations of tannins and anthocyanins, is essential to determine if and how much a wine can be exposed to oxygen. Besides total phenolic content, the type of phenols is also relevant, given that different compounds react differently with oxygen. Generally, the technique is indicated for highly tannic red wines, with a ratio of anthocyanins to tannins of at least 1:4 (5, 10, 15, 16).

Another key factor that can influence micro-oxygenation is **temperature**. Oxygen solubility tends to increase with lower temperatures. Conversely, higher temperatures increase the speed of the reactions, therefore a balance must be found. The recommended temperatures for micro-oxygenation tend to range from 15 to 20 ºC (2, 3, 5, 10).

Sulphur dioxide is usually added to wine in order to inhibit microbial growth. However, this anti-oxidant reacts with hydrogen peroxide and with acetaldehyde, both necessary for the occurrence of polymerization and condensation reactions. Moreover, it also binds to anthocyanins, preventing the production of its more stable forms (5, 10). Thus, the concentrations of SO_2 should also be taken into consideration when starting a micro-oxygenation treatment.

A factor that can also influence the overall outcome of micro-oxygenation is its **timing** (2), or in other words, the stage of winemaking where the treatment is introduced. This of course depends on the objective of the treatment, but it has been reported that it is often more effective after alcoholic fermentation (AF) and before malolactic fermentation (MLF) (2).

Similarly, the **rate** of oxygen addition can also influence the outcome of micro-oxygenation (10). For example, a rapid exposure to oxygen can cause the wine to become oxidized and browning can occur. But once again, different strategies can be chosen according to the goals of the treatment. However, it is important to keep in mind that the rate of oxygen addition should remain lower than the rate of its consumption when the oxidation phenomena are unwanted (2, 3, 5).

1.4. Timing and phases

As aforementioned, micro-oxygenation is mostly applied to red wines, and as such can be introduced at any stage of the winemaking process, according to the desired objective. There are three stages (5) in which micro-oxygenation is most commonly applied: a) early stages of AF; b) after AF but before MLF and c) after MLF.

On the basis of practical experience, most producers of table wines recommend introducing micro-oxygenation after alcoholic fermentation but before malolactic fermentation (8). It is suggested in the literature that right after alcoholic fermentation most tannins and anthocyanins are still in their monomeric forms and, as such, are more unstable (2, 17). Over time, these molecules suffer natural polymerization and condensation reactions. Some authors argue that if oxygen is introduced after these reactions occur, it can result in high molecular weight polymeric compounds that may precipitate, which in turn would leave the wine with lower color intensity (5). Additionally, the increased amount of acetaldehyde, which results from a micro-oxygenation treatment after AF, can be consumed by lactic acid bacteria (LAB) during MLF, producing mainly acetic acid and ethanol, whose increase should be sensorially insignificant. This is another advantage for most red wines that undergo malolactic fermentation. Nevertheless, the time between AF and MLF is often very short and insufficient to complete a micro-oxygenation treatment. Instead, an increasingly popular approach is to micro-oxygenate wine both before and after MLF (5).

For most wines, MLF is usually followed by the addition of SO_2 in order to protect wine against microbial spoilage. As previously mentioned in Section 1.3., SO_2 interferes with the polymerization and condensation reactions, slowing them down. However, some winemakers regard it as an advantage, since they claim that in this way it resembles more closely to barrel maturation (2).

Throughout micro-oxygenation, the phenolic composition of a wine undergoes a series of changes, which in turn result in alterations of its organoleptic characteristics. There is a model, commonly followed by winemakers, that defines these changes in three distinct phases – structuration, harmonization and over-oxygenation (13).

The **structuring** phase is characterized by a gain in the tannic character of the wine, with tannins becoming more aggressive and intense, increasing astringency and

eventually color intensity and decreasing the aroma complexity and intensity. These changes depend on the wine characteristics and procedural conditions (1, 4, 13).

As the treatment continues, the **harmonization** phase begins, in which a reversal of the previous changes occurs. Tannins become softer, astringency and herbaceous aromas decrease, mouthfeel improves and aromatic complexity increases (1, 13). However, unlike what happens during the structuration phase, all the changes that wine undergoes during harmonization are irreversible. It is also suggested that the longer the structuration phase is, the more necessary harmonization becomes. Also, a close monitoring of wine during this phase is critical, because wine becomes increasingly more sensitive to oxygen (4, 5).

If micro-oxygenation continues, **over-oxygenation** may occur and wine can deteriorate. This can result in an increase of dryness and astringency, decrease of fresh aromas and aromatic intensity and possibly the occurrence of oxidation aromas (1, 4, 5, 13).

This model has long been bringing winemakers to micro-oxygenation, but still has little support by scientific research. In fact, often, it is in disagreement with what is common knowledge about the oxidation reactions that happen during aging. This model does, however, highlight the risks of under or over-oxygenation, given that there is no standard protocol that winemakers can follow. It also accentuates the importance of monitoring the wine's evolution in order to adapt the treatment to the wine's needs and to correctly determine the micro-oxygenation endpoint.

1.5. Monitoring

Regardless of the micro-oxygenation timing or dosage, one of the key factors to achieve a beneficial micro-oxygenation is its constant and careful monitoring. Wines respond differently to micro-oxygenation, and while under-treatments are ineffective, over-oxygenation can completely spoil a wine. In order to avoid these outcomes, the control of several parameters has been suggested. Blaauw (5) has proposed three levels of monitoring – basic, intermediate and advanced.

Basic monitoring is strongly advised for all micro-oxygenation treatments and can easily be applied in most wineries, since it involves controlling basic parameters using simple procedures. Temperature control is fundamental for any micro-oxygenation treatment. An average temperature between 15 to 17 ºC is recommended in order to maintain a good relation between the oxygen solubility and the speed of oxidation reactions (2). The levels of free and total sulphur dioxide should also be monitored. As previously mentioned, concentrations above 20 mg/L of SO_2 are necessary to prevent bacterial spoilage, but can also be enough to inhibit oxidation reactions. On the other hand, since SO_2 reacts with any O_2 excess, its measurement is useful to determine if O_2 levels are too high (2, 4, 5). Also, micro-oxygenation treatments are usually accompanied by regular tastings. This is of extreme importance because it helps oenologists determine whether the treatment is damaging the wine and should be stopped. The frequency of the tastings depends on the treatment stage and should preferably be performed by a group of trained tasters (2, 4, 5, 10).

In addition to the aforementioned parameters, the **intermediate** monitoring includes the control of three other parameters. Considering that the amount of oxygen introduced into wine should be inferior to its consumption, the concentration of dissolved oxygen is a very important marker for micro-oxygenation. Indeed, dissolved oxygen levels should remain constant throughout the treatment (2, 4, 5). However, determining the content of dissolved oxygen in wine can be often difficult and not always accurate. This is usually explained by the fact that oxygen concentration in wine is not constant but rather distributed in layers. Moreover, turbidity and volatile acidity should also be monitored (4, 5, 18). Turbidity should be kept below 200 Nephelometric Turbidity Units (NTU), although below 100 NTU would be preferable (2). Volatile acidity is a good indicator of the acetic acid bacteria activity.

An **advanced** monitoring is very hard to employ in most wineries because it involves specialized and expensive equipment. In fact, often, it is the focus of most scientific studies involving micro-oxygenation. These tests include the analysis of color parameters and polyphenolic components, through ultraviolet-visible (UV-Vis) spectrophotometry and high performance liquid chromatography (HPLC), respectively.

1.6. Basic equipment and newer innovations

The fundamental principles of micro-oxygenation require the controlled addition of small quantities of pure oxygen into wine, during an established period of time. Additionally, it is imperative that the flow of oxygen addition be inferior to its consumption. These specifications were a challenge for industry, since that required the development of specialized equipment, not only to regulate the oxygen flow for very small quantities but also to ensure that oxygen is dispersed through the wine rather than just accumulate in the headspace (5).

The essential equipment for micro-oxygenation, as originally designed by OenoDev, includes three basic components: a source of oxygen, a dosing system and a delivery device (5). The oxygen source usually consists of a pressurized cylinder of food or medical grade oxygen. The use of oxygen is preferred to air, since it allows a more accurate regulation of the flow. The original dosing system developed by OenoDev involves the use of an apparatus with two chambers of known volume. After the first chamber is filled with a set value, fixed volumes of gas are transferred, at low pressure, to the second chamber. From this second chamber, oxygen is then carried to the delivery system (1). Newer dosing systems involve the use of sensors, which allow a precise measurement and regulation of the oxygen flow. Finally, the delivery of oxygen is achieved by the use of micro-porous filters, usually made of ceramic or stainless steel, which convert the gas flow into a flow of very fine bubbles (10, 15). These bubbles are readily dispersed throughout the wine as they rise towards the surface. However, this particular delivery system has an inconvenient. In order to ensure that the path length of the bubbles to the surface is sufficient for oxygen to diffuse into the wine, the container in which the wine is stored must have at least 2 to 2.5 meters in height. The use of smaller vessels can result in headspace accumulation, which in turn may lead to negative oxidation reactions and unwanted microbiological activity (10).

In recent years, new technological innovations have been introduced and a few alternatives to the traditional micro-oxygenation methods have emerged. For example, Memstar has developed a new diffusion method called O_2mate, which includes two systems – barrel(mate) and tank(mate) (19). Rather than use a traditional micro-porous diffuser, this delivery method uses a specialized polydimethylsiloxane tube that allows oxygen to permeate into the wine, mimicking more closely the process that occurs in

barrels. Another advantage of this method is that it can be used with vessels of smaller size. Since the bubbles produced are much smaller than those produced by traditional diffusion systems, a path length of 2.5 meters is not required and headspace accumulation is avoided. Another alternative that closely mimics oxygen permeation through the walls of wood barrels are the oxygen-permeable vessels (5). Developed in 2004 by Flextank, these vessels made of high-density polyethylene are permeable to oxygen, much like wood barrels. They represent a cheaper and less laborious alternative and were specially designed to inhibit bacterial spoilage. Oxygen addition depends only on the size of the tank and several sizes are available, from 100 to 1000 L. Finally, electrochemical micro-oxygenation introduced a new process of oxidizing wine, different from the molecular oxidation methods described above. This process is based on electrolysis, wherein a current is passed through wine using electrodes. It is an analogous process to the electrolysis of water, in which the compounds near the cathode (negative electrode) are oxidized (5, 20). In the case of wine, the result is oxidized polyphenols. In a study (20) where an electric current was passed through the wine, the authors reported an increase in SO_2 resistant pigments, less free anthocyanins and decreased astringency – changes that are similar to those expected from traditional micro-oxygenation. The absence of bubbles and the oxidation of ethanol to acetaldehyde without the production of hydrogen peroxide (H_2O_2) are the main attraction of this technique.

1.7. Effect on polyphenols

As previously mentioned, the response of wine to micro-oxygenation varies according to its phenolic composition. On this basis, most of the theory behind oxygen treatments is strongly based on scientific research about phenolic reactions during winemaking. Phenolic compounds play an important role in oenology, not only are they one of the main constituents of wine, but they also have an important role regarding the organoleptic characteristics of wine, such as structure, color and mouthfeel. Moreover, polyphenols determine the evolution of wine during its maturation. Their basic structure is composed of, at least, one benzene ring to which one or more hydroxyl groups (-OH) are linked to (15, 21).

Polyphenols can be categorized as flavonoids and non-flavonoids. **Flavonoids** are mostly found in the skin and seeds of grapes and are the group that most contributes to

the concentration of polyphenols in red wines. They have a 15 carbon basic structure, constituted by two aromatic rings (Ring A and Ring B) connected to each other by a 3 carbon chain. When the chain is closed by oxygen it forms a heterocyclic ring (Ring C) (21). Depending on the differences in Ring C, these compounds can be classified in various groups. Individual compounds in each class differ from each other by substitutions on Ring A and Ring B. The most important classes of flavonoids in wines are anthocyanins, flavanols and flavonols (15).

Anthocyanins are known for their important contribution to red pigments and are responsible for the color changes of red wines. In wine, they occur in four forms with different colors, which are dependent on pH and SO_2 concentrations. At wine pH, anthocyanins can be commonly found in the hydrated hemiketal form, which is colorless, or in the form of a red flavylium cation. Reactions between anthocyanins and SO_2 also produce colorless pigments. Anthocyanins also react with tannins and the product is a polymeric pigment, much more stable to pH changes and SO_2 content, usually present in the red form in a much higher percentage when compared with free anthocyanins (5, 10).

Tannins are hydrosoluble molecules that can react with proteins. Indeed, the complexes formed by these molecules with salivary proteins are the main cause for the sense of astringency and mouthfeel of wine (5). Tannins in wine can be classified as hydrolysable or condensed. Hydrolysable tannins are composed by gallotannins and ellagitannins, which by hydrolysis release gallic acid and ellagic acid, respectively (5, 21). In turn, condensed tannins, or proanthocyanidins, are the polymeric form of flavanols. The polymers differ from each other by the length of the chain and the type of link between the monomers.

The most important wine **flavanols**, or flavan-3-ols, are the monomeric components of procyanidins: *(−)*-epicatechin and *(+)*-catechin. *(−)*-Epigallocatechin and *(+)*-gallocatechin are the monomers of prodelphinidins and can also be found in wines. Flavanols are usually linked to the oxidative browning of white wine, especially due to the reaction with glyoxylic acid, forming yellow compounds known as xanthylium cations (21, 22). These reactions are favored by the pH and temperature increase (23).

Flavonols are yellow pigmented compounds, characterized by a double-bond on C-2 and a hydroxyl group on C-3, in the heterocyclic ring. The most important wine flavonols are kaempferol, myricetin and quercetin. Flavonols are partly responsible for

wine's antioxidant capacity. During aging and storage, they can react with anthocyanins to form co-pigments, decreasing their content in wine (21). Oxidative conditions and high temperatures are known to favor the co-pigmentation phenomena (24).

Non-flavonoid polyphenols are mainly found in grape pulp and include hydroxybenzoic acids, hydroxycinnamic acids and esters, and also stilbenes. **Phenolic acids**, such as syringic and vanillic acids, which are colorless, unflavored and odorless compounds, compose a great fraction of wine phenolics and are of particular interest because they are meaningful contributors to the antioxidant capacity of a wine (5). In turn, **stilbenes** are also of great importance, mainly due to *trans*-resveratrol. These compounds are defined by two aromatic rings linked together by a 3 carbon chain with a double-bond on C-2, and can exist as both the *cis*- or *trans*- isomer. The most abundant stilbene is *trans*-resveratrol, which is produced in grapes as a response to bacterial and fungal infections. It has gained interest due to its apparent benefits to human health, especially in the prevention of cancer and regulation of the synthesis of lipoproteins (25).

Polyphenols are in general highly reactive molecules due to the nucleophilic nature of the aromatic ring and the acid-base reactions of the hydroxyl groups. As it is known, wine undergoes several changes throughout maturation, involving changes on color intensity, aroma and flavor and decreasing of astringency, with softer mouthfeel. These changes occur due to various reactions that take place during this period (21). The most important reactions that occur during aging, and are consequently of particular interest to the topic of micro-oxygenation, will now be addressed. These reactions encompass chemical and enzymatic oxidation, direct or aldehyde-mediated condensation reactions between anthocyanins and tannins and cyclo-addition reactions.

In wine, phenols are the most important substrate for oxidation reactions, and can be involved in chemical and enzymatic oxidation. **Enzymatic oxidation** occurs early in the winemaking process due to oxidase enzymes, such as polyphenol oxidase (PPO) and laccase that convert phenols into quinones. The primary substrates for this type of oxidation are hydroxycinnamic acids and esters (5).

Chemical oxidation, on the other hand, is a slower process that results from the diffusion of oxygen, which usually occurs during barrel aging, and commonly results in a moderate oxidation of phenolic compounds. These are the reactions that micro-oxygenation intends to mimic. Chemical oxidation in wine starts with the formation of

oxygen reactive species (ROS), usually a hydroperoxyl radical, from the reduction of oxygen by iron and copper ions. It is this radical that then oxidizes hydrogen-donating phenols and transforms them into semi-quinones and quinones. These oxidation reactions also produce hydrogen peroxide, which in turn oxidizes ethanol into acetaldehyde (5, 15). The production of acetaldehyde plays an essential role in the reactions between anthocyanins and tannins. Condensation reactions between anthocyanins and tannins can be direct or mediated by an aldehyde. The resulting polymeric pigments are, at wine pH, mostly in the red colored form and since they can no longer react with SO_2 are also more resistant to discoloration (5). A direct condensation reaction involves two possible mechanisms. The first mechanism does not involve oxygen and takes place between the flavylium form of an anthocyanin (A^+) and a flavanol (T) to form a product denoted A-T. The other mechanism requires oxygen and occurs between a flavanol and the hydrated hemiketal form of an anthocyanin (AOH) leading to the formation of a T-A type product (5, 26, 27). In both cases the resulting polymers are colorless (15).

The most important mechanism for the condensation of anthocyanins and tannins, in oxidative conditions, is through indirect reactions that require an aldehyde, customarily acetaldehyde. Acetaldehyde is a product of fermentation, but can also be obtained from the oxidation of ethanol (5). This molecule is activated to its carbocation form, which reacts with a tannin molecule, forming a new carbocation, which, in turn, reacts with an anthocyanin (26). This reaction is much faster than direct condensation and the compounds formed are more stable and have an intense coloration. Dimeric and polymeric forms of tannins can also be involved in these reactions, originating larger polymeric complexes (5).

Cyclo-addition reactions are based on the addition of an ethylene chain to the heterocyclic ring of an anthocyanin. The chain is then oxidized to form a pyranoanthocyanin molecule. The importance of acetaldehyde is once again emphasized, as the addition of this compound to an anthocyanin molecule is one of the most important mechanisms to produce a pyranoanthocyanin. Pyranoanthocyanins are very stable and resistant to pH changes and reactions with SO_2 (10, 26). They have a red-orange coloration and are thought to play a key role in the color changes that occur during wine maturation (5, 15).

From these mechanisms, only aldehyde mediated condensations and cyclo-additions leading to the formation of pyranoanthocyanins are thought to benefit from exposure to micro-oxygenation treatments (26).

Given the importance of phenolic compounds on the characteristics of wine, most studies found on micro-oxygenation are focused on the impact of this technique on the phenolic composition and color of red table wines. In general, results tend to point towards an increase in polymeric anthocyanins, pyranoanthocyanins and ethyl-bridged compounds and subsequent decrease in free anthocyanins and tannin monomers (5). The effect of two different MOX treatments on the phenolic composition of a Sangiovese wine was studied by Castellari *et al.* (28), reporting a decrease in total phenols and an increase in the percentage of polymeric phenols. On the other hand, proanthocyanidins were only affected by an intensive MOX treatment, which caused a decrease in its concentration. The analysis of individual phenols showed that, while caftaric and coutaric acids did not seem to be affected, both MOX treatments resulted in lower concentrations of quercetin. Other low molecular weight compounds, such as gallic, caffeic and ferrulic acids, *(+)*-catechin, *(-)*-epicatechin and *trans*-resveratrol decreased significantly, but only due to the intensive treatment. Ganić *et al.* (29) also determined that the addition of oxygen to a Plavac Mali wine resulted in a decrease in total anthocyanins and an increase in the polymeric anthocyanins. Additionally, the authors noticed that, although the concentration of total phenolics increased over time, its level was slightly lower in the treated wines. González-del Pozo *et al.* (30), on the other hand, did not find significant differences in the content of total phenols between a Cabernet Sauvignon supplied with oxygen and the control. However, the authors verified a significant decrease in the concentrations of free and total anthocyanins.

Atanasova *et al.* (26) reported that micro-oxygenation favored the acetaldehyde dependent reactions, resulting in a decrease of the concentration of anthocyanins and a concomitant increase in ethyl-bridged compounds and pyranoanthocyanins. These results are similar to those found by Pérez-Magariño *et al.* (31), while studying the impact of oxygen addition on the phenolic composition of four monovarietal wines – Mencía, Tinta de Toro, Tinta del País and Tempranillo – during three consecutive vintages. They determined that, in general, MOX treated wines tend to have slightly lower levels of phenolic compounds, particularly total anthocyanins. On the other hand, these wines presented higher levels of polymeric anthocyanins, indicating a high influence of oxygen

on polymerization reactions. Cano-López *et al.* (32) supplied a Monastrell wine with oxygen for 3 months in order to determine if MOX could mimic the effects of barrel aging. The resulting wines were very similar to those aged in oak barrels for the same period, however, they had lower concentrations of monomeric anthocyanins and higher content of vitisin-related compounds, when compared to the control wine kept in a stainless steel tank.

Sartini *et al.* (33) evaluated the influence of a combined treatment of micro-oxygenation and chips on the phenolic composition of a Sangiovese wine. The MOX treatment, which lasted for 90 days, resulted in wines with lower content of total anthocyanins, as well as a decrease in the concentrations of *(+)*-catechin, *(-)*-epicatechin, quercetin, miricetin and caftaric acid. Similarly, Cejudo-Bastante *et al.* (34) studied how the addition of oak chips and oxygen before MLF influenced the color-related phenolic compounds of a Merlot wine. The wine was analyzed after the MOX treatment and again after MLF, revealing significantly lower concentrations of monomeric anthocyanins and anthocyanin-derived pigments, such as hydroxyphenyl-pyranoanthocyanins and anthocyanin-ethyl-flavan-3-ol adducts.

Ortega *et al.* (35) studied the effect of two oxidative aging models on the polyphenolic composition and color of a Sherry type wine. The study compared oak cask aging and stainless steel tank aging submitted to successive oxygen saturations, at different temperatures. In general, their results showed that the concentrations of individual polyphenols in oxygenated wines were lower than in wines aged in oak casks. Additionally, compounds such as vanillin and syringaldehyde, which are known to develop during aging in wood casks, were only found in trace amounts and showed no evolution in the wines aged in stainless steel tanks with oxygen addition.

1.8. Effect on color

As previously mentioned, changes in the polyphenolic composition and color characteristics are the main expected outcomes of oxygen exposure. As such, several studies have been developed, in which the impact of the micro-oxygenation treatments in the color evolution of red table wines is prominently featured.

Some researchers have suggested that micro-oxygenation stabilizes and increases the color intensity of red wines, protecting it from degradation and SO_2 bleaching. Atanasova *et al.* (26) treated a red wine (Cabernet Sauvignon and Tannat blend) with 5 mL/L O_2 and showed that the color intensity of the micro-oxygenated (MOX) wine decreased less than that of the control wine. Moreover, the same study also revealed a higher proportion of SO_2 bleaching resistant pigments in the micro-oxygenated wine, attributed to the formation of pyranoanthocyanins. Similar results were found by Cano-López *et al.* (32), who reported that SO_2 bleaching resistant pigments found in a Monastrell O_2 treated wine were in lower quantities compared to those found in a barrel aged wine, but higher than those found in the control wine. The authors also reported that, while the MOX wine evolved in a similar manner to the barrel aged wine, considerable differences arose after six months of bottle aging, in which the MOX wine became chromatically different, with higher values of tonality. This was justified by the protective effect on wine color, potentially developed by the wood phenolics. Pérez-Magariño *et al.* (31) also reported a slight decrease of the red component concomitant with a significant increase of the blue component, which allowed the stabilization of the color of four monovarietal micro-oxygenated red wines (Mencía, Tinta de Toro, Tinta del País and Tempranillo grape varieties).

In contrast to these findings, Llaudy *et al.* (36) revealed a decrease on the color intensity and the red, yellow and blue components, after a Carbernet Sauvignon wine was treated with 3 mg/L per month O_2 for three months, before oak aging.

Other authors, namely Cano-López *et al.* (37), Cano-López *et al.* (38), Ganić *et al.* (29), and Sánchez-Iglesias *et al.* (39), suggest that the impact of MOX on wine color usually follows a consistent pattern, in which treated wines suffer a smaller decrease in color intensity and develop higher quantities of SO_2 bleaching resistant pigments. This is in agreement with the proposed theory that micro-oxygenation favors the production of more stable polymeric pigments that benefit wine color.

It is also worth mentioning that two analogous studies carried out by Cejudo-Bastante *et al.* (34), with Merlot and Petit Verdot monovarietal wines, brought about different results. This led the authors to propose that grape variety has influence on the final results of micro-oxygenation, in particular on wine color and polyphenols. This was

subsequently supported by Pérez-Magariño *et al.* (40), after a study with Mencía and Tinta del País monovarietal wines.

1.9. Effect on aroma compounds

The effect of micro-oxygenation treatments on wine aroma compounds is still greatly unexplored. In fact, there are not many published studies on this subject (8, 41).

One of the first papers published on this topic was developed by Heras *et al.* (41), about two monovarietal wines, Tinta de Toro and Mencía, of two successive vintages. After alcoholic fermentation, the wines underwent two customized micro-oxygenation treatments based on their initial characteristics. The end of the treatments was determined by sensorial analysis and was followed by malolactic fermentation and a twelve month barrel aging period. Differences between the micro-oxygenated wines and the control wines were detected and seemed to increase with the time of maturation. Furthermore, it was observed that the composition of ethyl esters remained unchanged and that fatty acids revealed a small increase in their concentration, but not enough to bring about significant changes in the wine aroma. A varietal and vintage effect was also observed, which was impeditive for authors to draw conclusions about the effect of oxygen on the composition of terpene compounds, usually linked to fruity and floral notes, and C6 alcohols, proposed as being responsible for green and herbaceous aromas. MOX wines showed similar or higher concentrations of these compounds, leading authors to believe that the perceived decrease of green notes should be related to other compounds. Additionally, it was observed that, after the aging period, MOX wines possessed much lower concentrations of important compounds extracted from wood, such as furfural, 5-methylfurfural, eugenol and *cis*-whiskey lactone.

Hernández-Orte *et al.* (42) studied Tempranillo and Cabernet Sauvignon wines submitted to the addition of 60 mL/L per month of O_2 for fifteen days, prior to malolactic fermentation. Once again, a varietal effect was described. It was found that micro-oxygenation did not have much influence on the concentration of terpenes and C6 alcohols. The significant increase observed in ethyl esters and alcohol acetates right after the treatment disappeared after malolactic fermentation and during the following maturation period. The same attenuating effect was described for most compounds

affected by micro-oxygenation. Nevertheless, after 8 months in maturation, significant sensorial differences were perceived between the micro-oxygenated and the control wines.

Cejudo-Bastante *et al.* (34) applied a 30 mL/L per month flow of O_2 to a Merlot wine, before malolactic fermentation. A decrease in fatty acid esters, C13 norisoprenoids and benzenic compounds was described. This was accompanied by an increase of alcohols, with the exception of methanol and isoamylic alcohols. A significant increase in acetaldehyde was also observed. Acetaldehyde presented a similar behavior in a study with Cencibel red wines (43) that underwent micro-oxygenation prior to malolactic fermentation. After 5 months in stainless steel vats, the treated wines also showed a decrease in the concentration of short-chain esters, acetates and C6 alcohols, as well as an increase of long-chain esters and succinic acid derivatives. A sensorial analysis of these wines showed that MOX wines presented improved aromatic complexity and a decrease in herbaceous aromas.

Another study with Cencibel wines, also developed by Cejudo-Bastante *et al.* (8), revealed an increase in C6 alcohols, lactones, long-chain esters and terpenes, particularly eugenol, after being submitted to micro-oxygenation. No significant differences were noticed in the concentration of short- and medium-chain esters. An increase in acetaldehyde was once again reported, presumably due to the oxidation of ethanol. However, after the last micro-oxygenation step, the concentration of this aldehyde diminished to the same values as the control wines. It was suggested that this might have been caused by over-oxygenation, which could also be responsible for the disappearance of β-damascenone and α-ionol.

Most authors recommend the introduction of micro-oxygenation treatments right after alcoholic fermentation but before malolactic fermentation. Indeed very few studies have been found in which the effect of oxygen after malolactic fermentation or subsequent maturation periods was evaluated (11, 33, 36, 44-46). Yet, in order to improve color stability and sensorial characteristics of two monovarietal red wines, Touriga Nacional and Sousão, Trigo (47) applied two consecutive micro-oxygenation treatments after malolactic fermentation. Despite the notorious sensorial differences between oxygenated and control wines, the author was not able to find differences in the volatile profile of the wines.

Ugliano *et al.* (48) investigated the impact of oxygen exposure, pre- and post-bottling, on the evolution of volatile sulfur compounds of two Shiraz wines from different origins. According to the authors, the oxygen treatments had very limited effect on these compounds during bottle maturation, and only one of the wines showed an increased accumulation of hydrogen sulfide and methyl mercaptan.

A study on the forced aging of Port wine showed that the exposure of these wines to high temperatures (60 ºC) and different concentrations of oxygen (0, 1, 2 and 5 saturations, achieved by stirring the wine during 1 hour until a DO concentration of 8-9 mg/L) promotes the production of dioxane isomers (*cis*- and *trans*-5-hydroxy-2-methyl-1,3-dioxane), dioxolane isomers (*cis*- and *trans*-4-hydroxymethyl-2-methyl-1,3-dioxolane), furfural and 5-hydroxymethylfurfural, having impact on color, aroma and taste (49). Another study, in which the combination between different oxygen addition treatments (0, 3, 5 and 10 saturations) with different temperatures (20, 30, 35 and 40 °C) was tested, revealed that oxygen exposure and temperature increased the concentrations of sotolon, a key odorant in Port wine. Furthermore, it was reported that the combined effect of oxygen and temperature increased the rate of formation of this compound. As a result the wine that underwent 10 saturations at 40 °C had the highest amount of sotolon (6).

1.10. Effect on organoleptic characteristics

Changes in the aroma and mouthfeel characteristics are commonly indicated as benefits of micro-oxygenation, since the reduction of green and herbaceous aromas, the increase of fruity attributes and the decrease in astringency is observed. Moreover, it has also been suggested that the exposure to small and controlled doses of oxygen can help integrate the aroma from wood (41). However, once again, there are not many scientific studies focused on the impact of oxygen in the sensory characteristics of wine.

Some of the studies mentioned on Section 1.9., regarding the effect of micro-oxygenation on the aroma compounds, also include reports on its impact in the wine sensory characteristics. For example, Cejudo-Bastante *et al.* (34) described significant sensorial differences between the control and the MOX wine. The Merlot MOX wines contained higher red fruit and spicy attributes, developed nutty and sweet fruit flavors

and had reduced astringency. The olfactory analysis revealed a decrease in woody aromas after treatment and no significant changes in green aromas.

The same researchers reported that micro-oxygenated Cencibel red wines (43) (prior to malolactic fermentation followed by a five month storage period) presented less red fruit notes and higher liquorice and spicy attributes. The gustatory analysis revealed that, after the storage period, MOX wines presented decreased acidity, bitterness and green taste but higher astringency. This was justified by the higher degree of polymerization caused by over-oxygenation. They also concluded that MOX wines had improved complexity and quality.

Finally, Cejudo-Bastante *et al.* (8) also evaluated the effect of a micro-oxygenation treatment after malolactic fermentation in Cencibel wines. This time, they observed a reduction in liquorice notes and an increase in red fruit, spicy and plum/currant notes. However, the plum/currant scent diminished at the end of the treatment. In terms of gustatory attributes, a decrease in herbaceous notes, bitterness and acidity was once again reported, accompanied by a decrease in astringency. These results are similar to those obtain by Trigo (47) with Touriga Nacional and Sousão red wines. They reported a decrease in astringency in both wines. When treated with oxygen, Touriga Nacional also showed a decrease in herbaceous attributes and an increase in fullness and red fruit notes. It was concluded that both wines had higher global quality.

In 2006, du Toit *et al.* (11) studied the effect of micro-oxygenation on the quality of different South African red wines. They observed that tasters preferred young micro-oxygenated wines better than the control. However, they also noticed that when older wines underwent oxygen treatments they became over-aged and developed medicinal notes, which the authors correlated with an increase in *Brettanomyces*.

De Beer *et al.* (14) in turn, compared red wines treated with a low dose (2.5 mg/L per month) and a high dose (5.0 mg/L per month) of O_2 with a control wine. No significant changes in astringency were observed. MOX wines presented higher scores for fullness but a decrease in berry/plum notes, especially for the high-dose treated wines, which also seemed to have lower overall quality.

In a similar study with a Cabernet Sauvignon wine, a consumer preference test revealed that most people preferred the low-dose oxygen treated wine (25 mL/L per

month) over the high-dose oxygen treated wine (50 mL/L per month) and the control. A sensory analysis performed by experts showed a decrease in astringency, an increase in mouthfeel complexity and roundness and a more intense aroma (50).

1.11. Effect on the dissolved oxygen content

The concentration of dissolved oxygen (DO) in wine is a helpful tool that can give clues to see whether a wine is being under or over micro-oxygenated. Therefore, it is important to understand what other parameters can influence oxygen solubility and consumption. According to Lesica *et al.* (4), oxygen in wine reaches saturation levels at 7.7 mg/L at 20 ºC. Temperature has a dual effect on its concentration: higher temperatures decrease oxygen solubility but increase oxygen consumption. Ethanol content, pH and concentration of phenolic compounds are also known to affect oxygen consumption.

Ideally, the concentration of DO should remain constant throughout a micro-oxygenation treatment in order to ensure that the rate of supply is sufficient, but not superior to the rate of consumption. Consequently, the measurement of DO can be of key importance during a micro-oxygenation treatment.

In this regard, the homogeneity of the concentration of DO in wine during a micro-oxygenation treatment can be a problem. Nevares *et al.* (51) found that gradients of DO levels were developed during an oxygen treatment and that the column of wine right above the diffusing system possessed greater concentrations of DO. Consequently, this can result in wines with different levels of oxidation. These conclusions are of particular concern in cases where very small doses of oxygen are needed. Castellari *et al.* (52) determined that the addition of 5 mL/L per month of O_2 to wine maturing in tanks was enough to match the concentrations of DO to those measured in wine stored in casks.

Laurie *et al.* (53) performed two experiments with a Cabernet Sauvignon wine in order to determine the DO concentrations. In the first experiment, the wine was submitted to a flow rate of 5 mL/L per month of O_2 after malolactic fermentation. After one month, the DO concentration measured for the control wine ranged from 25 to 30 µg/L while for the treated wine ranged between 50 to 270 µg/L. In the second experiment, they applied micro-oxygenation before and after malolactic fermentation, varying flow rates from approximately 60 to 0.5 mL/L per month. Once again, the control wines had lower DO

concentrations (4 to 14 µg/L) than treated wines (220 to 2400 µg/L). However, when the oxygen flow reached 0.5 mL/L per month the DO values became much more similar to those of the control wine.

While trying to improve the dosage for the different phases of an accelerated-aging process, Nevares *et al.* (54) monitored the evolution of dissolved and consumed oxygen throughout a micro-oxygenation treatment. It was found that the levels of consumed oxygen were positively correlated with volatile and total acidity, pH, content of anthocyannins, color intensity and red color component. In addition, it was also found that consumed oxygen was inversely correlated to sugars, tartaric acid content, hue and yellow color component.

Martins *et al.* (6) submitted Port wines to different combinations of oxygen levels and temperatures in order to evaluate the impact of these two parameters on the wine quality. They observed that the rate of oxygen consumption was higher for wines with higher oxygen concentrations, whereas wines with lower oxygen levels presented lower consumption rates. It was also reported a more rapid oxygen consumption at the beginning of the experiment, which can be associated with the concentration decrease of substrate towards the end of the study.

2. MADEIRA WINE

Madeira wine is a fortified wine, with an alcohol content that ranges between 17 and 22 % (55), produced at the Island of Madeira, from where it got its name. It possesses distinctive, intense and complex aroma and flavor and, as such, is mostly consumed as an aperitif or dessert wine. Its origin dates back to the 15th century, time at which the island was first discovered by Portuguese sailors. It soon became a much appreciated wine worldwide and is still today a hallmark of the Island.

Madeira wine can be produced from both white and red recommended varieties of *Vitis vinifera* L. grapes. Nowadays, a small part of the production is obtained from the traditional varieties Sercial, Verdelho, Boal and Malvasia, which originate dry, medium-dry, medium-sweet and sweet wines, respectively. The vast majority of the production (around 80% of total production) is obtained, however, from the Tinta Negra grape

variety. This red grape variety, due to its versatility, allows the production of all styles (56-58).

In addition to its organoleptic characteristics, Madeira wine also stands out due to its winemaking process, in particular its aging process. Briefly, after grape processing, the must can ferment under two types of vinification processes: *bica aberta* or *curtimenta*. The difference between these two is that, while in *curtimenta* the must is left to ferment in contact with grape solids, in *bica aberta* the must is first pressed and the grape solids are removed before fermentation begins. In either case, when the desired level of sweetness is obtained, fermentation is stopped by the addition of natural grape spirit (96% (v/v) of ethanol) until the alcohol content reaches 17-22% (v/v) (55).

Typically, Madeira wine can undergo two unique aging processes: *canteiro* and *estufagem*. In the *canteiro* aging process the wine is kept in wooden casks, usually oak, and aged in the attics of the wine cellars, where the temperatures are higher, for a minimum period of at least two years. In the *estufagem* process the wine is placed in stainless steel tanks equipped with a coil system. As hot water passes through the coil, the wine is heated to temperatures that range from 45 to 50 ºC, for at least three months. After *estufagem*, the wine is subjected to another aging period, usually in wooden casks, until it is deemed ready to be bottled and sold (55, 58).

This oxidative aging process is usually related to the development of the wine main characteristics, which include complex aroma and flavor and color attributes, that can vary from very pale (typical of dry wines), to dark brown (typical of sweet wines). It is also thought to be responsible for the robustness and extended longevity that are typically associated to this wine (59).

In recent years, several studies have been published about Madeira wine, mainly focusing on volatile profile (18, 59-65), analytical characterization (66-70) and changes occurring during the winemaking process (56-58, 70, 71), especially during aging. The impact of the *estufagem* process on the chemical constituents of Madeira wine has been studied (58, 70). It was reported that the concentrations of most polyphenols decreased about 30% during *estufagem*, however, this process did not have a severe impact on the wines total polyphenolic content. Additionally, the wines submitted to this process adopted a similar color evolution trend, with an increase of the yellow tones (70). This is in agreement with the results obtained by Carvalho *et al.* (71) that studied the color

evolution of different Madeira wines during two years of aging under *estufagem* and *canteiro*. In this study, the authors found that the color of wines made from white grape varieties tend to evolve in a similar way, independently of their sweetness degree. On the other hand, Tinta Negra wines showed different color evolution trends depending on their sweetness degree, on the first two years of aging.

Pereira *et al.* (59) studied the effect of the *estufagem* process on the volatile composition of Madeira wine and was able to identify 190 compounds, predominantly esters and alcohols. The study showed that the process led to an increase in esters and furans and a decrease in acetates, alcohols and fatty acids. Moreover, authors concluded that *estufagem* resulted in a higher content of volatile compounds. In fact, 53 of the compounds identified were exclusively found after the heating process.

Câmara *et al.* (61) was the first to identify and determine the concentration of 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon) in Madeira wines. Sotolon is a chiral lactone, well known as a powerful odorant, which can impart a nutty, caramel, curry or rancid odour, depending on its concentration and enantiomeric distribution (72, 73). It has been identified in several wines, including Sherry (74), Port (72) and Madeira (61). Sotolon is usually associated to the premature oxidative aging of dry white wines, however, in fortified wines, such as Port, Madeira and Sherry, it is recognized as a key odorant and has, in fact, been classified as a potential aging and oxidation marker of these kind of wines (56, 72, 74). Several attempts have been made to identify the precursors and formation pathways of sotolon in different food matrices. Kobayashi (75) suggested that sotolon could be produced by an aldol condensation between acetaldehyde and α-ketobutyric acid (resulting from the deamination of threonine), followed by cyclization. It has also been suggested that, in fortified wines, sotolon could result from the condensation between pyruvic and glutamic acids (76). However, in Port and Madeira fortified wines, it is generally accepted that the connection between certain winemaking conditions, such as oxidation, sugar concentration, temperature and storage time influences the formation of sotolon, suggesting that its origin might involve a connection between oxidation and Maillard mechanisms (6, 77). Recently, using model systems, Pereira *et al.* (78) has demonstrated that the production of sotolon in Madeira wines can derive from the degradation of fructose, in acidic medium. Despite the formation pathways of sotolon not yet being completely understood, a relationship has been established between the concentrations of sotolon and other wine age indicators, such as

furfural, 5-methylfurfural and 5-hydroxymethylfurfural (HMF) (61, 72). Moreover, a correlation has also been found between the production of sotolon and heterocyclic acetals (49), such as *cis*- and *trans*-5-hydroxy-2-methyl-1,3-dioxane (1,3-*cis/trans*-dioxane) and *cis*- and *trans*-4-hydroxymethyl-2-methyl-1,3-dioxolane (1,3-*cis/trans*-dioxolane), which are also strongly correlated with aging and oxidation in fortified wines (7, 64). These kind of acetals are generated by the acid-catalysed condensation reaction between glycerol and acetaldehyde, at wine pH, and have also been reported as one of the indicators of Port (7) and Madeira (64) wines aging, under oxidative conditions. Additionally, they can also contribute to the aroma of very old fortified wines.

3. OBJECTIVES

Madeira wine is well known for undergoing an oxidative aging process. Thus, the main objective of this work was to determine how the addition of oxygen, by means of three MOX treatments, at different stages of the aging process (one treatment before and two other treatments during *estufagem*), would impact the main characteristics of a Tinta Negra sweet wine. The parameters proposed for this evaluation included dissolved oxygen content, general oenological characteristics (such as alcohol content, volumetric mass density, volatile acidity, total acidity, pH and reducing sugars), color, individual polyphenols and furans, volatile composition and sensory analysis. Additionally, given how sotolon and the heterocyclic acetals (*cis*- and *trans*-dioxane and *cis*- and *trans*-dioxolane), known as aging indicators for Madeira wines, have been shown to be affected by the addition of oxygen, the development of a simple and accessible extraction procedure to analyze these compounds and determine how their development was influenced by the MOX treatments was also proposed.

PART II – EXPERIMENTAL

4. PRELIMINARY STUDIES

4.1. Chemicals and standards

Ultra-pure water that was used throughout the experiment was obtained from a Millipore Simplicity UV purification system (Milford, MA, USA), 3-octanol (97%), used as an internal standard, was from Acros Organics (Geel, Belgium) and sodium chloride (99.5%), used in the solid-phase micro-extraction procedure, was from Panreac Química S.A. (Barcelona, Spain).

4.2. Samples and oxygenation treatment conditions

In order to verify whether oxygen had an impact on the characteristics of Madeira wine during its forced aging period (*estufagem*) a preliminary study was carried out. A 2013 wine made from the *Vitis vinifera* L. white grape variety Sercial was divided into nine 120 mL amber flasks and kept in an oven (Memmert, Schwabach, Germany) at 45 ºC for two months, in order to mimic the e*stufagem* process. Of the nine flasks, three corresponded to a control group and the other six to the oxygenation treatments.

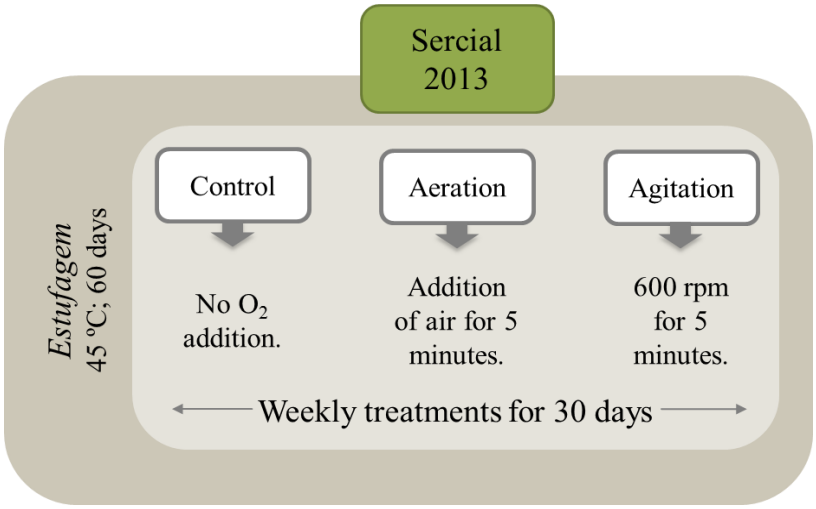

Figure 1: Schematization of the two oxygen addition treatments performed weekly during the first 30 days of the *estufagem* process.

Two different oxygenation treatments were tested – aeration and agitation (Figure 1). The aeration treatment consisted on adding air directly to wine, for 5 minutes, using a KNF Laboport vacuum pump (KNF, Freiburg, Germany). For the agitation treatment, samples were agitated using a magnetic stirrer (Labbox Labware, Barcelona, Spain) for 5 minutes at 600 rpm. Both oxygenation treatments were performed weekly for a month.

4.3. Dissolved oxygen and pH measurements

Dissolved oxygen levels and pH were measured weekly during the first month, both before and after oxygenation treatments, and once again at the end of the second month. The concentration of DO was measured, in mg/L, using an YSI 5000 Dissolved Oxygen meter (YSI, Yellow Springs, Ohio, USA), with a measurement range from 0.0 to 60.0 mg/L and an accuracy of ±0.1%. Prior to use, the equipment was calibrated according to the manufacturer's instructions. Wine pH was determined using a Metrohm pH meter (Metrohm, Switzerland), which was also calibrated before being used.

4.4. Color parameters

The chromatic characteristics were evaluated by the determination of the Glories parameters, namely color intensity (CI), tonality (To), yellow color component (A420 nm), red color component (A520 nm) and blue color component (A620 nm). The analysis of these parameters was carried out on a Shimadzu UV-vis 2600 spectrophotometer (Shimadzu, Kyoto, Japan). The readings were performed in triplicate, using quartz cells with a path length of 1 cm and ultra-pure water as blank. Prior to analysis, samples were filtered through 0.20 µm Chromafil Xtra (Macherey-Nigel, Düren, Germany) syringe filters.

4.5. Solid-phase micro-extraction and GC-MS analysis

For the extraction of volatiles by headspace solid-phase micro-extraction (HS-SPME) samples were previously prepared by adding 10 µL of internal standard (3-octanol, 500 mg/L) to 20 mL of wine sample. Of this solution, 5 mL were transferred to

a 20 mL vial containing 3 g of sodium chloride and 5 mL of ultra-pure water. The vials were properly capped and then homogenized.

The extraction was carried out in duplicate, exposing the SPME fiber, 50 µm/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxan (DVB/CAR/PDMS, bipolar, adsorbent), into the vial containing the sample for 30 minutes at 60 ºC, under constant stirring. The extracted compounds were then thermally desorbed for 5 minutes at 240 ºC in the GC injector inlet. Between extractions, the fiber was conditioned at 260 ºC for 10 minutes.

The analysis were carried out on a TRACE GC Ultra gas chromatograph equipped with an ISQ single quadrupole (on electronic impact mode, at 70 e^-V) and a TriPlus autosampler (on SPME mode) from Thermo Scientific (Hudson, NH, USA). The column used was a DB-WAXETR 30 m x 0.25 mm with 0.50 µm film thickness from Agilent J&W (Folsom, CA, USA). Injections were performed in splitless mode using helium (Air Liquide, Portugal) as carrier gas, at a constant flow rate of 1 mL/min. The transfer line and the ion source were both kept at 230 ºC. The oven temperature program was set at an initial temperature of 40 ºC for 5 minutes, increased 3 ºC/minute until 230 ºC and held at 230 ºC for 15 minutes. Chromatograms were acquired in total ion count (TIC), in the range m/z 30-300.

Data was recorded and processed using the Thermo Xcalibur 2.2 software and compounds were identified by comparing their mass spectra to those of authentic compounds or in the NIST08 and Wiley 6.0 spectral databases. In addition, Kovats indexes were determined, using a C_7-C_{30} n-alkanes mixture, and compared to those reported and compiled in the NIST Chemistry WebBook (79). The quantification of the identified compounds was expressed in terms of the internal standard (3-octanol).

5. IMPACT OF MICRO-OXYGENATION DURING MADEIRA WINE AGING

5.1. Chemicals and standards

Ultra-pure water was used and was obtained from a Millipore Simplicity UV purification system (Milford, MA, USA). Sodium chloride (99.5%) from Panreac Química S.A. (Barcelona, Spain) was used in the solid-phase micro-extraction procedure. Dichloromethane (analytical grade) from Fisher Scientific (Loughborough, UK) was used for the extraction of sotolon and the dioxane and dioxolane isomers. The sotolon (3-hydroxy-4,5-dimethylfuran-2(5H)-one, ≥97%) standard used to prepare the stock solution was from SAFC (St. Louis, MO, USA) and 3-octanol (97%), used as internal standard, was from Acros Organics (Geel, Belgium). Absolute ethanol and sodium hydroxide, both obtained from Panreac (Barcelona, Spain), and *L-(+)*-tartaric acid, from Merck Co. (Darmstadt, Germany), were used to prepare synthetic wine. Ethyl acetate (HPLC grade) from Fisher Scientific (Loughborough, UK), sodium citrate tribasic dehydrate (≥98%) from Sigma-Aldrich (St. Louis, MO, USA), sodium citrate dibasic sesquihydrate (99%) from Acros Organics (Geel, Belgium) and anhydrous magnesium sulfate (technical grade) from Panreac Química S.A. (Barcelona, Spain) were tested during the development of an extraction procedure of sotolon and the dioxane and dioxolane isomers. The standards used for the identification and quantification of individual polyphenols and furans were as follows: ellagic acid and sinapic acid were from Fluka Biochemika (Switzerland); caftaric acid, *(+)*-catechin, *(−)*-epicatechin, *(−)*-epigallocatechin, *(−)*-epigallocatechin gallate, ferulic acid, gallic acid, kaempferol, myricetin, *p*-coumaric acid, quercetin, rutin and *trans*-resveratrol were from Sigma-Aldrich (St. Louis, MO, USA) and caffeic acid, HMF, furfural, p-hydroxybenzoic acid, protocatechuic acid, syringaldehyde, syringic acid, vanillic acid, and vanillin were from Acros Organics (Geel, Belgium). Methanol and acetonitrile, used to prepare the mobile phases, were both HPLC gradient grade and were obtained from Chem-Lab (Zedelgem, Belgium). Phosphoric acid and ammonium dihydrogen phosphate, used to prepare the phosphate buffer solution, were both from Fisher Scientific (Loughborough, UK). All mobile phase solutions were filtered through 0.20 µm membrane filters from Pall (Ann Arbor, MI, USA).

5.2. Samples and oxygenation conditions

The impact of micro-oxygenation treatments during the aging period of a sweet Madeira wine, made from the *Vitis vinifera* L. red grape variety Tinta Negra was evaluated. After undergoing the typical winemaking process, the wine was transferred to six 200 L vats. Three micro-oxygenation treatments were employed at different stages of the aging process. The first treatment (t1) was applied before e*stufagem* and lasted 78 days. During this period, a continuous flow of 66 mg O_2/L per month was applied to two vats (MOX1 wine). Other two did not receive oxygen addition at this stage and were used as control. Two more were reserved for another experiment (MOX2).

When the t1 treatment was concluded all wines were transferred to 200 L stainless steel tanks and heated at 45 ºC for four months. The second micro-oxygenation treatment (t2) was introduced during the *estufagem* period. At this stage, the two wines previously reserved underwent micro-oxygenation and the wines that had previously been micro-oxygenated did not receive any more oxygen addition. In the t2 treatment, which lasted 60 days, a flow rate of 66 mg O_2/L per month was added to MOX2 wine. MOX2 wine underwent an additional period of micro-oxygenation for 65 days (t3), during which the oxygen flow rate was increased up to wine saturation: the flow rate was initially raised up to 660 mg O_2/L per month (increased by ten-fold) and was gradually increased up to a final flow rate of 1122 mg O_2/L per month, in order to compensate for the continuous decrease in DO levels.

The continuous oxygen (Air Liquide, Portugal) flow rate was delivered by an Enartis Microox 2 (Enartis, Windsor, CA, USA) micro-oxygenation system, fitted with two dosing points connected to two stainless steel diffusers (Enartis, Windsor, CA, USA). The diffusers were suspended inside the thanks, near to the bottom but without touching it. Figure 2 illustrates the sequence by which each wine was subjected to micro-oxygenation.

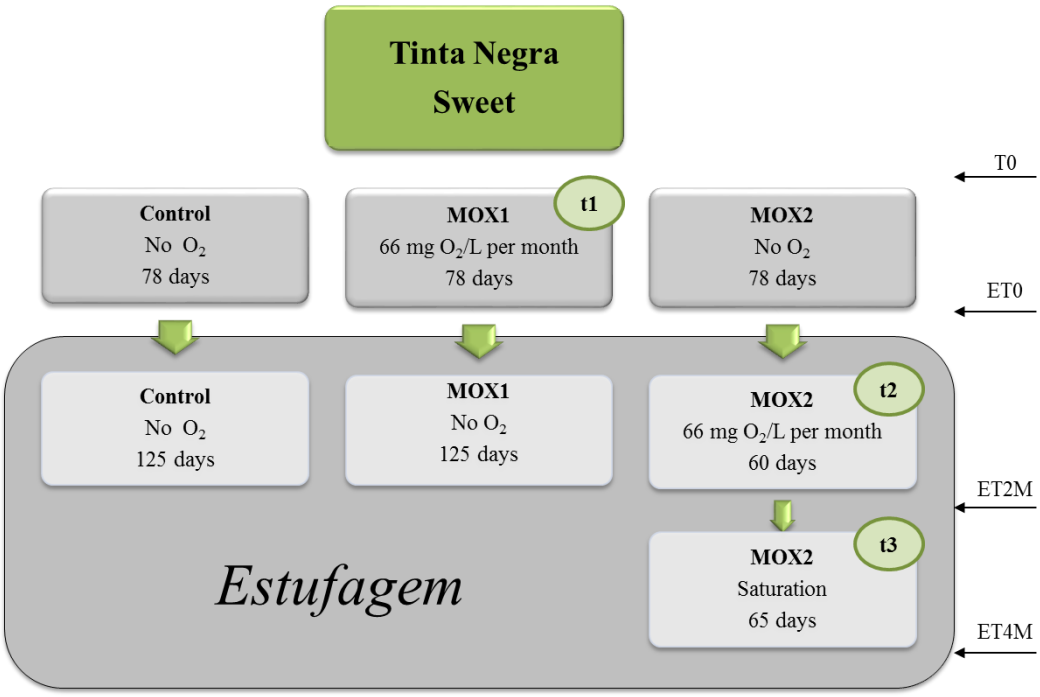

Figure 2: Schematization of the micro-oxygenation treatments introduced before and during the *estufagem* process. t1 – micro-oxygenation treatment 1; t2 – micro-oxygenation treatment 2 and t3 – micro-oxygenation treatment 3. Four key sampling points were established: T0 – before t1 treatment; ET0 – before t2 treatment and beginning of *estufagem*; ET2M – before t3 treatment; ET4M – end of t3 treatment and *estufagem*.

5.3. Dissolved oxygen

The concentrations of DO were measured weekly during the t1 and t2 treatments. During the t3 treatment DO levels in the MOX2 wine were only controlled in order to check if the saturation levels were maintained.

The determination of the DO concentrations (in mg/L) was performed using an YSI 5000 Dissolved Oxygen meter (YSI, Yellow Springs, Ohio, USA). The equipment was calibrated according to manufacturer's instructions prior to use. Measurements were carried out in triplicate for every tank.

5.4. General oenological and color parameters

General oenological parameters, namely alcohol content (%), volumetric mass density (g/cm^3), volatile acidity (g/L, expressed in terms of acetic acid), total acidity (g/L, expressed in terms of tartaric acid), pH and reducing sugars (g/L), were determined using

Bacchus 3 MultiSpec analyzer, fitted with an iD1 transmission accessory and patent from Tecnología Difusión Ibérica, S.L (Barcelona, Spain). The equipment includes a rapid-scanning infrared Fourier-transform spectrophotometer, Nicolet iS5 from Thermo Scientific (spectral zone range between 7800 – 350 cm^{-1}), with CaF_2 windows, a Czerny-Turner UV-Vis spectrophotometer (250-600 nm) fitted with 0.2 mm flow cells and an autosampler with previous thermostatization of the samples by Peltier effect at 27 ºC. The adjustment of the standard calibration provided by the Bacchus Analysis software was performed, recording a wide spectral zone that covers UV-Vis-IR (250-3000 nm) and using reference values of 503 Madeira wines, which were determined according to standard methods. The calibrations adjusted to a linear regression with an average R^2 of 0.96. Data acquisition and processing was done using the Bacchus 3 Analysis 2.4.0.8 software. Before analysis, all samples were filtered through 0.20 µm Chromafil Xtra (Macherey-Nigel, Düren, Germany) syringe filters. Readings were carried out in duplicate.

The equipment also allowed the reading of absorbances at 420, 520 and 620 nm, corresponding to the yellow, red and blue color components, respectively. The contribution of each color (in percentage) to the overall color of the wine is calculated by the ratio between the absorbance at the corresponding wavelength and color intensity. These values were also used to determine two other Glories parameters – color intensity and tonality. Color intensity is expressed as the sum of absorbances at 420, 520 and 620 nm and tonality is defined as the ratio between absorbances at 420 and 520 nm.

5.5. Individual polyphenols and furans

The analysis of individual polyphenols and furans was carried out by high performance liquid chromatography coupled with photodiode array detection (HPLC-PDA), following a methodology previously described by Pereira *et al.* (70). Briefly, after being filtered through 0.20 µm Chromafil Xtra (Macherey-Nigel, Düren, Germany) syringe filters, the samples were directly injected in a Waters Alliance liquid chromatographer (Milford, MA, USA) equipped with an auto-injector (Waters 2695) and a photodiode array detector (Waters 2996). An Atlantis T3 column (250 × 4.6 mm; 5 µm; Milford, MA, USA), set at 30 ºC, was used to separate the compounds. The elution

solvents used were: A – 10 mM of phosphate buffer solution, pH 2.70, B – acetonitrile and C – methanol, using the same gradient established by Pereira *et al.* (70) (Table 1).

Table 1: Gradient program for the analysis of individual polyphenols. Mobile phases used were: A - 10 mM phosphate buffer solution, pH 2.70; B - acetonitrile; and C – methanol (70).

Time (min)	Flow rate (mL/min)	%A	%B	%C
--	1.0	100	0	0
30.0	1.0	79	10	11
42.0	1.0	73	10	17
55.0	1.0	40	60	0
58.0	1.0	40	60	0
65.0	1.0	100	0	0

Data was recorded and processed using the Empower Pro 5 software from Waters Corporation. Compounds were detected at 210 (flavan-3-ols and benzoic acids), 280 (furans), 315 (hydroxycinnamic acids and stilbenes) and 360 nm (flavonoids) and were individually identified by elution order, retention time and the comparison of their UV-vis spectra with those of the standards. The external standard method was used for compound quantification. All samples were injected in duplicate.

5.6. Volatile profile

The analysis of volatile compounds was performed by HS-SPME/GC-MS, using experimental conditions similar to those described in Section 4.5., with slight modifications: the column used was a TG-WAXMS 60 m x 0.25 mm with 0.50 μm film thickness from Thermo Scientific (Hudson, NH, USA). The transfer line and the ion source were both kept at 240 ºC. The oven temperature program was set at an initial temperature of 40 ºC for 2 minutes, increased 4 ºC/minute until 230 ºC and held at 230 ºC for 15 minutes.

5.7. Sotolon and dioxane and dioxolane isomers

5.7.1. Optimization of the extraction procedure

The fist approach towards the development of a methodology for the extraction of sotolon and the dioxane and dioxolane isomers consisted on a conventional QuEChERS procedure: to 10 mL of wine (placed into 50 mL PTFE centrifuge tubes) 4 mL of ethyl acetate, 1 g of sodium citrate tribasic dehydrate, 500 mg sodium citrate dibasic sesquihydrate, 1 g of sodium chloride and 4 g of anhydrous magnesium sulphate were added. The tubes were vortexed for 1 min and centrifuged for 4 min at 4400 rpm (Centrifuge Eppendorf 5702, NY, USA). After separation, 3 mL of extract were concentrated by evaporation up to 1 mL, under a slow nitrogen stream. Taking into account the results of this extraction procedure the absence of salts and buffers were then studied to check its influence. Additionally, dichloromethane was also tested to check its feasibility to extract the target compounds.

For further optimization of the extraction method, rather than test one variable at a time, a Design of Experiments (DoE) strategy was employed, namely full factory design. The optimization procedure took into account three experimental variables, or factors, which included sample volume (3 levels: 8, 10 and 15 mL; and a center point at 11.5 mL), solvent volume (3 levels: 4, 5 and 8 mL; and a center point at 6 mL) and concentration of the extract (2 levels: 3-fold or 10-fold). Nineteen experiments were carried out in duplicate. The criterium used for the method optimization was the sotolon peak area due to its lower response. The extraction conditions that maximized the response were selected and are described in Section 5.7.2.

5.7.2. Extraction procedure and GC-MS conditions

The optimized extraction procedure was as follows: to 8 mL of wine sample, 4 µL of internal standard (3-octanol in synthetic wine with 18% of ethanol and pH adjusted to 3.5, 500 mg/L) and 5 mL of dichloromethane were added. The mixture was vortexed for 1 minute and then centrifuged (Centrifuge 5702, Eppendorf, NY, USA) for 10 minutes at 4400 rpm. After separation, 3 mL of the organic phase were transferred to graduated conic glass vials to evaporate the extract up to 0.3 mL, using a small nitrogen flow,

concentrating it up to ten-fold. Extractions and injections of each sample were carried out in duplicate.

The GC-MS analysis was carried out in a TRACE GC Ultra gas chromatograph equipped with an ISQ single quadrupole (on electronic impact mode, at 70 eV) and a TriPlus autosampler (on liquid mode) from Thermo Scientific (Hudson, NH, USA). The column used was a TR-5MS 60 m × 0.25 mm with 0.25 μm film thickness from Thermo Scientific. The injection (1 μL) was performed in splitless mode using helium (Air Liquide, Algés, Portugal) as carrier gas, at a constant flow rate of 1 mL/min. The GC injector port was kept at 230 ºC and the transfer line and ion source were kept at 290 and 250 ºC, respectively. The oven temperature was initially set to 40 ºC for 2 minutes, and then raised 12 ºC/minute until 196 ºC, raised again 30 ºC/minute until 260 ºC and held at 260 ºC for 5 minutes. Initial tests were performed with the sotolon standard and old fortified wines in TIC mode, in the range m/z 30–300, to ensure the retention time of the target compounds. All other chromatograms were acquired in selective ion monitoring (SIM) mode using the ions m/z 57, 87, 88, 103 and 117 for the dioxane and dioxolane isomers and the ions m/z 59, 83, 101, 113 and 128 for sotolon. For quantification purposes, the ion m/z 103 was used for the dioxane and dioxolane isomers and the ion m/z 83 was used for sotolon. The quantification of dioxane and dioxolane isomers was expressed in terms of the internal standard (3-octanol), since there were no standards available for these compounds.

5.7.3. Method validation

The methodology described above was assessed in terms of linearity, sensitivity, precision and accuracy for sotolon. The method precision was also evaluated for the dioxane and dioxolane isomers.

Selectivity was assessed by the analysis of several fortified wines to ensure the absence of chromatographic interferences at the retention time of sotolon (SIM at m/z 83), which could compromise its quantification.

In order to evaluate the method linearity, a young fortified dry wine (Sercial) was spiked with known amounts of sotolon standard at seven different concentration levels (10, 25, 50, 100, 200, 500 and 2000 μg/L). Each solution was prepared in duplicate and

the extractions and injections as well (a total of eight injections for each concentration level). The matrix matched calibration curve was obtained by plotting the peak area ratio of each calibration solution against the corresponding concentration, determining the method linearity (R^2).

The method sensitivity was appraised by determining the limit of detection (LOD) and the limit of quantification (LOQ), which were calculated as follows: $LOD=3.3\ \sigma/b$ and $LOQ=10\ \sigma/b$, in which σ is the standard deviation of the *y* intercept and *b* the slope.

Precision was evaluated based on inter- and intra-day analysis of two fortified sweet wines from different vintages. In order to verify intra-day repeatability ten successive extractions were performed for each wine and the results analyzed. Inter-day reproducibility was verified by the results of five extractions of each wine in three different days. Both parameters were expressed as relative standard deviation (%RSD).

Accuracy was evaluated by a recovery study, in which one of the wines used in the precision study was spiked with known amounts of sotolon standard at three different concentration levels (25, 200 and 2000 μg/L). The extractions were carried out in triplicate and recovery was calculated by comparing the mean values of the 3 replicates with theoretical concentrations of each one.

To test the method applicability, the extraction procedure was performed in duplicate on all 22 samples from the micro-oxygenation study and then injected, also in duplicate, into the GC-MS.

5.8. Sensory analysis

In order to evaluate if there were any perceptible olfactory differences between the control wine and each of the wines that underwent micro-oxygenation treatment, two triangle tests were performed. In each test, the participants were presented with three glasses, each containing a different wine. The participants were then asked to identify which wine was the different one. In one triangle test it was asked to differentiate between the Control and the MOX1 wine and in the other between the Control and the MOX2 wine.

An olfactory preference test was also carried out. In this test, three glasses containing the Control, MOX1 and MOX2 wines were presented to the participants, to whom was asked to assign a number to each glass (from 1 to 3), according to their preference. The number 1 represented the least preferred wine and the number 3 represented the most preferred.

All tests were carried out by placing 30 mL of wine in dark tinted glasses to avoid participants being influenced by the wine's chromatic attributes. The glasses were then covered with petri dishes. An untrained panel composed by 10 members was used. Both triangle and preference tests were done in duplicate to ensure that the panels' choices were not random.

5.9. Data Analysis

Most data was treated using Microsoft Office Excel 2013. The statistical software SigmaPlot 12.0. was used to evaluate significant differences by the analysis of variance (One-way ANOVA, Holm-Sidak method).

PART III - RESULTS AND DISCUSSION

6. PRELIMINARY STUDIES

6.1. Dissolved oxygen and pH

DO concentrations, in mg/L, were determined every week during the first month of *estufagem* and at the end of the second month. Measurements were carried out before and after oxygen addition treatments and the results are presented in Table 2. It is important to mention that the DO concentration of each replicate was only measured once since, due to sample volume, the stirring caused by the measurement device was enough to promote variations on the DO levels.

Table 2: Dissolved oxygen (mg/L), measured before and after the agitation and aeration treatments, during the *estufagem* period.

		Agitation		**Aeration**		**Control**	
		DO (mg/L)	**±SD**	**DO (mg/L)**	**±SD**	**DO (mg/L)**	**±SD**
Week 0	Initial	7.1	0.1	7.1	0.1	7.1	0.1
	Final	8.5	0.1	8.1	0.2		
Week 1	Initial	5.6	0.4	4.8	0.7		
	Final	8.0	0.2	7.3	0.3		
Week 2	Initial	5.2	0.3	5.4	0.9		
	Final	8.3	0.2	7.8	0.5		
Week 3	Initial	6.1	0.2	6.2	0.6		
	Final	8.1	0.1	7.8	0.5		
Week 4	Initial	7.4	0.1	7.4	0.4		
	Final	8.6	0.1	8.3	0.2		
Week 8	Initial	3.2	0.3	5	3	0.3	0.1
	Final	8.0	0.4	7.7	0.9		

DO – dissolved oxygen; SD – standard deviation

The results show that both treatments present very similar effects. It is possible to infer that both treatments caused an immediate increase in DO concentrations up to wine saturation levels (7.7 mg/L at 20 ºC) (4). Results also show that there is a consumption of oxygen between treatments. The DO variations tend to progressively decrease along the four weeks period, consequently, less oxygen was being consumed.

In regards to the control, the flasks remained sealed during the experiment. Oxygen measurements showed that DO concentration decreased about 96% after two months of *estufagem*. Since there was no oxygen addition, it can be concluded that almost all the oxygen dissolved in the wine was consumed during the *estufagem* period.

The evolution of pH was also evaluated with the same frequency of DO levels. The pH values remained between 3.06-3.08 throughout the two month period and were not affected by both treatments. This is in agreement with what has been reported by other authors, who found that oxygen addition did not have great impact on wine pH (8, 34, 38, 39, 43).

6.2. Color parameters

One of the most important sensorial characteristics of a wine is the color as it can provide important clues about its age and quality. Several factors can have an impact on wine color, mostly the winemaking procedures. Because some of the proclaimed effects of micro-oxygenation are lower color intensity decrease and, consequently, higher color stability, the evaluation of this element was essential. In this case, color parameters were determined according to the method proposed by Glories. This is the most common and easily accessible analytical procedure to determine wine color, as it consists in simply measuring absorbances at 420, 520 and 620 nm. Color intensity, tonality and the contribution of the yellow, red and blue components to the overall wine color can be then calculated. These parameters were evaluated weekly, before and after the oxygen addition treatments, during the first month of *estufagem*. The average values are presented in Figure 3. The results show that the evolution of the Glories parameters during the *estufagem* process is not linear, but it is identical for the two experiments (aeration *vs.* agitation). It is also noticeable that at the end of the *estufagem* process, there are differences between the control wine and the wines that underwent oxygen addition. According to Cabrita *et al.* (21), the absorbance at 420 nm is characteristic of the development of a yellow/brown color, due to the presence of yellow pigmented flavonols and to the degradation reactions of anthocyanins and tannins (5), while the absorbance at 520 nm is representative of the red color, due to the presence of anthocyanins and pigmented complexes formed with tannins and other anthocyanins. At the end of the *estufagem* process the percentage of the yellow color, measured in the treated wines, was

about 66%. In the case of the red color component, despite the changes that occurred during the first month of *estufagem*, its contribution at the end of this process in the treated wines was about 29%, which was the same as in the initial wine, and 31% in the control wine. The oxygen addition treatments did not introduce great differences on the blue color component. The contribution of the blue color component is usually attributed to the quinone forms of free and combined anthocyanins (21).

The evolution of color intensity, expressed as the sum of the absorbances at 420, 520 and 620 nm, strongly resembles to what was observed for the red tone. The color intensity values of the treated wines were the same as those observed for the initial wine (1.4). However, after *estufagem*, color intensity of the control wine was slightly higher (1.8). A similar trend was also verified for the evolution of tonality (calculated by the ratio between absorbances at 420 and 520 nm) in treated wines, which after two months of *estufagem* was the same as for the initial wine (2.3). The *estufagem* process caused a slight decrease on the control wine's tonality.

During the month that oxygenation treatments were carried out several changes were observed in color parameters. However, the treated wines (agitation and aeration), after *estufagem*, presented similar color parameters to those of the initial wine. On the other hand, the duration of the oxygenation treatments might not have been sufficient for wine color to be fully developed. Nonetheless, this preliminary study was enough to conclude that oxygen addition may influence the behavior in Madeira wine color, encouraging to continue studying its effect on the features of this wine type.

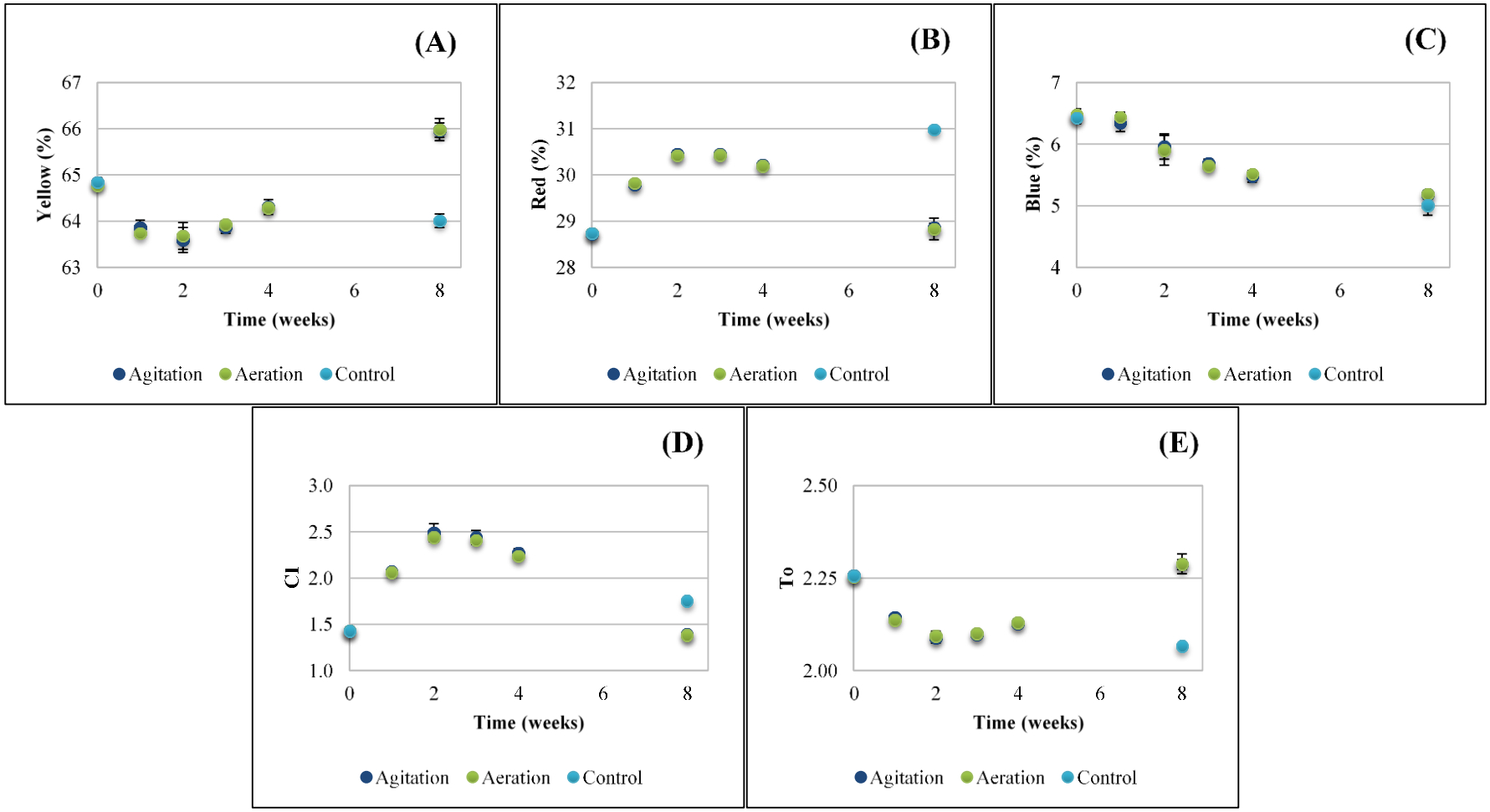

Figure 3: Evolution of the Glories colorimetric parameters during the different oxygenation experiments: agitation, aeration and control. (A) – yellow color component (%); (B) – red color component (%); (C) – blue color component (%); (D) – color intensity and (E) – tonality.

6.3. Volatile profile

The volatile composition of a wine is another parameter of great relevance for its characterization. As such, and taking into account the possibility that oxygen addition might influence important volatile compounds, this was also studied. The analysis of the volatile composition of the wine samples allowed the identification of about 50 compounds, which were then organized according to their chemical family – alcohols, carbonyl compounds, esters, fatty acids, terpenes and other compounds which cannot be placed on the previous categories. The abundance of each chemical family in the volatile composition of the four wine samples (Initial, Control, Agitation and Aeration) is shown in Figure 4. The values were given by the sum of the concentrations of each individual compound belonging to a determined chemical family.

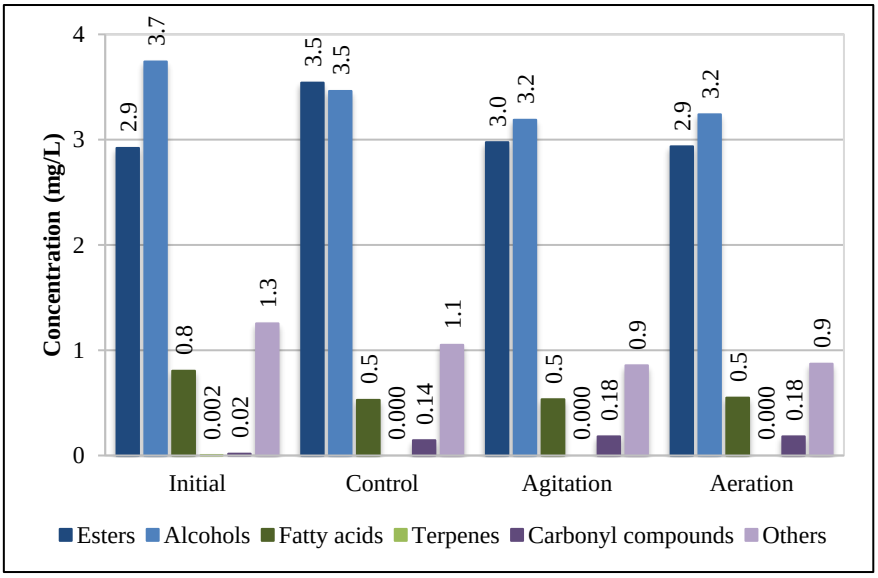

Figure 4: Important chemical families found in the volatile profile of the initial wine and in the control, agitated and aerated wines after *estufagem*.

In the volatile composition of the initial wine the presence of alcohols and esters stands-out. Fatty acids, carbonyl compounds, terpenes and other classes of compounds were also found but with lower abundance. After *estufagem*, the volatile composition of the control wine evolved, resulting in an increase in the contribution of esters and carbonyl compounds, in about 17 and 88%, respectively. On the other hand, the contribution of alcohols, fatty acids and other classes of compounds decreased, in about 7, 34 and 16%, respectively. Also, it was observed that *estufagem* promoted the disappearance of terpenes. In general, these findings are in agreement with those

described by Pereira (59), who observed a similar behavior after submitting different Madeira wines (Malvasia, TN sweet and TN dry) to 3 months of *estufagem* at 45 ºC.

Once again, and following the observations previously described for DO and color parameters, both oxygenation treatments resulted in wines with very similar volatile profiles. When compared to the control, the abundance of the total volatiles found in these wines was lower (about 11%). This is mostly caused by a lower content of esters, which were affected by the addition of oxygen. In fact, the levels of esters found in the treated wines were very similar to those found in the initial wine. The opposite was found for the control wine. Also, the concentrations of alcohols and of other classes of compounds diminished during the accelerated aging more than in the control wine, up to 15% and 32% respectively. The concentrations of fatty acids were very similar to those of the control wine, so it can be inferred that their behavior was only influenced by temperature and not by the oxygenation treatments. Terpenes also remained absent from the volatile composition of the treated wines so it is impossible to determine how this class of compounds might have been affected by the addition of oxygen.

It seems that the changes found between the treated wines and the control suggest that the addition of oxygen has an impact on the volatile composition of Madeira wines and, as such, further studies are required.

7. IMPACT OF MICRO-OXYGENATION DURING MADEIRA WINE AGING

7.1. Dissolved oxygen

The knowledge of the DO levels is a valuable tool for regulating and maintaining a correct micro-oxygenation treatment, providing important clues about oxygen consumption and giving valuable information about whether the oxygen addition flow rate needs to be adjusted. The DO determination during the three micro-oxygenation periods was, therefore, one of the goals of this work.

As previously mentioned, during the first MOX treatment (t1) the DO levels were measured weekly. Figure 5 shows that, at the beginning of the experiment, DO

concentrations found in the control and MOX1 wine were quite similar. However, after one week and up to the end of t1, noticeable changes arose between them. As expected, the control's DO concentrations gradually decreased throughout the treatment, since oxygen present was consumed. The same was previously observed in the experiments with Sercial.

On the other hand, the measurements performed after the first week revealed that the oxygen flow rate (66 mg/L per month) applied to the MOX1 wine was enough to elevate the DO concentration towards levels equal or superior to those described as corresponding to wine saturation (about 7.7 mg/L at 20 ºC) (4). It was observed that DO levels were maintained up to the end of t1.

The measurements performed at the beginning of t2 (ET0) showed that the DO concentration in MOX2 wine was comparable to that found for the control wine. Right after the first week of the t2 treatment, results show that DO levels continued to gradually decrease for all wines under analysis, including for MOX2 wine. This result suggests that the flow rate applied (the same used in t1) was no longer sufficient to maintain wine saturation. This is mostly due to the effect promoted by the temperature to which wine is submitted during *estufagem*, since at 45 ºC not only is expected a decrease of oxygen solubility in wine, but also an increase of the polyphenols reaction kinetics, leading to a greater consumption of oxygen. In the same way, that can also explain the great decrease in DO levels detected for MOX1. In this case, the DO concentration of MOX1 decreased from the saturation levels to 0.07 mg/L, in just one week of *estufagem*.

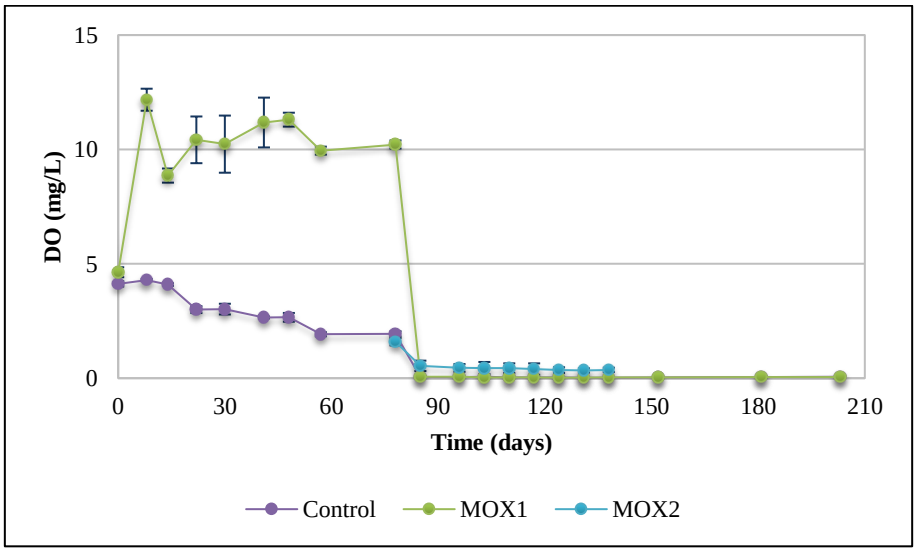

Figure 5: Evolution of the dissolved oxygen levels during the micro-oxygenation treatments.

With the t3 treatment, oxygen supply was increased up to wine saturation levels. While the DO measurement was no longer possible to perform, the MOX2 wine was controlled in order to maintain DO values always above 7 mg/L. To do so, several adjustments had to be made to the oxygen supply, as it seemed that even at such high flow rates, DO concentrations kept decreasing. On the other hand, the DO concentrations found for control and MOX1 wines remained stable throughout t3, albeit at very low levels. This is in agreement to what Martins *et al.* (6) found while studying the aging process of Port wine under oxidative conditions. The authors reported that wines that had higher DO concentrations seemed to consume oxygen more rapidly, while wines with lower DO levels consumed oxygen at a slower rate.

7.2. General oenological and color parameters

General oenological parameters were evaluated during the extent of all micro-oxygenation treatments. Table 3 presents the results of the general oenological parameters determined at T0, ET0, ET2M and ET4M.

Table 3: General oenological parameters determined at T0, ET0, ET2M and ET4M.

		ABV (%)	± SD	ρ (g/mL)	± SD	VA (g/L)	± SD	TA (g/L)	± SD	pH	± SD	RS (g/L)	± SD
T0	Control	18.46	0.05	1.01965	0.00007	0.15	0.01	5.44	0.03	3.345	0.005	90.9	0.5
	MOX1	18.44	0.04	1.01959	0.00007	0.16	0.03	5.46	0.06	3.346	0.008	91.1	0.7
	MOX2	-	-	-	-	-	-	-	-	-	-	-	-
ET0	Control	18.40	0.02	1.01981	0.00007	0.23	0.01	5.65	0.04	3.424	0.005	92.6	0.5
	MOX1	18.36	0.04	1.02006	0.00007	0.22	0.01	5.62	0.02	3.417	0.007	91.9	0.3
	MOX2	18.29	0.06	1.0199	0.0001	0.216	0.007	5.60	0.03	3.422	0.008	91.7	0.7
ET2M	Control	18.01	0.08	1.0206	0.0001	0.421	0.008	6.05	0.03	3.583	0.008	95.4	0.5
	MOX1	17.90	0.06	1.0209	0.0001	0.414	0.005	6.10	0.02	3.573	0.006	95.2	0.7
	MOX2	18.0	0.1	1.0211	0.0002	0.43	0.01	6.09	0.03	3.561	0.007	92.8	0.5
ET4M	Control	18.12	0.04	1.0205	0.0001	0.40	0.01	6.06	0.02	3.550	0.004	96.0	0.8
	MOX1	18.11	0.09	1.0204	0.0002	0.38	0.01	6.05	0.03	3.548	0.004	95.4	0.5
	MOX2	18.24	0.06	1.0203	0.0002	0.41	0.02	6.22	0.04	3.538	0.006	95.3	0.5

ABV – alcohol by volume (%), ρ - volumetric mass density (g/cm^3), VA - volatile acidity (g/L), TA - total acidity (g/L, expressed in terms of tartaric acid), pH and RS - reducing sugars (g/L).

The oenological parameters analyzed were within the expected values and differences cannot be observed between the control and the oxygenated wines (MOX1

and MOX2), which suggests that the oxygen treatments did not have a great impact on the basic physicochemical characteristics of the wines. These results are in concordance with those found by other authors (8, 34, 38, 39, 43). In the particular case of volatile acidity, the values are also very similar for all wines and are well within the local legal limit of 1.2 g/L (*portaria nº. 302/2011, artigo 3º de 2011*).

The Glories color parameters, namely the yellow, red and blue components, color intensity and tonality were also evaluated on a weekly basis during all micro-oxygenation treatments and the results are presented in Figure 6. The overall evolution of all wines during the 203 days of the experiment reflects what has previously been described for Tinta Negra sweet wines (71), namely an increase of the yellow color component and tonality and a decrease of the red and blue components and color intensity. These changes were in general more pronounced during *estufagem*. The t1 treatment had a very subtle effect on the chromatic characteristics of the MOX1 wine, which remained very similar to the control throughout the whole experimental period. In this sense, oxygen addition appears to have slightly enhanced the natural evolution of the wine color described above. The results for the control wine, at the end of *estufagem* (ET4M), show that the value for color intensity was 2.1, tonality was 2.2 and the contribution of the yellow, red and blue components were around 66.2, 29.7 and 4.1%, respectively. At the same time, the values determined for these parameters in the MOX1 wine showed that the yellow percentage (69.4%) and tonality (2.5) were slightly higher than those found in the control. On the other hand, the percentage of red (27.4%) and blue (3.3%) and color intensity (1.9) were lower. The t2 treatment had an identical effect on the MOX2 wine and the evolution of the Glories parameters was, once again, similar to the control and MOX1 wines. Measurements of the MOX2 wine at the end of this treatment (ET2M) showed that color intensity had reached values of 2.1, tonality of 1.8 and the yellow, red and blue contributions were 61.1, 33.7 and 5.2%, respectively. These results are analogous to those determined for the control wine at the same time, with color intensity being 2.4, tonality 1.7 and the yellow, red and blue percentages 58.9, 35.6 and 5.5%, respectively.

The impact of the t3 treatment on the MOX2 wine was evident and caused more pronounced changes in the evolution of the Glories parameters, with higher increase of the yellow color component (79%, at ET4M) and decrease of the red (20%) and blue (1%) color components. Consequently, these alterations also resulted in a greater increase of tonality (4.00) and decrease of color intensity (1.22).

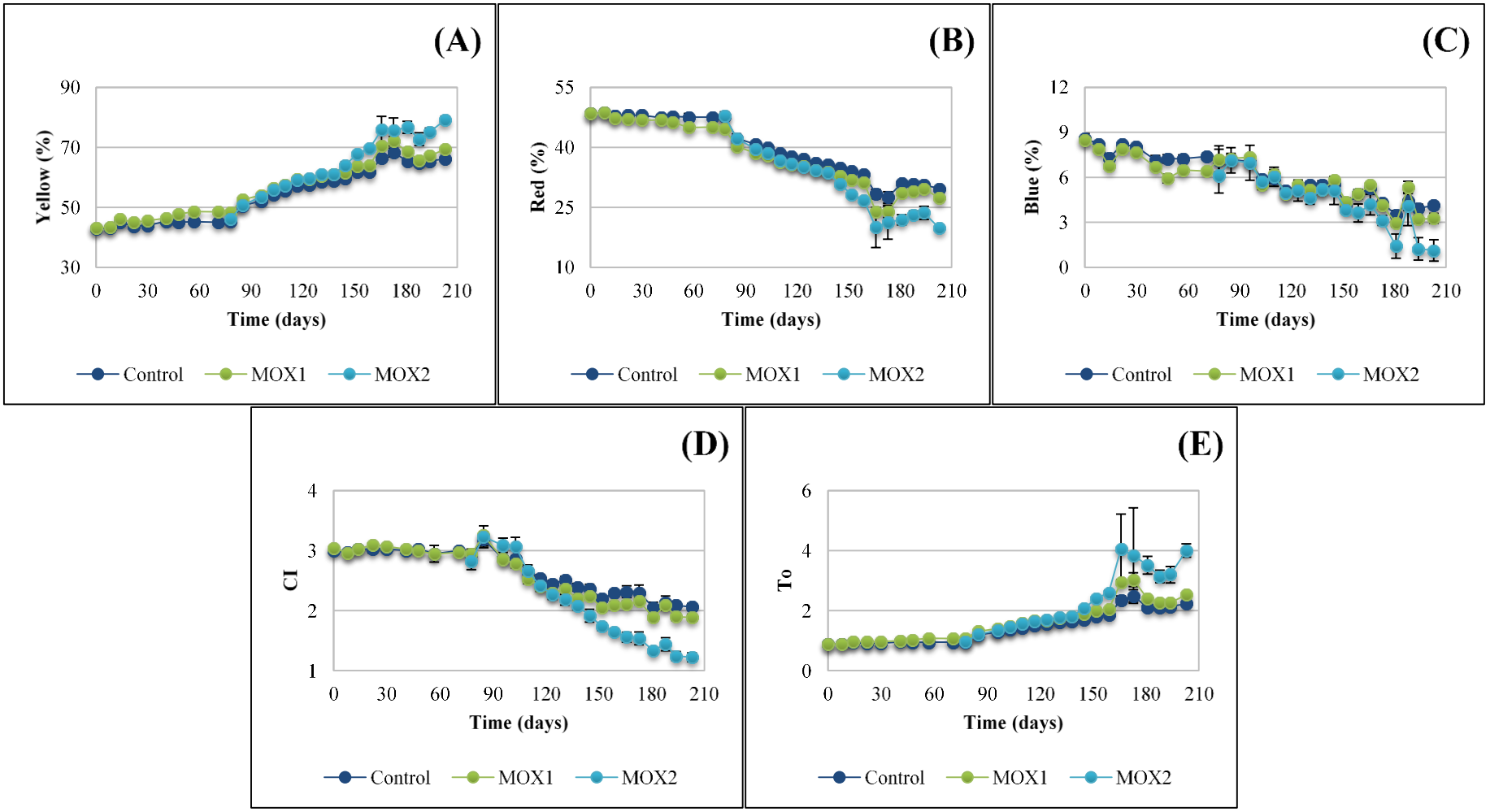

Figure 6: Evolution of the Glories colorimetric parameters during the three micro-oxygenation treatments: (A) – yellow color component (%); (B) – red color component (%); (C) – blue color component (%); (D) – color intensity and (E) – tonality.

These results evidence that rather than promote a lesser decrease in color intensity, as it is often proposed and described by other authors (1, 13, 26), micro-oxygenation had the opposite effect on Madeira wine. At the end of *estufagem* MOX1 and MOX2 wines showcased lower values of color intensity and higher values of tonality. This trend is similar to what Llaudy *et al.* (36) described for an oxygen treated Carbernet Sauvignon wine. Differences were also similar to what had previously been observed during the preliminary study. The greater decrease in color intensity in MOX2 wine, after being subjected to saturation levels of oxygen during the t3 treatment, might be indicative of over-oxygenation, which is known to cause wine deterioration. This result is due to the formation of high molecular weight polymeric compounds that precipitate, and lowers color intensity.

7.3. Individual polyphenols and furans

Phenolic compounds play a very important role in wine characteristics. They are also suggested as the most affected compounds in the presence of oxygen. It was therefore essential to understand the effect of each micro-oxygenation treatment on the polyphenolic composition. The individual polyphenols identified were organized according to their classification and are presented in Table 4.

In general, the evolution of these compounds did not follow a linear trend, despite the occurrence of variations. Taking into consideration that all wines were kept first in vats and then in stainless steel tanks, these variations did not include the contribution of phenols from wood. Notwithstanding, the total concentration of polyphenols after the 203 days of aging was lower in all wines, when compared to the initial wine. In regards to flavonoids, both flavan-3-ol and flavonol compounds were found. Flavan-3-ols, namely *(+)*-catechin and *(-)*-epicatechin, did not present a similar behavior during *estufagem*. Despite decreasing in the first two months of *estufagem* (between ET0 and ET2M), the content of *(+)*-catechin increased in all wines after the thermal processing. This behavior was less noticeable in MOX2 (about 35%). On the other hand, *(-)*-epicatechin was no longer detected in all wines after *estufagem*, regardless of oxygen addition. This result has previously been described and is thought to be related to oxidation reactions that lead to the development of yellow/brown notes (33, 70, 80). Despite decreasing during t1, quercetin, the only flavonol found, increased during *estufagem*, except when oxygen was

introduced during this stage (t2 and t3), which could be due to its high reactivity with oxygen (28, 33).

In regard to non-flavonoids, vanillin was the only hydroxybenzaldehyde identified and its concentration decreased with accelerated aging, with or without oxygen addition. Although present in small amounts in grapes, the occurrence of vanillin is usually associated to the degradation of lignin in oak casks (81). However, since the wine did not have contact with wood, variations in the content of vanillin could be the result of oxidative degradation (82). In general, e*stufagem* promoted an important increase of hydroxybenzoic acids, especially in protocatechuic and vanillic acids, up to 60 and 74%, respectively.

Conversely, the total concentration of hydroxycinnamic acids decreased with *estufagem*, mostly in micro-oxygenated wines. The increase of oxygen exposure during t3 enhanced this effect (up to 75%). Similar to what was described for flavan-3-ols, it has been suggested that the decrease of hydroxycinnamic acids during *estufagem* could be caused by oxidative phenomena (70). The only stilbene identified was *trans*-resveratrol in the initial wine. However, with *estufagem* its occurrence was not observed, similar to previous findings (70).

In general, it was observed that micro-oxygenated before *estufagem* and *estufagem* by itself, slightly increases the polyphenolic content in about 10%. A different behavior has previously been described (70). The combination of *estufagem* with an intense micro-oxygenation treatment (t3) caused a decrease of the polyphenolic content in about 29%.

Table 4: Individual polyphenolic compounds determined at key points of the micro-oxygenation treatments. Significant differences ($p < 0.050$) are identified by different letters in the same row, for each key sampling point.

	T0		**ET0**						**ET2M**						**ET4M**					
	Initial mg/L	**± SD**	**Control mg/L**	**± SD**	**MOX1 mg/L**	**± SD**	**MOX2 mg/L**	**± SD**	**Control mg/L**	**± SD**	**MOX1 mg/L**	**± SD**	**MOX2 mg/L**	**± SD**	**Control mg/L**	**± SD**	**MOX1 mg/L**	**± SD**	**MOX2 mg/L**	**± SD**
Flavonoids																				
Flavan-3-ols																				
(+)- Catechin	9.05	0.05	4.69 **a**	0.03	3.56 **b**	0.08	4.65 **a**	0.09	n.q.	-	n.d.	-	n.d.	-	9.8 **a**	0.2	9.6 **b**	0.1	7.2 **c**	0.4
(-)- Epicatehin	2.67	0.02	1.36 **a**	0.03	1.02 **b**	0.02	1.36 **a**	0.06	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
Total	**11.7**		**6.1**		**4.6**		**6.0**								**9.8**		**9.6**		**7.2**	
Flavonols																				
Quercetin	9.2	0.5	2.5 **a**	0.2	0.70 **b**	0.04	2.12 **c**	0.01	5.9 **a**	0.2	3.27 **b**	0.02	1.5 **c**	0.7	4.5 **a**	0.7	3.2 **b**	0.6	n.d.	-
Non-flavonoids																				
Hydroxybenzaldehydes																				
Vanillin	2.12	0.03	1.31 **a**	0.05	1.23 **b**	0.06	1.33 **a**	0.05	0.65 **a**	0.04	0.56 **b**	0.05	0.40 **c**	0.02	0.89 **a**	0.01	0.964 **b**	0.002	1.14 **c**	0.03
Hydroxybenzoic acids																				
Gallic acid	5.45	0.05	4.67 **a**	0.02	4.63 **b**	0.01	4.67 **a**	0.02	5.1 **a**	0.1	6.4 **b**	1.9	7.3 **b**	0.1	8.2 **a**	0.1	8.1 **a**	0.2	3.6 **b**	0.1
p-Hydroxybenzoic acid	4.32	0.03	0.50 **a**	0.01	0.51 **a**	0.01	0.517 **b**	0.002	0.554 **a**	0.006	0.606 **b**	0.005	0.57 **c**	0.03	0.39 **a**	0.02	0.366 **b**	0.004	0.49 **c**	0.04
Protocatechuic acid	4.37	0.05	3.12 **a**	0.02	3.09 **a**	0.02	3.12 **a**	0.05	3.7 **a**	0.3	3.3 **b**	0.6	2.65 **c**	0.02	7.2 **a**	0.7	7.7 **b**	0.2	5.79 **c**	0.04
Syringic acid	1.93	0.01	2.87 **a**	0.02	3.12 **b**	0.03	2.90 **c**	0.01	4.00 **a**	0.05	4.34 **b**	0.08	5.1 **c**	0.3	4.1 **a**	0.1	4.35 **b**	0.03	5.5 **c**	0.2
Vanillic acid	2.683	0.001	1.857 **a**	0.005	1.64 **b**	0.02	1.826 **c**	0.009	1.581 **a**	0.009	1.56 **a**	0.02	1.77 **b**	0.05	6.32 **a**	0.06	6.27 **a**	0.02	4.6 **b**	0.1
Total	**18.8**		**13.0**		**13.0**		**13.0**		**14.9**		**16.3**		**17.4**		**26.3**		**26.8**		**20.1**	
Hydroxycinnamic acids																				
Caffeic acid	1.05	0.01	1.068 **a**	0.001	1.050 **b**	0.007	1.067 **a**	0.005	1.85 **a**	0.07	1.87 **a**	0.06	2.16 **b**	0.05	1.8 **a**	0.1	1.733 **b**	0.006	0.71 **c**	0.03
Caftaric acid	26.33	0.08	25.0 **a**	0.1	24.83 **a**	0.07	25.0 **a**	0.3	16.6 **a**	0.8	15.9 **a**	0.1	16.4 **a**	1.6	11.2 **a**	0.9	10.27 **b**	0.05	4.6 **c**	0.4
p-Coumaric acid	0.842	0.001	0.897 **a**	0.003	0.856 **b**	0.004	0.882 **c**	0.004	1.24 **a**	0.02	1.22 **b**	0.01	1.32 **c**	0.02	1.41 **a**	0.08	1.379 **a**	0.001	1.62 **b**	0.06
Ferulic acid	0.367	0.002	0.343 **a**	0.006	0.33 **b**	0.01	0.327 **b**	0.007	0.42 **a**	0.02	0.373 **b**	0.009	0.342 **c**	0.005	n.q.	-	n.q.	-	n.q.	-
Total	**28.6**		**27.3**		**27.1**		**27.3**		**20.1**		**19.4**		**20.2**		**14.4**		**13.4**		**6.9**	
Stilbenes																				
trans-Resveratrol	0.205	0.003	n.d.	-	n.d.	-	n.d.		n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
Total	**70.6**		**50.2**		**46.6**		**49.8**		**41.5**		**39.5**		**39.5**		**55.9**		**53.9**		**35.3**	

SD – standard deviation; n.d. – not detected; n.q. – not quantified

The methodology used to analyze individual polyphenolic compounds has also enabled the evaluation of the evolution of furfural and 5-hydroxymethylfurfural (HMF). These furanic derivatives are well known to be correlated with wine aging. The evolution of furfural, in the three wines, during the key points of the process are shown in Figure 7 (A). Furfural was detected only after the second month of *estufagem* (ET2M). These results are in agreement with what has previously been reported (70). At this stage, the concentrations of furfural were quite similar between all wines (approximately 1.8 mg/L). The results pointed out that the most important factor for the development of furfural is temperature. Even though this compound was found in lower concentrations in the MOX2 wine, micro-oxygenation does not seem to have a relevant effect on its formation.

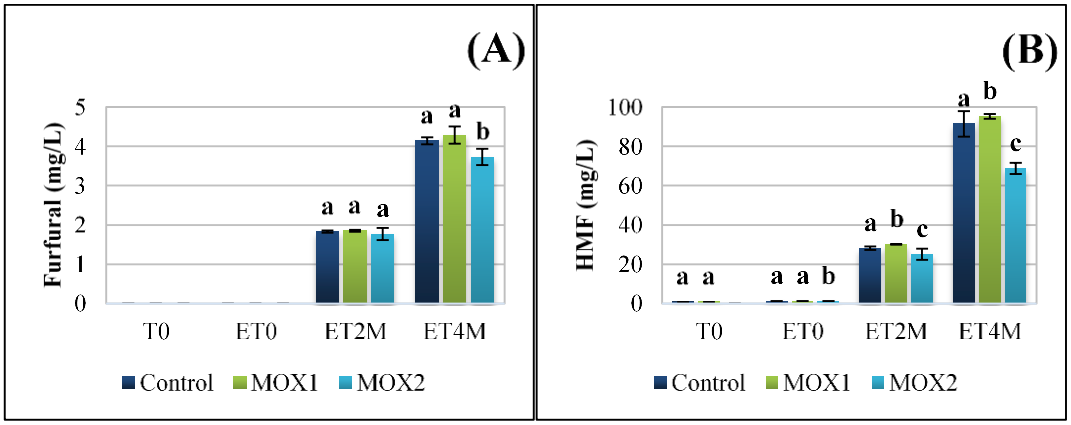

Figure 7: Concentrations of Furfural (A) and HMF (B) during the three micro-oxygenation treatments. Different letters on the results of each sampling point means significant differences, at $p < 0.050$.

The behavior displayed by HMF, Figure 7 (B), was very similar to furfural during the t1, t2 and t3 treatments, although the developed content was much higher for HMF, in average 15-fold at ET2M and 20-fold at ET4M.

A study with a Port wine has shown that under oxidative conditions, that included both high temperature and oxygen exposure, concentrations of furfural increase with time (49). The present study revealed that oxygen introduction during *estufagem* diminishes the formation of furans. Heras *et al.* (41), also described lower concentrations of furfural when Tinta de Toro and Mencía wines were exposed to oxygen.

7.4. Volatile Profile

The control, MOX1 and MOX2 wines, after t3, were analyzed in terms of their volatile composition, and compared against the initial wine. Once again, the compounds identified were organized according to their chemical family (as alcohols, carbonyl compounds, esters, fatty acids, terpenes and other compounds) and the abundance of each chemical family in each wine was determined (Figure 8).

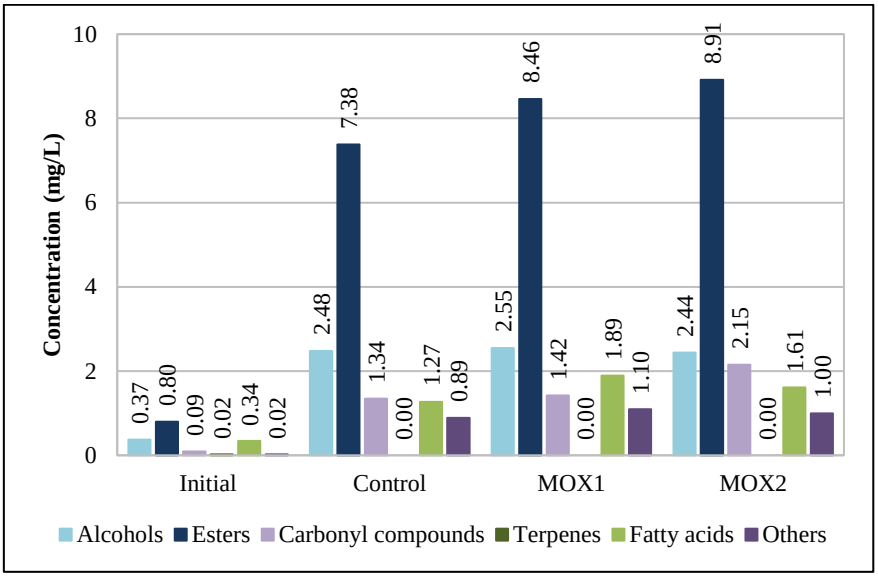

Figure 8: Important chemical families found in the volatile profile of the initial wine and in the control, MOX1 and MOX2 wines after *estufagem*.

Fifty three compounds were identified in the initial wine, which corresponds to a total concentration of 1.64 mg/L. Most of these compounds were esters (49%), alcohols (23%) and fatty acids (21%), followed by carbonyl compounds (5%), terpenes (1%) and other classes of compounds (1%).

After *estufagem*, after t3, all wines showed an increase in their volatile composition when compared to the initial wine. As such, the content of volatile compounds was 88% higher in the control wine, 89% in MOX1 and 90% in MOX2. The total content of volatile compounds identified in the control wine increased to 13.35 mg/L, most of which corresponded to esters (55%) and alcohols (19%). The presence of carbonyl compounds (10%), fatty acids (10%) and other classes of compounds (7%) was also detected. However, at this point, terpenes were not detected.

The treated wines, MOX1 and MOX2, showed a similar behavior to the control wine. The total concentration of volatile compounds in the MOX1 wine was 15.42 mg/L, slightly higher than the control, which reflects an increase in the concentration of all classes of compounds identified. Esters remain the most prominent class of compounds identified (55%), followed by alcohols (17%), fatty acids (12%), carbonyl compounds (9%) and other classes of compounds (7%). The total concentration of volatile compounds in MOX2 wine was 16.11 mg/L, higher than in MOX 1 and the control wines. This is mostly due to a higher content in esters (55%) and carbonyl compounds (13%). The concentration of fatty acids (10%) was also slightly higher than in the control wine, but lower when compared to what was found in MOX1 wine. On the other hand, the concentrations of alcohols (15%) and other classes of compounds (6%) were inferior to what was found in the control and MOX1 wines.

A discussion about the impact of micro-oxygenation on some sensory-active and accelerated aging (*estufagem*) related compounds is now presented (Table 10 - Section 10.). β-damascenone, said to contribute to the aroma of oak aged Madeira wines (59), was negatively affected only by the most intense MOX treatment (t3). As a result, MOX2 wine showed a significantly ($p < 0.001$) lower concentration of this compound (37 µg/L) when compared to the control and the other MOX wine (60 and 58 µg/L, respectively). Likewise, the concentrations of vitispirane in treated wines were also negatively impacted by the addition of oxygen. In this case, both MOX1 (190 µg/L) and MOX2 (130 µg/L) showed lower concentrations of this compound than the control (210 µg/L). Ethyl hexanoate and diethyl succinate were also affected by both MOX treatments, however, not in the same way. The concentrations of ethyl hexanoate found in MOX1 (450 µg/L) and MOX2 (390 µg/L) are lower than those found in the control (490 µg/L), with significant difference at $p < 0.001$. On the other hand, diethyl succinate benefited from oxygen exposure, increasing its concentration from 1.43 mg/L in the control wine, to 1.64 mg/L in MOX1 and 1.95 mg/L in MOX2. The same trend was observed for ethyl acetate, which increased from 1.90 mg/L in the control wine, to 2.2 mg/L in MOX1 and 3.4 mg/L in MOX2. The concentrations of ethyl 2-methylbutanoate and ethyl 3-methylbutanoate also increased significantly ($p < 0.001$) due to t3 and as a result MOX2 had higher concentrations of these esters (33 and 45 µg/L, respectively). 5-methylfurfural, an age indicator for Madeira wine (61), has also been affected by the t3. As a result, the concentration of this compound in MOX 3 wine (23 µg/L) was considerably lower than

what was found in the control and MOX1 wines (31 µg/L in both). This behavior is analogous to what was described for furfural and HMF in Section 7.3.. Oxygen exposure had a beneficial impact on the formation of benzaldehyde and, at the end of *estufagem*, the concentrations of this compound in MOX1 and MOX2 wines (660 µg/L and 1.28 mg/L, respectively) were higher than in the control (610 µg/L). Additionally, the evolution of acetaldehyde, which can be produced by the oxidation of ethanol and is well known for playing an important role in the reactions between anthocyanins and tannins, was also evaluated. Results show that, while the concentrations of acetaldehyde are very similar in the control (200 µg/L) and MOX1 (210 µg/L) wines, its content is significantly higher ($p < 0.001$) in MOX2 (310 µg/L). Similarly, some authors (8, 34, 43) have also reported an increase in acetaldehyde as a consequence of micro-oxygenation.

7.5. Sotolon and dioxane and dioxolane isomers

7.5.1. Method optimization and validation

Up to date, most procedures for the analysis of heterocyclic acetals in wines are based on liquid-liquid extraction (LLE) (7, 56). Similarly, the analysis of sotolon in wines has usually been based on LLE (83, 84), even though other miniaturized methods, such as micro-extraction by packed sorbent coupled with ultra-high pressure liquid chromatography (MEPS/UHPLC) have recently been proposed (85). However, the goal was to develop a simple and inexpensive methodology that allowed the analysis of both sotolon and heterocyclic acetals in Madeira wines.

Taking into account the several advantages that the QuEChERS approach presents – user-friendly, inexpensive, effective, robust and secure – it was initially chosen for the extraction of the target compounds from fortified wines. However, the salts and buffers used in the conventional QuEChERS procedure (described in section 5.7.1.), appeared to interfere in the extraction of the compounds of interest, reducing the extraction yield of sotolon. Therefore, they were removed from the extraction procedure. The choice of dichloromethane as the extraction solvent over ethyl acetate was based on the better peak shapes and less interferences at the retention time of sotolon this solvent yielded.

This methodology for the extraction of sotolon was then optimized using a DoE, which evaluated sample volume, solvent volume and concentration of the extract to determine the combination that led to the best GC-MS response. Based on the analysis of the results of the DoE it was determined that the best extraction conditions were: 8 mL of sample (spiked with 4 µL of internal standard), to which 5 mL of dichloromethane were added, and a final concentration of the extract of 0.3 mL (ten-fold). The exact optimized procedure for the extraction of the target compounds is described in section 5.7.2.

Sotolon

In terms of validation, the extraction methodology described in Section 5.7.2. was fully evaluated. The results of the parameters used for method validation are summarized in Table 5. The selectivity of the proposed methodology was evaluated by the analysis of several fortified wines, from which a wine, absent of significant interferences at sotolon retention time, was then chosen to be used to generate the matrix-matched calibration.

Table 5: Results of the parameters used to validate the extraction methodology for sotolon.

	Parameter	**Result**
Linearity	Linear regression	$y = 0.0027926x - 0.0105862$
	R^2	0.999
Sensitivity	LOD	2.3 µg/L
	LOQ	6.8 µg/L
Accuracy	Recovery:	
	25 µg/L sotolon	99.3 %
	200 µg/L sotolon	114.9 %
	2000 µg/L sotolon	102.1 %
Precision	Repeatability	6.6 - 7.4 %
	Reproducibility	6.7 - 9.0 %

R^2 – correlation coefficient; LOD – limit of detection; LOQ – limit of quantification

To evaluate linearity a calibration curve was constructed, with a range between 10-2000 µg/L, and a linear regression applied. The results gave a correlation coefficient (R^2) of 0.999, showing good linearity. Additionally, the method also showed high sensitivity, with a LOD of 2.3 µg/L and a LOQ of 6.8 µg/L.

Accuracy was verified by means of a recovery study. A sweet fortified wine was spiked with known amounts of the sotolon standard at three concentration levels of 25, 200 and 2000 µg/L. Results show that recovery was of 99.3, 114.9 and 102.1 % for the three concentration levels, respectively. This sets the average recovery at 105.4 %.

Method precision was evaluated through repeatability and reproducibility. In this regard the method proved to be suitably precise with repeatability and reproducibility inferior to 8 and 9 %, respectively.

***Cis*- and *trans*-dioxane and *cis*- and *trans*-dioxolane**

The extraction procedure was also evaluated in terms of precision for the dioxane and dioxolane isomers. Repeatability and reproducibility for *cis*- and *trans*-dioxane and for *cis*-dioxolane had relative standard deviations lower than 13 %. In the case of *trans*-dioxolane repeatability and reproducibility were a little higher, reaching values of 17 %. The results of the inter- and intra-day analysis are detailed in Table 6 for all four compounds.

Table 6: Results of the repeatability and reproducibility assays for *cis*- and *trans*-dioxane and *cis*- and *trans*-dioxolane.

	Repeatability	**Reproducibility**
***cis*-Dioxane**	4.9 - 8.9 %	7.1 - 9.5 %
***trans*-Dioxane**	4.6 - 9.4 %	10.6 - 12.8 %
***cis*-Dioxolane**	5.8 - 9.9 %	8.9 - 8.9 %
***trans*-Dioxolane**	6.3 - 11.4 %	16.6 - 16.8 %

7.5.2. Evolution of sotolon and the dioxane and dioxolane isomers during micro-oxygenation

After optimized and validated, the new methodology was applied in order to determine the concentrations of sotolon in the wine samples used for this study. Figure 9 shows the typical GC-MS/SIM chromatogram of the wine extract, obtained for sotolon and 3-octanol. The evolution of the concentrations of sotolon during the three micro-oxygenation treatments is presented in Figure 10. During the 78 days preceding *estufagem* the levels of sotolon present in the wine samples remained mostly unchanged, however, during *estufagem* the concentration of sotolon in all three wines increased considerably. These results are as expected, since the formation of sotolon is known to be favored by oxidative conditions such as temperature and oxygen exposure.

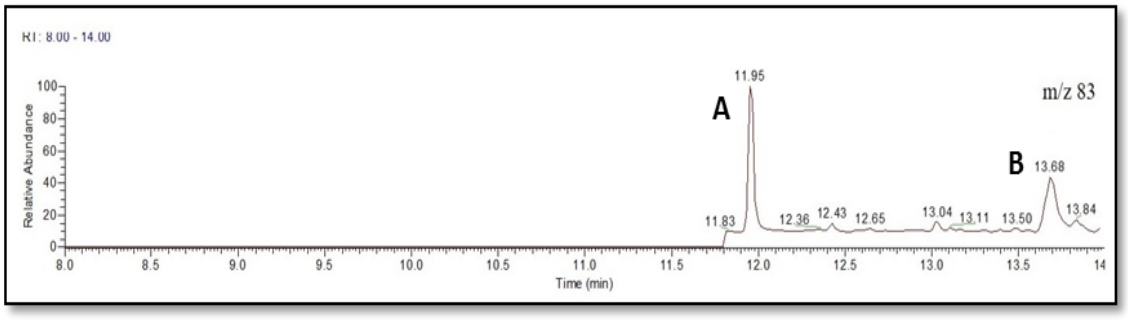

Figure 9: Typical GC-MS/SIM chromatogram of the wine extract obtained by the optimized methodology. Peak identification: A – 3-octanol; B – sotolon.

The flow rate of 66 mg O_2/L per month applied during the t1 treatment did not have an immediate impact. As a matter of fact, it appears that the effect of the first treatment only became noticeable during *estufagem*, when the concentration of sotolon increased and reached values of 85.69 and 273.20 µg/L at two and four months, respectively. These concentrations are higher than those detected for the control wine (69.58 and 191.20 µg/L) at the same periods.

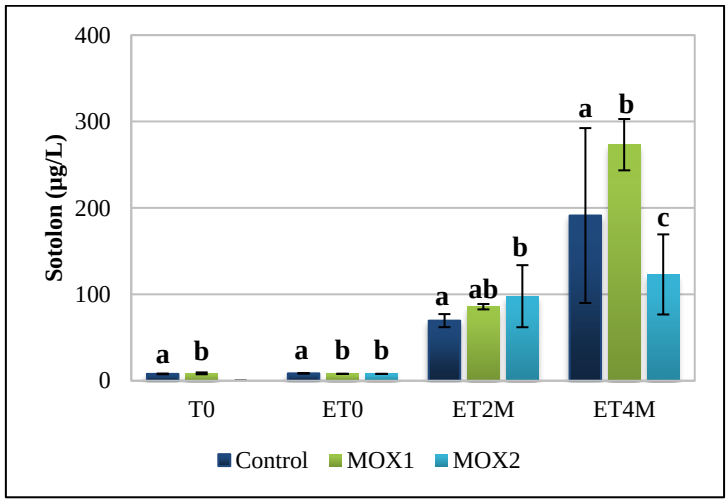

Figure 10: Evolution of Sotolon during the three micro-oxygenation treatments. Different letters on the results of each sampling point means significant differences, at $p < 0.050$.

When a similar flow rate was applied during the first 60 days of *estufagem* (t2) the combination of oxygen exposure and higher temperature had a positive effect on the formation of sotolon, increasing its concentration from 7.98 µg/L at ET0 to 97.85 µg/L. During the t3, when wine saturation was reached, the levels of sotolon continued to increase, however, its evolution was much slower and the concentration of sotolon measured in MOX2 at ET4M was only 123.04 µg/L, much lower than those of the control

and MOX1 wines at the same stage. These results are unexpected since the formation of sotolon has been found to be very dependent upon temperature and oxygen consumption (6). On the other hand, the evolution trend displayed by sotolon is consistent with what was observed for furfural and HMF and a correlation (R^2>0.98) was found between them. In fact, several authors have found a correlation between the concentrations of sotolon, furfural and HMF (61, 72).

The impact of the micro-oxygenation treatments on the evolution of *cis*- and *trans*-dioxane and *cis*- and *trans*-dioxolane was also evaluated and the typical GC-MS/SIM chromatogram of the wine extract, obtained for these compounds, is shown in Figure 11. The evolution of the heterocyclic acetals during the MOX treatments is presented in (Figure 12). Results show that *cis*-dioxane was the most predominant isomer, at all measuring stages. Moreover, the concentrations of the *cis*- isomers were always superior to the concentrations of the *trans*- isomers. These results are somewhat similar to those described in previous studies for Madeira wines made from the white grape varieties (64). In regards to the effect temperature and oxygen exposure had on these compounds, the first observation that is worth mentioning is that all four isomers presented a very similarly behavior.

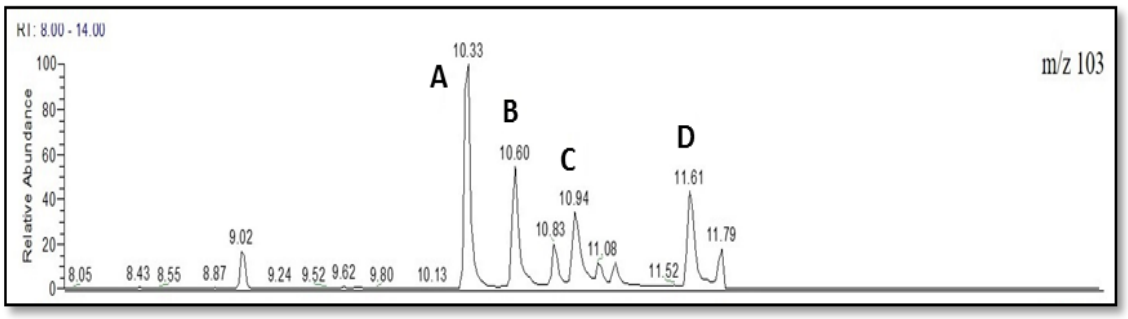

Figure 11: Typical GC-MS/SIM chromatogram of the wine extract obtained by the optimized methodology. Peak identification: A – 1,3-*cis*-dioxane; B – 1,3-*cis*-dioxolane; C – 1,3-*trans*-dioxolane; D – 1,3-*trans*-dioxane.

Although their overall evolution did not appear to be linear, their formation seemed to be affected by both temperature and oxygen exposure. In this since, *estufagem* had a considerable impact on the formation of these molecules, greatly increasing their concentrations. Exposure to oxygen also influenced the formation rate of these compounds. Once again, the t1 treatment had a very subtle impact, however, during *estufagem* the MOX1 wine developed higher concentrations of all isomers when

compared to the control wine. At the end of *estufagem* the concentrations of these compounds in the MOX1 wine were 0.56 µg/L for *cis*-dioxane, 0.23 µg/L for *trans*-dioxane, 0.28 µg/L for *cis*-dioxolane and 0.11 µg/L for *trans*-dioxolane.

The introduction of a 66 mg O_2/L per month flow rate during the t2 treatment was also beneficial for the formation of all four isomers and at the end of this treatment their concentrations were clearly higher than those determined for the control and MOX1 wines, ranging from 0.76 µg/L for *cis*-dioxane, 0.19 µg/L for *trans*-dioxane, 0.34 µg/L for *cis*-dioxolane and 0.08 µg/L for *trans*-dioxolane.

Conversely to what was observed for sotolon, the extreme conditions provided during the t3 treatment favored the formation of all four molecules and their concentrations in the MOX2 wine after four months of *estufagem* averaged 0.83 µg/L for *cis*-dioxane, 0.34 µg/L for *trans*-dioxane, 0.45 µg/L for *cis*-dioxolane and 0.19 µg/L for *trans*-dioxolane.

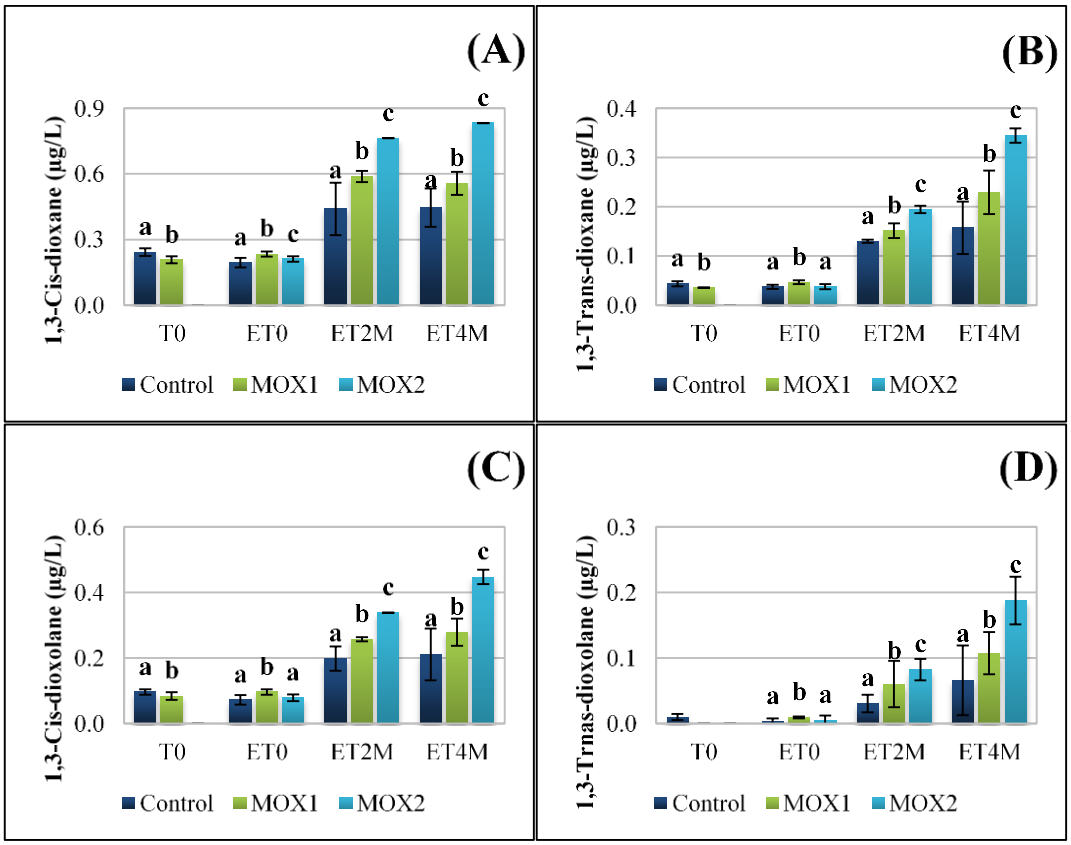

Figure 12: Evolution of the dioxane and dioxolane isomers during the three micro-oxygenation treatments: A – *cis*-dioxane; B – *trans*-dioxane; C – *cis*-dioxolane and D – *trans*-dioxolane. Different letters on the results of each sampling point means significant differences, at $p < 0.050$.

The fact that the formation of *cis*- and *trans*-dioxane and *cis*- and *trans*-dioxolane showed a positive dependence upon temperature and oxygen is in agreement with other findings in the literature (7, 49) where it was determined that extreme oxidative conditions favored the production of these compounds. Considering that the formation of these compounds is known to be the result of condensation reactions between glycerol and acetaldehyde and that micro-oxygenation is supposed to result in an increase of acetaldehyde concentrations, it stands to reason that the concentration of these compounds should in fact be higher after being subjected to oxygen treatments (7).

On the other hand, when comparing the evolution of the dioxane and dioxolane isomers with that of sotolon, furfural and HMF, it is very clear that these compounds behaved differently when exposed to the flow rates necessary for wine saturation during the t3 treatment. While these extreme conditions greatly favored the formation of *cis*- and *trans*-dioxane and *cis*- and *trans*-dioxolane, they led to a slower formation rate for sotolon, furfural and HMF.

7.6. Sensory Analysis

In order to further investigate how the micro-oxygenation treatments sensorially affected the wines, an olfactory analysis was carried out, by an untrained panel, in the form of two triangle tests (Table 7) and a preference test (Table 8).

The triangle tests were important to determine if tasters could detect any perceivable olfactory differences between the treated wines and the control, after *estufagem*. Considering this, the two triangle tests were set up in a way that allowed the comparison between MOX1, MOX2 and the control wines. Moreover, to ensure that tasters did not choose randomly, both tests were carried out in duplicate and the answers were only accepted as valid if a correct identification was achieved in both replicates.

Table 7: Results of the triangle tests for MOX1 and MOX2 in comparison to the control. Score results: 0 - taster did not successfully choose the different wine in both repetitions of the test; 1 - taster successfully chose the different wine in both repetitions of the test

	MOX1	MOX2
Taster 1	0	1
Taster 2	0	0
Taster 3	0	0
Taster 4	0	1
Taster 5	0	1
Taster 6	0	1
Taster 7	0	1
Taster 8	0	0
Taster 9	0	0
Taster 10	0	0
Total	**0**	**5**

The results show that all tasters were unable to differentiate between MOX1 and the control. On the other hand, when comparing MOX2 to the control wine, 50 % of the participants were able to correctly differentiate between the two. This seems to indicate that the t1 treatment did not cause enough changes for the panel to be able to detect any olfactory differences between MOX1 and the control. Conversely, t2 and t3 treatments had enough impact on the aromatic characteristics of MOX2 to allow half of the participants to detect the differences.

Table 8: Results of the preference test. Score results: 1 – Least preferred wine; 2 – intermediate preference; 3 – most preferred wine.

	Control	MOX1	MOX2
Taster 1	-	-	1
Taster 2	1	-	-
Taster 3	2	-	-
Taster 4	3	2	1
Taster 5	-	2	-
Taster 6	-	-	3
Taster 7	-	-	1
Taster 8	1	3	2
Taster 9	1	-	-
Taster 10	-	-	3

In the preference test, participants had to score the three wines on a scale of 1 to 3, by crescent order of preference. The results indicate that the preference test was strongly influenced by the panel's difficulty in detecting olfactory differences between the wines presented to them. In fact, most participants commented that they found it very hard to detect differences between the wines, which made it harder to score them by preference. Consequently, the results of the preference test are very unclear and inconclusive, as even the tasters who consistently gave the same scores in both repetitions of the test have very divergent opinions.

Ideally, the triangle tests and the preference test should have been performed by a trained panel to verify if more subtle differences exist between the wines and to perhaps elucidate if micro-oxygenation brought positive changes to Madeira wine.

PART IV – CONCLUSIONS

8. CONCLUSIONS

Three different micro-oxygenation treatments were employed in order to examine the impact of oxygen addition on important parameters of a Tinta Negra sweet Madeira wine, usually evaluated. Treatment 1 (t1) was employed before *estufagem*, using a flow rate of 66 mg/L per month. Treatment 2 (t2) was applied during the first 60 days of *estufagem*, using the same flow rate as t1. During the last 65 days of *estufagem*, treatment 3 (t3) was introduced and the oxygen flow rate was adjusted in order to maintain the DO level above 7 mg/L (over-oxygenation).

The *estufagem* process (control), by itself, had an impact on Madeira wine characteristics, causing an increase of the polyphenolic content (up to 10%), a decrease of color intensity and an increase of tonality. The formation of furfural, HMF, sotolon and dioxane and dioxolane isomers is favored during *estufagem*.

The introduction of oxygen, before and during *estufagem*, did not promote an impact on the basic oenological parameters but it was found that it had an effect on Madeira wine's composition:

- In general, standard oxygen flow addition (t1 and t2) did not have a noticeable impact on the color parameters of Madeira wine, however, when elevated to extreme conditions (t3), the effect of *estufagem* was amplified, causing a considerable decrease of color intensity and an increase of tonality.
- Micro-oxygenation only affected the polyphenolic composition of Madeira wine when oxygen addition was combined with *estufagem*, decreasing its concentration up to 29%.
- Standard oxygen flow addition (t1 and t2) did not reveal a relevant effect on the concentrations of the furans analyzed, furfural and HMF, and their formation was mostly affected by *estufagem*. The intense oxygenation (t3), somehow slowed the development of these compounds in about 10% for furfural and 25% for HMF, when compared to the control.
- The impact of micro-oxygenation on the concentrations of sotolon and dioxane and dioxolane isomers was verified using a new optimized methodology, based on a miniaturized liquid-liquid extraction followed by a GC-MS/SIM analysis. In general, exposure to oxygen favors the formation of these compounds. However,

extreme oxygenation conditions slows the natural evolution of sotolon. When compared to the control, the concentration of sotolon in the over-oxygenated wine was roughly 36% lower.

- The sensory analysis of the wines revealed a difficulty to differentiate between the oxygenated wines and the control, which might indicate that micro-oxygenation did not have enough impact on the olfactory characteristics to cause a clear difference between them.

In general, t1 had a very subtle impact on the main characteristics of the wine. Extreme oxidative conditions (t3) had a greater impact, and the resulting wine showed signs of over-oxygenation. On the other hand, despite the decrease on polyphenolic composition (about 21% at ET2M), t2 did not have a noticeable impact on color and on the content of furfural and HMF. Furthermore, it also accelerated the formation of sotolon and the heterocyclic acetals. Therefore, the conditions of t2 could be used as a basis to further develop new experiments to find the best conditions to conduct micro-oxygenation in Madeira wine.

Finally, the methodology optimized for the extraction of sotolon and the heterocyclic acetals, used an eco-friendly, fast and low-cost approach, and proved to be effective for the analysis of the target compounds, providing good sensitivity, linearity, precision and accuracy for the analysis of sotolon, simultaneously allowing the adequate control of heterocyclic acetals.

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10. APPENDIX

Table 9: Volatile compounds identified in the Initial wine and in the Control, Aerated and Agitated wines after *estufagem*. Significant differences ($p < 0.050$) are identified by different letters in the same row, for each key sampling point.

KI	*Compound*	Initial		Control		Aeration		Agitation	
		(mg/L)	±SD	(mg/L)	±SD	(mg/L)	±SD	(mg/L)	±SD
	Alcohols (16)								
1103	*2-methyl-1-propanol*	0.211 **a**	0.003	0.19 **b**	0.01	0.182 **c**	0.008	0.179 **c**	0.009
1159	*1-butanol*	0.0013 **ab**	0.0002	0.0014 **a**	0.0001	0.0014 **a**	0.0002	0.0011 **b**	0.0003
1220	*3-methyl-1-butanol*	1.99 **a**	0.09	1.9 **b**	0.1	1.73 **c**	0.07	1.69 **c**	0.08
1340	*3-methyl-1-pentanol*	0.00101 **a**	0.00006	0.0010 **a**	0.0002	0.0010 **a**	0.0001	0.0010 **a**	0.0001
1367	*1-hexanol*	0.210 **a**	0.003	0.194 **b**	0.005	0.182 **c**	0.003	0.183 **c**	0.003
1377	*(E)-3-hexen-1-ol*	0.00156 **a**	0.00009	0.0014 **a**	0.0001	0.0015 **a**	0.0002	0.0015 **a**	0.0002
1398	*(Z)-3-hexen-1-ol*	0.013	0.002	n.d.		n.d.		n.d.	
1468	*1-heptanol*	0.0076 **a**	0.0009	0.008 **a**	0.001	0.0060 **b**	0.0002	0.0078 **a**	0.0006
1500	*2-ethyl- hexanol*	0.0425 **a**	0.0007	0.041 **a**	0.003	0.042 **a**	0.003	0.047 **b**	0.001
1570	*1-octanol*	0.0224 **a**	0.0004	0.0226 **a**	0.0002	0.0211 **b**	0.0008	0.0206 **b**	0.0009
1671	*1-nonanol*	0.027 **a**	0.001	0.025 **b**	0.001	0.022 **c**	0.001	0.021 **c**	0.001
1773	*1-decanol*	0.054 **a**	0.001	0.042 **b**	0.002	0.036 **c**	0.001	0.036 **c**	0.002
1892	*Benzyl Alcohol*	0.0036 **a**	0.0001	0.0033 **b**	0.0003	0.0096 **c**	0.0004	0.0034 **ab**	0.0002
1926	*2-phenylethanol*	1.12 **a**	0.02	1.05 **b**	0.03	0.98 **c**	0.02	0.96 **c**	0.03
1977	*1-dodecanol*	0.0274 **a**	0.0002	0.025 **a**	0.002	0.025 **a**	0.002	0.04 **b**	0.02
2388	*1-hexadecanol*	0.0024 **a**	0.0003	0.0047 **b**	0.0006	0.0033 **c**	0.0004	0.0038 **d**	0.0002
	Carbonyl compounds (5)								
1303	*Octanal*	n.d.		0.0048 **a**	0.0005	0.0050 **a**	0.0006	0.0046 **a**	0.0007
1513	*Decanal*	0.0078 **a**	0.0009	0.012 **b**	0.002	0.015 **c**	0.002	0.016 **c**	0.002
1544	*Benzaldehyde*	n.d.		0.106 **a**	0.007	0.140 **b**	0.003	0.140 **b**	0.005
1835	*β-damascenone*	n.d.		0.0123 **a**	0.0006	0.0108 **b**	0.0007	0.0098 **c**	0.0005
1868	*Geranyl acetone*	0.0091 **a**	0.0003	0.0092 **a**	0.0005	0.0112 **b**	0.0009	0.0122 **c**	0.0005
	Esters (22)								
801	*Ethyl acetate*	0.24 **a**	0.02	0.27 **b**	0.02	0.26 **b**	0.01	0.26 **b**	0.01
1070	*Ethyl 3-methylbutanoate*	0.0080 **a**	0.0008	0.021 **b**	0.002	0.014 **c**	0.001	0.0152 **c**	0.0009
1135	*Isoamyl acetate*	0.065 **a**	0.004	0.0161 **b**	0.0004	0.0137 **c**	0.0008	0.0132 **c**	0.0009
1248	*Ethyl hexanoate*	0.53 **a**	0.02	0.55 **b**	0.01	0.41 **c**	0.03	0.44 **d**	0.01
1286	*Hexyl acetate*	0.0045	0.0004	n.d.		n.d.		n.d.	
1316	*Ethyl (Z)-3-hexenoate*	0.00111 **a**	0.00008	0.0012 **b**	0.0001	0.0010 **c**	0.0001	0.0011 **ac**	0.0001
1348	*Ethyl heptanoate*	0.0024 **a**	0.0003	0.0037 **b**	0.0004	0.0025 **a**	0.0002	0.0025 **a**	0.0003
1360	*Ethyl lactate*	0.022 **ab**	0.003	0.025 **c**	0.001	0.024 **ac**	0.001	0.025 **bc**	0.003
1440	*Ethyl 2-hydroxy-3-methylbutanoate*	0.0015 **a**	0.0002	0.0026 **b**	0.0001	0.0028 **b**	0.0001	0.0027 **b**	0.0002
1448	*Ethyl octanoate*	1.36 **a**	0.06	1.1 **b**	0.1	0.77 **c**	0.04	0.82 **c**	0.04
1594	*Diethyl malonate*	n.d.		0.0020 **a**	0.0001	0.0026 **b**	0.0003	0.0025 **b**	0.0002
1641	*Ethyl furoate*	0.0103 **a**	0.0001	0.0145 **b**	0.0006	0.0124 **c**	0.0003	0.0124 **c**	0.0004

1650	*Ethyl decanoate*	0.088 **a**	0.006	0.03 **b**	0.01	0.018 **c**	0.001	0.0186 **c**	0.0007
1683	*Ethyl benzoate*	0.018 **a**	0.002	0.02 **a**	0.01	0.0123 **b**	0.0007	0.0119 **b**	0.0009
1691	*Diethyl succinate*	0.393 **a**	0.004	1.38 **b**	0.04	1.29 **c**	0.03	1.24 **d**	0.03
1702	*Ethyl 9-decenoate*	0.064 **a**	0.002	0.0173 **b**	0.0009	0.0101 **c**	0.0006	0.0114 **d**	0.0006
1795	*Methyl salicylate*	0.040 **a**	0.002	0.032 **b**	0.002	0.032 **b**	0.001	0.031 **b**	0.002
1800	*Ethyl phenylacetate*	0.0140 **a**	0.0003	0.032 **b**	0.002	0.0299 **c**	0.0006	0.0285 **d**	0.0008
1832	*2-phenylethyl acetate*	0.053	0.002	n.d.		n.d.		n.d.	
1832	*Ethyl salicylate*	n.d.		0.010 **a**	0.003	0.010 **a**	0.003	0.009 **a**	0.003
1853	*Ethyl dodecanoate*	0.0039 **a**	0.0002	0.0022 **b**	0.0002	0.0017 **b**	0.0002	0.025 **c**	0.002
2263	*Ethyl hexadecanoate*	0.0066 **a**	0.0008	0.0046 **b**	0.0003	0.0055 **c**	0.0004	0.007 **a**	0.001
	Fatty acids (6)								
1478	*Acetic acid*	0.0034 **a**	0.0002	0.0025 **b**	0.0005	0.0028 **c**	0.0002	0.0046 **d**	0.0006
1864	*Hexanoic acid*	0.037 **a**	0.002	0.019 **b**	0.002	0.025 **c**	0.001	0.019 **b**	0.002
2074	*Octanoic acid*	0.66 **a**	0.03	0.41 **b**	0.02	0.43 **b**	0.02	0.41 **b**	0.03
2298	*Decanoic acid*	0.085 **a**	0.005	0.067 **b**	0.004	0.068 **b**	0.003	0.068 **b**	0.006
2360	*9-decenoic acid*	0.0102 **a**	0.0001	0.0059 **b**	0.0005	0.0065 **c**	0.0005	0.0068 **c**	0.0008
2511	*Dodecanoic acid*	0.0130 **a**	0.0001	0.019 **b**	0.001	0.0160 **c**	0.0004	0.023 **d**	0.003
	Terpenes (2)								
1205	*Limonene*	0.00075	0.00008	n.d.		n.d.		n.d.	
1560	*Linalool*	0.00142	0.00009	n.d.		n.d.		n.d.	
	Others (4)								
1484	*Furfural*	0.0065 **a**	0.0004	0.046 **b**	0.002	0.037 **c**	0.002	0.038 **c**	0.002
2323	*2,4-di-tert-butylphenol*	1.21 **a**	0.04	0.98 **b**	0.03	0.80 **c**	0.03	0.77 **c**	0.06
2558	*Diisobutyl phthalate*	0.0088 **a**	0.0003	0.0088 **a**	0.0007	0.0084 **a**	0.0005	0.0099 **b**	0.0008
2713	*Dibutyl phthalate*	0.029 **a**	0.003	0.020 **b**	0.001	0.031 **a**	0.005	0.037 **c**	0.004

Table 10: Volatile compounds identified in the Initial wine and in the Control, MOX1 and MOX2 wines after *estufagem*. Significant differences ($p < 0.050$) are identified by different letters in the same row, for each key sampling point.

KI	***Compound***	**Initial**		**Control**		**MOX1**		**MOX2**	
		(mg/L)	±SD	(mg/L)	±SD	(mg/L)	±SD	(mg/L)	±SD
	Alcohols (19)								
913	*Methanol*	n.d.		0.061 **ab**	0.007	0.055 **a**	0.009	0.07 **b**	0.01
1109	*2-methyl-1-propanol*	0.0117 **a**	0.0005	0.15 **b**	0.03	0.16 **c**	0.02	0.15 **bc**	0.02
1220	*3-methyl-1-butanol*	0.098 **a**	0.006	0.6 **b**	0.1	0.75 **c**	0.09	0.75 **c**	0.08
1318	*2-heptanol*	0.0010	0.0002	n.d.		n.d.		n.d.	
1363	*1-hexanol*	0.092 **a**	0.007	0.40 **b**	0.04	0.46 **c**	0.03	0.45 **c**	0.03
1361	*(E)-3-hexen-1-ol*	0.00068	0.00007	n.d.		n.d.		n.d.	
1400	*(Z)-3-hexenol*	0.0056 **a**	0.0005	0.030 **b**	0.003	0.030 **b**	0.002	0.025 **c**	0.001
1421	*(E)-2-hexen-1-ol*	0.0064 **a**	0.0004	0.039 **b**	0.003	0.048 **c**	0.004	0.041 **b**	0.004

1444	*1-octen-3-ol*	0.0022	0.0002	n.d.		n.d.		n.d.	
1468	*1-heptanol*	n.d.		0.027 **a**	0.002	0.026 **a**	0.002	0.021 **b**	0.002
1500	*2-ethyl-1-hexanol*	0.0056 **a**	0.0008	0.06 **b**	0.01	0.084 **c**	0.01	0.060 **b**	0.005
1555	*2,3-butanediol*	0.0017 **a**	0.0002	0.016 **b**	0.003	0.011 **c**	0.002	0.018 **d**	0.002
1669	*1-nonanol*	0.014 **a**	0.001	0.08 **b**	0.01	0.078 **b**	0.009	0.068 **c**	0.003
1773	*1-decanol*	0.016 **a**	0.002	0.11 **b**	0.01	0.13 **c**	0.02	0.13 **c**	0.01
1849	*1-undecanol*	0.0023	0.0003	n.d.		n.d.		n.d.	
1909	*Benzyl Alcohol*	n.d.		0.05 **a**	0.01	0.031 **b**	0.006	0.026 **c**	0.004
1948	*2-phenylethanol*	0.089 **a**	0.009	0.46 **b**	0.05	0.49 **c**	0.04	0.45 **b**	0.03
1979	*1-dodecanol*	0.029 **a**	0.004	0.24 **b**	0.04	0.058 **c**	0.01	0.06 **c**	0.01
2391	*1-hexadecanol*	n.d.		0.14 **a**	0.02	0.14 **a**	0.02	0.12 **b**	0.02
	Carbonyl compounds (18)								
711	*Acetaldehyde*	0.0069 **a**	0.0003	0.20 **b**	0.02	0.21 **b**	0.03	0.31 **c**	0.04
930	*2-methyl-butanal*	n.d.		n.d.		n.d.		0.019	0.002
935	*3-methyl-butanal*	n.d.		0.033 **a**	0.003	0.042 **b**	0.003	0.069 **c**	0.005
1070	*Hexanal*	0.0037	0.0002	n.d.		n.d.		n.d.	
1087	*1,1-diethoxy-3-methylbutane*	n.d.		n.d.		0.017 **a**	0.001	0.025 **b**	0.002
1124	*2-methyl-2-butenal*	n.d.		0.016 **a**	0.002	0.020 **b**	0.001	0.011 **c**	0.002
1307	*3-hydroxy-2-butanone*	n.d.		0.017 **a**	0.003	0.019 **a**	0.002	0.022 **b**	0.004
1334	*6-methyl-5-hepten-2-one*	0.00047	0.00004	n.d.		n.d.		n.d.	
1412	*2-nonanone*	n.d.		0.012 **a**	0.002	0.0096 **b**	0.0006	0.0091 **b**	0.0007
1418	*Nonanal*	n.d.		0.057 **a**	0.01	0.069 **b**	0.01	0.07 **b**	0.01
1523	*Decanal*	0.025 **a**	0.003	0.15 **bc**	0.02	0.17 **b**	0.03	0.14 **c**	0.02
1566	*Benzaldehyde*	0.030 **a**	0.001	0.61 **b**	0.04	0.66 **c**	0.04	1.28 **d**	0.04
1626	*Undecanal*	0.0031 **a**	0.0003	0.028 **b**	0.004	0.026 **b**	0.005	0.025 **b**	0.003
1681	*4-(1,1-dimethylethyl)-cyclohexanone*	n.d.		0.10 **a**	0.01	0.10 **a**	0.005	0.062 **b**	0.009
1732	*Dodecanal*	n.d.		n.d.		n.d.		0.045	0.003
1861	*β-damascenone*	0.015 **a**	0.002	0.06 **b**	0.01	0.058 **b**	0.008	0.037 **c**	0.002
1881	*Geranyl acetone*	n.d.		0.06 **a**	0.01	0.031 **b**	0.006	0.023 **c**	0.003
1906	*Tetradecanal*	0.0034	0.0003	n.d.		n.d.		n.d.	
	Esters (28)								
902	*Ethyl acetate*	0.143 **a**	0.009	1.9 **b**	0.2	2.2 **c**	0.2	3.4 **d**	0.2
980	*Ethyl isobutyrate*	n.d.		0.10 **a**	0.01	0.12 **b**	0.02	0.09 **c**	0.01
1054	*Ethyl butanoate*	n.d.		0.064 **a**	0.004	0.073 **a**	0.01	0.07 **a**	0.01
1070	*Ethyl 2-methylbutanoate*	n.d.		0.021 **a**	0.003	0.019 **a**	0.002	0.033 **b**	0.002
1084	*Ethyl 3-methylbutanoate*	n.d.		0.026 **a**	0.003	0.026 **a**	0.002	0.045 **b**	0.005
1141	*Isoamyl acetate*	0.014 **a**	0.001	n.d.		0.012 **b**	0.002	0.012 **b**	0.001
1247	*Ethyl hexanoate*	0.032 **a**	0.001	0.49 **b**	0.04	0.45 **c**	0.02	0.39 **d**	0.02
1256	*Hexyl acetate*	0.0067	0.0004	n.d.		n.d.		n.d.	
1310	*(Z)-3-hexenyl acetate*	0.00096	0.00005	n.d.		n.d.		n.d.	
1346	*Ethyl heptanoate*	0.00150 **a**	0.00005	0.048 **b**	0.003	0.051 **c**	0.005	0.069 **d**	0.004
1360	*Ethyl lactate*	0.0045 **a**	0.0002	0.044 **b**	0.006	0.047 **b**	0.007	0.045 **b**	0.006
1452	*Ethyl octanoate*	0.5 **a**	0.1	1.1 **b**	1.2	2.1 **c**	0.1	1.5 **b**	0.2
1523	*Ethyl nonanoate*	0.0016	0.0001	n.d.		n.d.		n.d.	

1651	*Ethyl decanoate*	0.034 **a**	0.003	1.4 **b**	0.2	1.1 **c**	0.2	0.5 **d**	0.1
1692	*Diethyl succinate*	0.023 **a**	0.002	1.4 **b**	0.1	1.6 **c**	0.1	1.95 **d**	0.08
1701	*Ethyl benzoate*	0.0033 **a**	0.0004	0.032 **b**	0.004	0.031 **b**	0.002	0.084 **c**	0.006
1705	*Ethyl 9-decenoate*	n.d.		0.021 **a**	0.003	0.023 **b**	0.003	n.d.	
1798	*Diethyl glutarate*	n.d.		n.d.		0.043 **a**	0.006	0.066 **b**	0.005
1818	*Ethyl phenylacetate*	0.013 **a**	0.001	0.20 **b**	0.02	0.24 **c**	0.02	0.28 **d**	0.01
1859	*Ethyl dodecanoate*	0.019 **a**	0.002	0.11 **b**	0.02	0.11 **b**	0.01	0.049 **c**	0.006
1843	*Ethyl salicylate*	0.0034	0.0008	n.d.		n.d.		n.d.	
1847	*2-phenylethyl acetate*	0.034	0.004	n.d.		n.d.		n.d.	
1922	*Ethyl dihydrocinnamate*	n.d.		n.d.		n.d.		0.041	0.005
2050	*Isopropyl tetradecanoate*	n.d.		0.039	0.007	n.d.		n.d.	
2065	*Ethyl tetradecanoate*	n.d.		0.10 **a**	0.01	n.d.		0.08 **b**	0.01
2235	*Methyl hexadecanoate*	n.d.		0.07	0.01	n.d.		n.d.	
2270	*Ethyl hexadecanoate*	n.d.		0.12 **a**	0.02	0.15 **b**	0.02	0.11 **a**	0.02
2448	*Butyl hexadecanoate*	0.0011 **a**	0.0002	0.058 **b**	0.008	0.060 **b**	0.009	0.037 **c**	0.007
	Fatty acids (7)								
1481	*Acetic acid*	0.016 **a**	0.002	0.12 **b**	0.02	0.12 **b**	0.01	0.23 **c**	0.08
1868	*Hexanoic acid*	n.d.		0.037 **a**	0.007	0.031 **b**	0.002	0.032 **b**	0.003
2082	*Octanoic acid*	0.083 **a**	0.007	0.35 **b**	0.07	0.51 **c**	0.08	0.48 **c**	0.04
2293	*Decanoic acid*	0.17 **a**	0.03	0.57 **b**	0.09	1.0 **c**	0.1	0.7 **d**	0.1
2504	*Dodecanoic acid*	0.043 **a**	0.007	0.20 **b**	0.04	0.23 **c**	0.04	0.17 **d**	0.03
2681	*Tetradecanoic acid*	0.015	0.001	n.d.		n.d.		n.d.	
2934	*Hexadecanoic Acid*	0.0109	0.0006	n.d.		n.d.		n.d.	
	Terpenes (4)								
1175	*Limonene*	0.00089	0.00007	n.d.		n.d.		n.d.	
1539	*Linalool*	0.0062	0.0004	n.d.		n.d.		n.d.	
1686	*(-)-α-terpineol*	0.013	0.001	n.d.		n.d.		n.d.	
1831	*Geraniol*	0.00160	0.00009	n.d.		n.d.		n.d.	
	Others (9)								
1127	*2,2,6-trimethyl-6-vinyltetrahydropyran*	n.d.		0.024 **a**	0.004	0.026 **b**	0.002	0.021 **c**	0.002
1293	*Tridecane*	n.d.		0.016 **a**	0.002	0.025 **b**	0.002	0.026 **b**	0.003
1496	*Furfural*	0.0014 **a**	0.0001	0.35 **b**	0.03	0.39 **c**	0.06	0.30 **d**	0.02
1564	*Vitispirane*	n.d.		0.21 **a**	0.02	0.19 **b**	0.01	0.13 **c**	0.02
1608	*5-methylfurfural*	n.d.		0.031 **a**	0.005	0.031 **a**	0.003	0.023 **b**	0.002
1738	*1,3-dimethoxybenzene*	0.0040	0.0003	n.d.		n.d.		n.d.	
2325	*2,4-di-tert-butylphenol*	n.d.		0.17 **a**	0.03	0.33 **b**	0.03	0.30 **c**	0.05
2545	*5-(hydroxymethyl)furfural*	n.d.		0.09 **a**	0.01	0.10 **b**	0.01	0.20 **c**	0.02
2672	*Dibutyl phthalate*	0.017	0.002	n.d.		n.d.		n.d.	